2012

PCR Protocol (50µl) – UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols

Buffer Pfu 5 µl

Upper primers 2.5 µl

Lower primers 2.5 µl

MgCL2 1 µl

DNA (depends on concentration, usually 0.5µl)

Enzime Taq Polymerase 1 µl

DNTP's 8 µl

Water 29.5 µl

Cycles :)

95ºC 4 min

95ºC 1 min

55ºC :30 secs

70ºC 1 min

goto 2:4 30 times

72ºC 5 min

Hot start PCR protocol – UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols

Preheated 105ºC

Heated Lid ON

Pause OFF
In denat 94ºC 5 min.
Hot start OFF

30 cycles
94 ºC 30 secs.
55ºC 30 secs.
70ºC 45 secs.
72ºC 5 min
hold 10ºC

Ligation – UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols

These are done in 1 mL eppendorf tubes and normally have a T.V. of 20 µl.

•Add A µl of the insert (plasmid digested with corresponding enzymes).
•Add B µl of vector (plasmid digested with corresponding enzymes and complementary so that the ligation can be done properly) and properly DEPHOSPOHATED.
•Add 2 µl T4 DNA ligase buffer.
•Add 20-A-B-3-1 µl H2O miliQ.
•Add 1 µl T4 DNA ligase enzyme.

Dephosphorylation protocol **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

-It is done to plasmids digested with the corresponding enzymes that will serve as a "vector" in a ligation.
1. Add 1 µl alkaline phosphatase.
2. Add 2 µl buffer.

3.. Add 20 µl of Digestion.

4. Leave 1 hour at 37ºC.

5. Inactivate at 70ºC for 10 minutes.

Transformation **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

•Pipette the plasmid to a 1.3 mL eppendorf with competent cells and mix with the pipette.

•Incubate tubes on ice for 42 minutes.

•Heatshock at 42ºC for 2 minutes.

•Incubate for 5 minutes on ice.

•Add 1 mL LB broth to each tube.

•Mix by inversion 2-3 times.

•Incubate at 37ºC for an hour with shaking.

•During this hour prepare LB agar plates (30 mL) containing the appropriate antibiotics.

•After the hour Plate 100 µl.

•Centrifuge at 10000 rpm for 2 minutes.

•Drain 700-800 µl of supernatant, mix pellet with the rest and plate. Add to the plate.

•Let grow overnight at 37ºC.

liquid culture **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

•Once the bacteria has grown, add 4 mL LB broth to a glass tube.

•Add the appropriate antibiotic.

•With a wooden toothpick take one colony per tube. Place the toothpick in the tube and vortex.

•Incubate the tube in the shaker 37ºC for at least 6 hours.

Digestion protocol (20 µl) **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

H_2O 9.5 µl

Enzyme 0.5 µl

Buffer 10x 2 µl

DNA (it depends on the concentration but we usually put 8 µl)

37ºC at least 4 hours

For double digestions add 0.5 µl of the other enzyme and 0.5 µl less of H_2O.

Gel extraction protocol **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

(Fermentas Gene Jet Gel extraction kit)

The extraction and purification of a DNA band from a gel electrophoresis is done when we digest and want to recover a specific fragment.

1. Cut the band of the fragment you wish to purify.
2. 400 µl of Binding Buffer, 10 min. at 65ºC with shaker so that the agarose melts.
3. 200 µl deisopropanol after 5 min in the shaker.
4. Pass through column and centrifuge. Throw out supernatant.
5. 700 µl wash buffer, centrifuge, throw it out, centrifuge.
6. Pass column to clean tube, add 40 µl de elution buffer.

Lysis protocol **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

1. Cultivate strain of interest in 5 µl LB with antibiotics.
2. Centrifuge culture at 14000rpm 2 minutes. Remove supernatant and repeat.
3. Add 200 µl solution I, incubate 5 minutes at room temperature. Mix well to dissolve pellet.
4. Add 400 µl of solution II and incubate 5 minutes at room temperature. Mix by inversion 3-6 times.
5. Add 300 µl of solution III. .
6. Mix by inversion and leave in ice 10 minutes. .
7. Centrifuge 10 minutes at 14000 rpm. .
8. Place supernatant in clean tubes and add 500µl 100% ethanol. Centrifuge 15 minutes at 14000 rpm and throw away supernatant. .
9. Add 1 ml 70% ethanol, centrifuge 5 minutes at 1200 rpm and throw out supernatant. Do this twice. .
10. Dry in SAVANT 5 minutes. .
11. Resuspend in 50 µl water MQ and add 5 µl RNAse. .

Gel electrophoresis **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

-Sample preparation
•Add 10 µl of DNA in each well.
•Add 3 µl loading buffer in the same well
•There are special markers that contain a mix of molecules of known size to compare and determine the size of our sample.
•Add 1 µl marker (ladder).

-Gel preparation and loading
•Add 1% agarose to an electrophoresis tank and let it become a gel.
•Introduce the tank in the chamber.
•In each well, with the pipette mix the content and load it in its corresponding well-
•Run with the appropriate Voltage, miliampers (mA) and time, corresponding to the size.

-Visualization

•Once completed, remove gel from chamber and place in special plastic container.

•Dye with ethidium bromide (5 minutes approximately).

•Rinse 10 minutes approximately with water.

Competent cells protocol **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

•Grow cells in LB all night.

•Dilute 100x in LB 50 µl and let grow for 2 hours till an OD of 0.45-0.55 (40 ml X 4 tubes).

•Leave 10 minutes on ice, centrifuge 10 minutes at 6000 rpm at 4ºC.

•Resuspend in 16ml of ice cold TfbI.

•Leave 5 minutes on ice, centrifuge as before.

•Resuspend in 1.6 ml of cold TfbII.

•Leave 10 minutes on ice.

•Make 200 microliters aliquots and store at -70ºC.

BUFFERS **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

TfbI: Mw-> 100ml

30mM Kac 98.146-> 0.294

100mM RbCl 120.9->1.2092

10mM CaCl2 147.020->0.147

50mM MnCL2 197.9-> 0.9895

15% glycerol 15 ml (30ml glycerol at 50%)

-Adjust pH at 5.8 with acetic acid 0.2M (2.4ml p/300ml)

-Sterilize in autoclave

TfbI:100ml 10mM MOPS or PIPES 209.27->0.20927

75 mM CaCl2 110.99-> 0.8324->0.410

10mM RbCl 120.92->0.1292->.60

15% glycerol 15ml(gl 50%)

-Adjust pH at 6.5 with 1 M KOH

-Sterilize by filtration.

PCR purification – **UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

- Add 500 μl solution I "green lid" from kit of PCR purification to eppendorf tube.
- Mix by inversion 5-6 times.
- Drain content to silicium column.
- Repeat band extraction protocol without heating to 65ºC since it is not necessary

GLYCEROL PROTOCOL – **UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

- Add 500 μl glycerol at 70% to a 1.5 mL eppendorf tube.
- Add 1 mL LB broth.
- Take respective colonie with a wooden toothpick.
- Place in eppendorf tube and mix with toothpick.
- Incubate at -80ºC and properly label tube.

PLASMID EXTRACTION 2 – **UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

Once grown in liquid culture:

Obtain the pellet:

•Add 1 mL liquid culture to a 1.5mL eppendorf.

•Centrifuge at 13000 rpm for 2 minutes.

•Drain supernatant.

•Repeat 4 times.

•If you will continue with the extraction continue, if not store pellets (1.5 mL eppendorf tubes) at -20ºC.

•Add 250 mL solution one “white lid” RNase.

•Vortex.

•Add 250 mL solution 2 “red lid” SDS NaOH.

•Mix gently by inversion:

-50 times slow inversion.

-Let rest 1 minute.

-Repeat 5 minutes.

•Add 350 mL solution 3 “green lid” KAc.

•Mix by inversion 20 secs.

•Incubate on ice 10 minutes.

•Centrifuge 13000 rpm for 15 minutes.

•During these 15 minutes place a silicium column inside a recollection tube for each eppendorf tube.

•Drain supernatant in the silicium column and close lid.

•Centrifuge at 13000 rpm for 1 minute.

•Drain supernatant from recollection tube.

•Add 700 mL solution 5 “blue lid” absolute ethanol in the center of the column and close.

•Centrifuge at 13000 rpm for a minute.

•Throw out column.

•Close tube and store -20ºC.

Escherichia coli MC1061 competent cells protocol **– UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols**

1.Plate the cells on LB, incubate at 37°C overnight.

2.Prepare the solutions.

3.Choose a colony and inoculate in 10 mL of LB media, incubate with vigorous shaking for about 16 hours.

4.Subculture in 1L of LB media and incubate at 37°C until it reaches late logarithmic phase reading 0.4 OD600 nm.

5.Incubate the culture on ice immediately after the desire concentration is reached. 6.Note: Incubate on ice all the solutions too.

7.Spin-dry 1000 g & 4°C for 30 minutes. Decant the supernatant.

8.Resuspend gently on 250 mL of 0.1M MgCl2 solution.

9.Spin-dry 1000 g & 4°C for 25 minutes. Decant the supernatant.

10.Resuspend gently on 250 mL of 0.075M CaCl2 solution. Incubate on ice for 20 minutes.

11.Spin-dry 1000 g & 4°C for 25 minutes. Decant the supernatant.

12.Resuspend gently on 50 mL of 0.075M CaCl2+15% glycerol solution (vol/vol). Incubate on ice for 20 minutes.

13.Alicuote (100 microliters) in sterile and cold eppendorff tubes.

14.Store at -80°C.

Escherichia coli MC1061 heat shock transformation protocol – UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols

1.Add the DNA solution to the competent cells and let them 5-20 minutes on ice

. 2.Put the cells on a 42°C termoblock for 5 minutes.

3.Immediately get them back to ice for one

minute.
4.Add 1 mL of LB, mix gently and incubate for one hour at 37°C & 250 rpm .
5.Plate 100 microliters onto selective media.
6.Pellet the cells 1 min at 1200 rpm and resuspend in 100 microliters.
7.Plate cells onto selective media.

Two-step Bacillus subtilis Transformation Procedure – UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols

Preparation of *Bacillus subtilis* competent cell

1.Streak out the strain to be made competent on a LB agar plate as a large patch and incubate overnight at 28°C

2.The following morning wash the cell growth of the plate with 3 ml of Spc and add 3 drops of the solution to inoculate fresh, pre-warmed, SpC medium (30 ml) in a klett flask and measure to give an OD600 reading of about 0.5 using a green filter.

3.Incubate the culture at 37°C with vigorous aeration and take periodic OD readings (OD600) to assess cell growth. (measure every 15 minutes)

4.When the rate of cell growth is seen to depart from exponential (i.e. no significant change in cell density ~5% over 15 min), dilute cell culture 1:10; inoculate 300 ml of pre-warmed, SpII medium with 30 ml of stationary-phase culture and continue incubation at 37°C with slower aeration.

5.After 90 min incubation, pellet the cells by centrifugation (8,000 g, 10 min) at 4°C.

6.Carefully decant the supernatant into a sterile container and save.

7.Gently resuspend the cell pellet in 30 ml of the saved supernatant and add 3 ml of sterile glycerol to a 10% concentration; mix gently.

8.Aliquot the competent cell (0.35 ml) in sterile eppendorf tubes, freeze rapidly in liquid nitrogen bath and store -80°C.

Transformation

1.Add one volume of SpII + EGTA to the competent cells; mix gently

2.Add the DNA solution (500 & 1000 ng) and incubate at 37°C for 1 hour at 200 rpm.

3.Plate 100 microliters immediately onto selective media.

4.Pellet the cells 1 min at 1200 rpm and resuspend in 100 microliters.

5.Plate onto selective media.

NOTE: if you were not able to select you Bacillus, maybe it is because you kill them before they activate their resistance gene. So, made the 5th step different, plate but the 1/40 of the antibiotic, to active the gene and incubate for 1 hour. Then put soft-agar until the petri dish is cover and put the original amount of antibiotic. Leave them overnight (16-24 hours) to grow.

Media for two-step trasformation procedure
T base per liter:
$(NH_4)_2SO_4$ 2 g
K_2HPO_4 13.96 g
KH_2PO_4 6 g
trisodium citrate•$2H_2O$ 1g
-> Autoclave

SpC Made fresh on the day of use from the following sterile solution:
T base 20 ml
50% (w/v) glucose 0.2 ml
1.2% (w/v) $MgSO_4$•$3H_2O$ 0.3 ml
10% (w/v) Bacto yeast extract 0.4 ml 1% (w/v) casamino acids 0.5 ml

SpII Made fresh on the day of use from the following sterile solutions:
T base 200 ml
50% (w/v) glucose 2 ml
1.2% (w/v) $MgSO_4$•$3H_2O$ 14 ml
10% (w/v) Bacto yeast extract 2 ml

1% (w/v) casamino acids 2 ml

0.1 M $CaCl_2$ 1 ml

SpII + EGTA SpII (200 ml) with 4 ml EGTA (0.1 M, pH 8.0) but without $CaCl_2$. SpII + EGTA can be frozen at -20 in small ℃ aliquots, although repeated freeze-thawing should be avoided.
Note: filter EGTA.

SEM Analysis (Scanning Electron Microscope)[1] – UNAM Geonomics Mexico – 2012 http://2012.igem.org/Team:UNAM_Genomics_Mexico/Notebook/Protocols

Exponentially growing cells were plated on LB. Cells were incubated for 3hr at 37°C and then EM grids (FCF200-Cu) were placed on top of the growing cells. Plates were incubated for additional 3 hr and EM grids in the plate was fixed with 2% glutaraldehyde in sodium cacodylate buffer (0.2 M, [pH 7.2]) for 12 hr at 25°C. Cells attached to the grids were washed with 0.1 M sodium cacodylate buffer Na $(CH_3)_2$ AsO_2 , $3H_2O$) (pH 7.2), 3X for 5 min each wash.

Next, cells were postfixed by incubation with 1% osmium tetroxide for 30 min at 25°C and then dehydrated by exposure to a graded series of ethanol washes (30%, 40%, 50%, 60%, 70%, 80%, 90%,; 5 min each and two times with: 100%; 10 min each).

Finally, the grid-attached cells were washed with a graded series of liquid CO_2. The liquefied gas must be miscible with the solvent in the cells. After sufficient equilibration time, the liquefied gas replaces the alcohol. The temperature of the pressure chamber is then raised above the critical point of the gas and the liquefied gas returns to the gaseous state without a change in volume or density and without surface tension forces. After drying, the specimens are mounted on a stub. Specimens were coated with gold-palladium with a Sputter Coater and cells observed with a SEM.

[1]Dunlap, M. Introduction to the Scanning Electron Microscope, 1997, FACILITY FOR ADVANCED INSTRUMENTATION

- Preparation of *E. coli* Calcium Chloride competent cells – UANL Mty-Mexico – 2012 http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

1. Inoculate a single colony into 5 mL of LB media without any antibiotics and grow overnight at 37 °C with vigorous shaking.

2. Inoculate 1 mL of the desired strain into 100 mL of fresh LB, use a 500 mL flask.
3. Incubate at 37 °C with vigorous shaking until 0.3 - 0.4 sideOD_{600}
4. Put the flask on ice. Pre-chill 50 mL centrifuge tubes and the centrifuge itself at 4°C.
5. Centrifuge 50 mL of the culture at 8,000 rpm for 5 minutes at 4 °C.
6. Remove the supernatant and add 10 mL of cold $CaCl_2$ 0.1 M. Vortex until the pellet is resuspended.
7. Incubate on ice for 30 minutes, shake the tube once in a while.
8. Centrifuge at 8,000 rpm for 5 minutes at 4°C. Remove the supernatant and add 2 mL of $CaCl_2$ 0.1 M. Resuspend carefully using a micropipette. Keep always on ice.
9. Mix the two preparations in a tube and store on ice, or use for transformation.

Note: The competent cells can be stored on ice up to two weeks.

- Preparation of *E. coli* Rubidium Chloride competent cells – UANL Mty-Mexico – 2012 http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

1. Inoculate 5 mL of LB broth with DH5α and incubate the culture overnight at 37°C with vigorous shacking.
2. Use small culture to inoculate 100 mL of LB broth. Incubate at 37°C with shacking until the culture reaches an optical density (OD595) 0.4-0.6.
3. Transfer to two 50 mL centrifuge tubes.
4. Spin at maximum speed for 5 minutes at 4°C.
5. Remove supernatant.
6. Add 20 mL of TBF1 and resuspend the pellet.
7. Incubate on ice for 20 minutes.
8. Pour off supernatant.
9. Centrifuge 5 minutes at 8000 rpm/4°C.
10. Resuspend cellular pellet with 4 mL of TBF2.
11. Place aliquots of 100 µL at -70°C.

- Heat-shock transformation of *E. coli* competent cells

1. Add 50 µL of Ca^{+2} competent cells to a pre-chilled centrifuge tube. Keep always on ice until step 4.
2. Add plasmid DNA (100 ng) or ligation (up to 5 µL) depending on DNA concentration.
3. Use 1 µL of a 1 ng/µL DNA sample as positive test in a separate tube. It is recommended to use a DNA-free negative test tube as well.
4. Chill the tube on ice for 20 - 30 minutes.
5. Expose the reaction mixture to a 42ºC 1 minute heat-shock.
6. Put the tube on ice for 2 minutes.
7. Add 200 µL of antibiotic-free LB media.
8. Incubate at 37ºC for 20 - 30 minutes.

9. Spread the appropriate quantity of cells (50-200 µL) on selective LB agar plates.
10. Incubate overnight at 37º C.
11. The positive plate must have around 1,000 colonies as an optimal ($1X10^6$ transformants per µg supercoiled DNA).

Notes: Until heat-shock, handle the tubes from the upper part to avoid warming the cells. Low temperature is critical for successful transformation. Avoid transforming with more than 5 µL of ligation mixture, as ligation buffer may reduce transformation efficiency.

- Preparation of Electrocompetent *E. coli* cells – UANL Mty-Mexico – 2012

http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

1. Inoculate a single colony of *E. coli* in 5 mL of LB media. Grow overnight or for 5 hours at 37°C with shaking at 250 rpm.
2. Inoculate 2.5 mL of the previous culture in 200 mL of LB media in a 2 L flask. Grow at 37 °C shaking at 300 rpm until the culture reaches an OD of 0.5 - 0.7.
3. Chill the cells on ice for 10 - 15 minutes and then transfer the cells into a pre-chilled centrifuge bottle.
4. Centrifuge at 4,200 rpm for 10 minutes at 2 °C (Beckman J-6M).
5. Remove the supernatant and resuspend the pellet in 5 mL of cold water. Add 200 mL of cold water and mix well. Centrifuge at 4,200 rpm for 10 minutes at 2 °C.
6. Remove the supernatant and resuspend the pellet by shaking gently in the remaining liquid volume.
7. Add 200 mL of cold water, mix well and centrifuge at 4,200 rpm for 20 minutes at 2°C.
8. Add 20 mL of 10% cold glycerol and mix well. Centrifuge at 4,200 rpm for 20 minutes at 2 °C.
9. Add 10 mL of 10% cold glycerol to each tube. Resuspend and gather all the content of the tubes in a single tube, centrifuge and remove the supernatant.
10. Estimate the pellet volume and add an equal volume of 10% cold glycerol. Resuspend the cells.
11. Divide the final volume into pre chilled tubes (100 µl) and store at -80 °C.

Note: Pre-chill all the materials that will be in contact with the cells.

- Electroporation of *E. coli* competent cells – UANL Mty-Mexico – 2012

http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

1. Take a tube with 50 µL of electrocompetent *E. coli* cells, thaw on ice.
2. Add a volume containing 100 ng of DNA.
3. Carefully transfer the cell/DNA mix into a pre-chilled electroporation cuvette. Make sure to deposit the cells at the bottom and not to introduce any air bubbles.
4. Electroporate under the following conditions:

5. Immediately add 250 µL of SOC media to the cuvette.
6. Incubate with vigorous shaking (250 rpm) at 37 °C for 1 hour.
7. Add 750 µL of LB media and mix by pipetting up and down.
8. Spread 200 µL of cells onto a selective LB agar plate.

Note: All must be performed on ice. Electroporation cuvettes are previously chilled on ice. DNA and bacteria must be thawed on ice too.

▪ Mini preparation of plasmid DNA – UANL Mty-Mexico – 2012
http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

1. Pour 1.5 mL of the culture in a 1.5 mL microcentrifuge tube and centrifuge at 14,000 rpm for 30 seconds. Remove carefully the supernatant.
2. Add 200 µL of Solution I. Resuspend the pellet by using vortex briefly or by pipetting up and down. Incubate at room temperature for 5 minutes.
3. Add 200 µL of Solution II and mix gently by inverting and rotating the tube several times. Do not vortex. Incubate at room temperature for 5 minutes.
4. Add 200 µL of Solution III and mix gently by inverting and rotating the tube several times. Incubate the tube on ice for 5 minutes.
5. Centrifuge at 14,000 rpm for 5 minutes.
6. Transfer the supernatant to a fresh tube containing 1 mL of 100% ethanol.
7. Incubate at -20 ºC for 10 minutes. (Max. 2 h)
8. Centrifuge at 14,000 rpm for 10 minutes. Remove the supernatant.
9. Add 200 µL of 70% ethanol and vortex gently for 10 seconds.
10. Centrifuge at 14,000 rpm for 5 minutes. Remove the supernatant by pipetting. Aspirate off any residual supernatant.
11. Dry at 37ºC for 5 minutes.
12. Add 20 µL of H_2O + 20 µg/mL of RNase. Resuspend by using vortex briefly.
13. Run an agarose gel (0.8%) or store at 4 ºC.

▪ Midi preparation of plasmid DNA (AXYPREP ™ PLASMID MIDIPREP KIT)

1. Collect 30 of overnight LB culture for the preparation of the high-copy plasmid, or 100 mL of overnight LB culture for the preparation of the low copy-plasmid. Centrifuge at 3000X G for 8 minutes to pellet the bacteria. Discard the supernatant. Invert the centrifuge tube on a paper towel for 1 minute to drain off residual medium.
2. Resuspend bacterial pellet with 4.5 mL of Buffer S1.

a. Make sure that RNase A has been added into Buffer S1.

b. Incompletely resuspension will reduce lysis efficiency, plasmid yield and purity.

3. Add 4.5 mL of Buffer S2, and mix by gently inverting tube 6-8 times (do NOT vortex)

a. Buffer S3K must be added within 5 minutes.

b. Check the Buffer S2 for precipitation before each use. If precipitation occurs, incubate at 37°C.

4. Add 4.5 mL Buffer S3K pre-chilled at 4°C, invert the tube 10 times. Incubate at room temperature for 5 minutes.
5. Add 4.5 mL of Buffer B (pre-chilled at 4°C), gently invert the tube 10 times.
6. Centrifuge at 6000 G for 10 minutes.
7. Attach the AxyPrep Vacuum Manifold base to a vacuum pump. Position the manifold top with luer-type fittings on the manifold base. Attach a Midiprep Plasmid Column to a complimentary fitting on the manifold top. Make sure that the column is firmly seated.
8. Transfer supernatant from step 5 to the Midiprep Syringe Filter. Carefully insert the plunger into the syringe filter and push with a steady motion. Discharged the filtrate into the Midiprep Plasmid Column.
9. Switch on the vacuum source and continue to apply vacuum to the column until lysate remains. Do not turn the vacuum off.
10. Keep vacuum, add 7 ml of Buffer W1 and draw the solution through the Midiprep Plasmid Column.
11. Add 8 mL of Buffer W2, and draw through the Midiprep Plasmid Column.

a. Make sure that ethanol has been added into Buffer W2 concentrate

12. Use the plastic wrench to detach the end component from the Midiprep Plasmid Column assembly and place it into a 1.5 mL microfuge tube. Add 300 µL of Buffer W2 and centrifuge at 12 000 G for 2 minutes.
13. Transfer the end component to a fresh tube 1.5 mL microfuge tube. Add 300 µL of distilled water to the center of the membrane. Let it stand at room temperature for 1 minute and centrifuge at 12 000 G for 1 minute to elute the plasmid DNA.

Note: This protocol was taken from AxyPrep Midi and Maxi Plasmid Kits, available at http://static3.jadedpixel.com/s/files/1/0001/7488/files/axyprep_midi_maxi_plasmid_protocol.pdf

- Solutions for Mini preparation of Plasmid DNA – UANL Mty-Mexico – 2012 http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

Solution I (200 mL)	**milliliters or grams**
- Tris HCl 1 M (pH 8.0)	5 mL
- EDTA 0.5 M (pH 8.0)	4 mL
- Distilled H_2O	Bring the final volume up to 200 mL
Solution II (200 mL)	
- NaOH 10N	4 mL

- SDS (powder) 2.0 gr
- Bidistilled H_20 Bring the final volume up to 200 mL

Soll III (200 mL)

- Potassium acetate (CH_3CO_2K) 58.8 gr
- Acetic acid (CH_3-COOH) 23.0 mL
- Distilled H_20 Bring the final volume up to 200 mL

Notes: When preparing Solution II, first add a little bidistilled water, then add NaOH and dissolve carefully SDS. Finally, bring the final volume up to 200 mL with bidistilled water.

When preparing Solution III, first add 100 mL of H_2O and then the potassium acetate. Once it has been add the acetic acid and finally bring the final volume up to 200 ml with bidistilled water.

- Agarose Gel Electrophoresis Protocol – UANL Mty-Mexico – 2012
http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

To be Loaded:

- DNA molecular size marker (λ *Pst*I): 2 - 3 μL
- Plasmid DNA: 2 - 3 μL
- Enzyme restrictions: 10 μL
- PCR products: 5 μL

Procedure:

5. Prepare an agarose gel of the desired concentration (see Agarose gels section).
6. Add the necessary SB 1X buffer into the electrophoresis tank to cover the gel.
7. Load the first well with marker, and then load the DNA samples mixed with loading buffer into the wells.
8. Plug in the anode and cathode cables so that the DNA samples can move through the gel toward the anode.
9. Run the electrophoresis at 200 volts.
10. Wait approximately 20 - 30 minutes or until the bromophenol blue reaches the end of the gel and stop the electrophoresis.

Note: DNA moves toward the positive electric field (anode, usually red cable) due to the negative charges.

- Agarose gel

Concentration for supercoiled and plasmid DNA: **0.8%**
For digestion reaction fragments over 1,000 bp: **0.8%**
For digestion reaction fragments below 500 bp: **1.5%**
DNA size marker (λ + *Pst*I): **Use 2 or 3 μL per gel.**
Note: Not needed when running supercoiled DNA samples, like plasmid DNA.

- SB buffer 20X

SB (Sodium Borate) electrophoresis buffer, 20X Stock:

0. In 700 mL of distilled H2O, dissolve 8 gr of NaOH.
1. Weight 51 of Boric Acid and dissolve ¾ parts in the NaOH solution
2. Dissolve the remaining Boric Acid until the buffer reach pH 8.0.
3. Complete to 1 L with distilled H2O and store in a sterile flask.

Note: Use SB 1X as buffer to run agarose gels up to 200 volts

- Ethidium Bromide Gel Staining – UANL Mty-Mexico – 2012
http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

0. Dilute the stock to 20 μg/mL in a special container with the gel buffer.
1. Put the gel into the container.
2. Let it stain for 3 - 5 minutes.
3. Take the gel out of the container and soak the stained gel in water for 5 minutes or more to clear background ethidium bromide from the gel.
4. View the gel under a UV light source or on a UV transilluminator.

Note: If you want to use ethidium bromide, confine its use to a small area of your laboratory. Wear gloves when staining, handle stained gels, and dispose of any waste.

- Lambda/PstI Molecular Size Marker

Mix:

- phage λ DNA (500 ng/μL) 50.0 μL
- *Pst*I 2.5 μL
- Buffer 10X 6.0 μL
- H_20 1.5 μL

Procedure:

4. Mix the ingredients listed above
5. Incubate at 37ºC / 45 minutes
6. Again add 2.5 μL *Pst*I
7. Incubate 37ºC / 45 minutes
8. Add 6.0 μL Loading buffer 6X

Check on agarose gel.

Notes: Final concentration: 0.30 μgr/μL. Final Volume: 66.0 μL.

- Restriction enzyme digestion of DNA – UANL Mty-Mexico – 2012 http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

Mix for 1 reaction, final volume of 20 μL

Add the following to a microcentrifuge tube:

DNA	2-3 μg
Buffer 10x	2.0 μL
Enzyme (10 U/μL)	0.3 μL (1 enzyme unit per μg DNA)
H_2O	Until 20.0 μL

Incubate the mixture at 37 °C (it may change, check enzyme specifications) for 1 - 1.5 hours.

Note: Prepare a mix when possible to minimize enzyme handling.

- PCR – UANL Mty-Mexico – 2012 http://2012.igem.org/Team:UANL_Mty-Mexico/Notebook/protocols

PCR reaction mix:

DNA template	Total 100 ng (In 25 μl)

Buffer 10x	2.5 µL
Mg^{++} 50 mM	0.75 µL
dNTPs 20 mM	0.25 µL
Primer Fwd 100 ng/µL	0.50 µL
Primer Rv 100 ng/µL	0.50 µL
Taq Pol 5 U/µL	0.25 µL
H_2O	To bring the volume up to 25 µL

- **Procedure:**

0. Add the corresponding H_2O to a sterile PCR tube.
1. Add the rest of the components but the enzyme and DNA.
2. Add the enzyme, mix gently.
3. Add the respective DNA sample and mix gently.
4. Spin the tube briefly.
5. Place the sample in the thermocycler and start your PCR program.

Notes:

Put on gloves before taking the PCR mix components out of the freezer.

DNA must be added at last because it may form complexes with Mg^{++} and inhibit the reaction.

When possible, make a mix with all the common components to minimize enzyme waste.

- Antibiotics

Antibiotic	Final concentration	Stock concentration	µL per mL
Spectinomycin (Sp)	100 µg/mL	20 µg/µL	5
Ampicillin (Amp)	50 µg/mL	10 µg/µL	1
Kanamycin (Kan)	50 µg/mL	50 µg/µL	1
Chloramphenicol (Cm)	34 µg/mL	34 µg/µL	1
Tetracycline (Tet)	10 µg/mL	5 µg/ µL	2

Notes:

Always verify stock concentration, in case of unknown assume the one indicated above.

When using more than one antibiotic simultaneously use half the concentration for each antibiotic.

PLASMID DNA ISOLATION - BY MINI PREP PROTOCOL - Panama INDICASAT – 2012 http://igem.org/Results?year=2012®ion=All&division=igem

1. Clean the benches and pipettes.
2. Centrifuge 1mL of bacterial culture (in log phase of growth) for 7 minutes at 13000rpm in a microcentrifuge.
3. Pour off the supernatant, leaving abput 50 - 100uL.
4. Resuspend the bacterial pellet with 300uL of P1 buffer (50mM Tris-HCl, 10uM EDTA and 10ug/mL of RNAse)
5. Add 300uL of P2 buffer (0.2 M NaOH, 1% SDS) and mix by inversion.
6. Incubate the tubes for 5 minutes at room temperature.
7. Add 300uL of P3 buffer (3M potassium acetate solution (pH 5.5)), this solution neutralizes NaOH in the previous lysis step while precipitating the genomic DNA and SDS in a insoluble white, rubbery precipitate.
8. Mix by inversion.
9. Place on ice during 5 minutes.
10. Centrifuge for 10 minutes at 13000rpm.
11. Carefully, transfer the supernatant to a new microcentrifuge tube and discard the pellet.
12. Add 600uL of Phenol: chloroform: isoamyl alcohol (24:1:1).
13. Mix by inversion and centrifuge for 5 minutes at 13000rpm.
14. Transfer the aqueous phase (?700uL) to new properly labeled microtube.
15. Add an equal volume of cold isopropanol (?700uL) to the recovered supernatant. Mix by inverting the tube.
16. Pellet the DNA by centrifugation at 4°C for 15 minutes at 13000rpm.
17. Pour off isopropanol, being carefully not to loose pellet. Use a pipette tip to remove remaining isopropanol without dislodging the DNA pellet.
18. Rinse the pellet with 500uL of 70% ethanol. Mix by inversion and centrifuge briefly (5 minutes at 13000rpm).
19. Remove the residual ethanol and dry the DNA in the DNA SpeedVac.
20. Resuspend the DNA in 30uL of ultrapure water.

POLYMERASE CHAIN REACTION (PCR) - FOR AMPLIFICATION OF RH1AB GENE COMPLEX - Panama INDICASAT – 2012

http://igem.org/Results?year=2012®ion=All&division=igem

1. Clean the bench and the pipettes.
2. Thawed the PCR mix, nuclease-free water, the primers, and the DNA samples. Place them on ice.

3. Mix the reagents in each PCR tube, in the following order: (If you planned to make a "Master Mix", calculate the volumes for the amount of samples plus 1 tube). Finally add 2uL of the DNA isolate.

Reagent	Volume for 1 tube
Nuclease free water	5.5uL
Platinum® Blue PCR Super Mix 2X	12.5uL
rht 1bF forward primer	2.5uL
Rht 2bR Reverse primer	2.5uL
Premix volume (per tube)	23uL
Amount of DNA sample (per tube)	2uL

4. Turn the thermal cycler on and set the Protocol: rh1AB.amp (within iGEM user).
5. Put the PCR tubes on the thermal cycler. Insert volume data and begin the program.
6. When the equipment reaches the last temperature setting (at 4°C), take off the tubes and turn off the equipment.
7. Use PCR products immediately. Otherwise, keep them at 4°C if they will be used within the first 24 hours or at -20°C to keep for longer periods.

AGAROSE GEL ELECTROPHORESIS - Panama INDICASAT – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

1. Assemble the gel casting tray and comb. The comb should not touch the bottom of the tray.
2. Add 0.7g of agarose to 70 mL of 1X TAE Buffer. Using a microwave, melt the agarose solution (for 1 minute).
3. When the agarose solution has cooled (to about 50°C), add 2uL of ethidium bromide solution and mix it well.
4. Pour solution directly into the casting tray, ensuring that no bubbles get into the gel. (rinse the flask immediately).
5. Allow the gel to cool. It will solidify and become slightly opaque within 20 to 30 minutes. Remove the gel casting and set in the correct position.
6. Submerge the gel by adding approximately 800mL of 1X TAE running buffer to cover the gel by about a half a centimeter.
7. Carefully remove the comb by lifting it gently at one end, tilting the comb as it comes out. Pulling the comb straight up creates a vacuum in the wells,

which tends to lift the whole gel out of the tray. Ensure that the wells are submerged and filled with buffer.

8. Prepare the 2uL DNA ladder (marker) for loading using 2uL of loading buffer.
9. Load a maximum of 10uL of each sample into individual wells with a gel loading tip.
10. Once all the samples are loaded place the cover on the gel apparatus. Connect the leads so that the red (positive) lead is at the end of the gel to wich DNA will migrate and the black (negative) lead is is at the side of the gel containing the wells. Turn on the power supply and set at 100V. Check the gel after minutes.
11. When the blue tracking dye (which runs in these gels along with a DNA fragment of about 200-400bp) has migrated about 50% of the distance to the end of the gel (usually within 60 minutes), turn off the power supply and disconnect the power leads.
12. Transfer the gel to the transilluminator and visualize DNA with UV light. The DNA fragment band corresponding rhlAB gene complex has 2343bp.

TRANSFORMATION Panama INDICASAT – 2012

http://igem.org/Results?year=2012®ion=All&division=igem

1. Thaw the competent cells on ice for 5 minutes (competent cells are extremely susceptible to heat, work with them always on ice.
2. Meanwhile the cells are thawing out, add 2-4uL of DNA to a 2 mL microtube on ice (use molecular grade H2O for your negative control).
3. Add 50uL of the thawed out competent cells to 2mL microtube.
4. Incubate 5 minutes on ice.
5. Add 200uL of room temperature SOC medium to the microtubes. (It is no longer necessary to keep the cells on ice from this step onwards).
6. Incubate for 1 - 2 hours at 37°C with vigorous shaking.
7. Spread 100uL to the transformants onto an agar plate with the appropriate antibiotic.
8. Incubate at 37°C "overnight" with the top side looking downwards

AMPICILLIN STOCK - Panama INDICASAT – 2012

http://igem.org/Results?year=2012®ion=All&division=igem

1. Weight the appropriate amount of antibiotic powder for the amount of H20dd to be used. (we use 0.25g in 5mL of H20 to make a 50mg/ml solution).
2. Absorb your solution with the sterile syringe.
3. Filter your solution into the sterile 15mL centrifuge tube.

PREPARING CHEMICALLY COMPETENT CELLS

1. Grow an overnight cell culture to the strain you want to turn competent.
2. Make a 1:100 dilution of the overnight cell culture and grow to a OD600 of 0.3 - 0.5 (we inoculate 1mL of overnight into 100 mL of LB)
3. Split the cell culture into two 50 ml centrifuge tubes.
4. Centrifuge at 4000rpm at 4°C for 10 minutes (Always keep the cells on ince from this point onward, this is very important).
5. Discard the supernatant.
6. Gently resuspend the cells using 20mL of cold 0.1M CaCl2 solution for each centrifuge tube.
7. Incubate on ice for 30 minutes.
8. Centrifuge at 4000rpm at 4°C for 10 minutes
9. Discard supernatant.
10. Resuspend each tube using 3mL of cold CaCl2 0.1M 15% glycerol solution.
11. Aliquot however you seem fit in 2mL microtubes or cryovials.
12. Store at -80°C as soon as you are done.

AGAR PLATES - Panama INDICASAT – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

1. Mix 20g of agar powder for every 1000mL of LB Broth being used.
2. Autoclave
3. Wait till the temperature has gone down to a point where it won't burn to touch and add the appropriate antibiotic at the desired concentration.
4. Serve 25ml of agar + LB Solution per petri dish.

GLYCEROL STOCK - Panama INDICASAT – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

1. Grow an overnight cell culture of the desired sample.
2. Add 1ml of overnight culture into a 2mL microtube or cryovial.
3. Add 1mL of 40% glycerol solution into the same tube.
4. Store at -80°C (To reactivate simple inoculate 50uL into fresh LB Broth with the appropriate antibiotic).

RESTRICTION DIGEST - Panama INDICASAT – 2012

http://igem.org/Results?year=2012®ion=All&division=igem

1. Quickly vortex all ingredients (Buffer, BSA, DNA from Miniprep) before beginning
2. Add the following in a microcentrifuge tube:
1. 5uL of Buffer (NEBuffer2).
2. 1uL of BSA.
3. 0.5 picomoles DNA, normally uses 10uL of miniprep or 5uL of purified PCR product.
4. Water to make 48uL
3. Vortex enzymes and add 1uL of each to the tube.
1. If you are digesting purified PCR products, add 1uL of Dpnl to the reaction.
4. Incubate reaction a 37°C water bath for at least one hour.
1. If your digesting a "vector", add 1uL Antarctic Phosphatase and 6uL of Phosphatase buffer after 2 hour of incubation and incubate for another hour.
2. See Bio-Brick Assembly Schedule for more details.
3. 5 Heat kill the digest for 20 minutes at 80°C.
4. Sore digested DNA in the refrigerator (4°C) for use in the very near future.

Bacterial (Xam) DNA extraction protocol – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

1. Shaking culture overnight.
2. Centrifuge twice at 10,700 rpm in 1.5 mL tube 2 or 3 minutes. On the same tube centrifuge all the cultures and remove the supernatant.
3. Wash the pellet twice with 1 mL NaCl 1 M and Resuspend.
4. Centrifuge 2 minutes at 10,700 rpm and remove the supernatant.
5. Resuspend the pellet on 567 µL TE (10 mM Tris-HCl pH 8; 1 mM EDTA pH 8).
6. Add 3 µL proteinase K (20 mg/mL) and 30 µL SDS (10%). Resuspend gently.
7. Incubate 2 hours 30 minutes at 37℃.
8. Add 100 µL NaCl 5 M and 80 µL of the mix NaCl-CTAB (NaCl 0.7 M and CTAB 10%). Preheat the mix to solve it.

9. Incubate 10 minutes at 65℃.
10. Add 600 µL phenol.
11. Gently shake manually for 10 minutes.
12. Centrifuge 10 minutes at 10,700 rpm.
13. Transfer the upper aqueous phase to a new tube and add an equal volume of chloroform-alcohol isoamyl (24:1).Gently mix by vortex.
14. Centrifuge 5 minutes at 10,700 rpm.
15. Transfer the upper aqueous phase to a new tube.
16. Add 0.6 V of isopropyl alcohol and incubate at least 15 minutes at -80℃ or overnight at -20℃.
17. Centrifuge 20 minutes at 10,700 rpm. Remove supernatant.
18. Wash the pellet with 1 mL ethanol 70% (2 or 3 times).
19. Centrifuge 15 minutes at 10,700 rpm. Remove supernatant with pipette.
20. Dry the pellet under vacuum for 20 minutes.
21. Resuspend the pellet on 30 µL TE or H_2O.
22. Add 2 µL RNAse and incubate 2 hours 30 minutes at 37℃.
23. Store the tubes at -20℃.

Miniprep – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

We are using the GenElute™ Plasmid Miniprep Kit of Sigma-Aldrich according to the manufacturer directions.200px|thumb|center

Preparation of electrocompetent E. coli cells – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

1. Grow a colony of culture overnight on 5mL LB at 37℃ shaker.
2. Put the content of the tube on an erlenmeyer with 250mL LB.
3. Shake at 37℃ - 200rpm and continuously read the OD.

4. When the OD reach 0,7 the cells must be kept on ice and all the implements must be cold.
5. Centrifuge 10 minutes at 8500 rpm and 4℃. Discard supernatant.
6. Wash the cells with deionized distilled H_2O and mix by vortex. Completing then the volume of the tube with more H_2O.
7. Centrifuge 10 minutes at 8500 rpm and 4℃. Discard supernatant.
8. Repeat steps 6 and 7 once again.
9. Repeat steps 6 and 7 but using sterile 10% glycerol.
10. Resuspend the product of the last centrifugation on 1mL 10% glycerol.
11. Aliquote 40µL on Eppendorf tubes and store cells at -80℃.

Electroporation – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

We electroporate E. coli DH5α Electro-competent cells at 1.25 mV, then we resuspend the cells in SOC medium and incubate on a shaker at 37℃ for one hour, then, we plate the cells in a solid medium containing antibiotic selection.

Primer Design – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

Primers were designed manually using OligoAnalyzer of IDT, secondary structures were also calculated using mfold and annealing conditions usign FAST PCR.

PCR - Pfu DNA polymerase – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

One reaction.

Reactives	**Volume(µL)**
H_2O	17.95
Buffer	2
$MgSO_4$	1.6
dNTP's	0.4
Primer Fw	0.4
Primer Rv	0.4
Taq	0.06
Pfu	0.19
DNA	2
Total	25

PCR - Taq DNA polimerase Invitrogen – Columbia – 2012

http://igem.org/Results?year=2012®ion=All&division=igem

One reaction.

Reactives	**Volume(µL)**
H_2O	6.15
Buffer	1
$MgCl_2$	0.5
dNTP's	0.25
Primer Fw	0.5
Primer Rv	0.5
Taq	0.1
DNA	1
Total	10

If needed add 1 µL of DMSO per reaction, and adjust the amount of H_2O to a final volume of 10 µL.

PCR – Colony – Columbia – 2012

http://igem.org/Results?year=2012®ion=All&division=igem

Based on the protocol used with Taq polimerase, add 1 µL more of H_2O and take a few cells from the strain pricking it with a toothpick and mixing them with the other PCR reactives instead of using purified DNA.

PCR – Boiling – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

We make a lysate of the cells and add 1 µL of that lysate instead of purified DNA using the Taq polimerase protocol. To obtain the lysate, scrape the growth solid culture and resuspend the cells on 50 µL of H_2O, then, put at 95℃ for 10 minutes.

Digestion of DNA with 2 different enzymes – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

One reaction

Reactives	Volume(µL)
H_2O	28,6
Buffer	4
BSA	0.4
Enzyme 1	1
Enzyme 2	1
DNA	5
Total	40

To prepare this reaction you first pipet together DNA, buffer and water, vortex the mixture and add the enzyme. Mix by pipetting and incubate for 3 hours at the temperature optimal for the enzyme (generally at 37℃).

DNA ligation with T4 DNA ligase – Columbia – 2012
http://igem.org/Results?year=2012®ion=All&division=igem

Reactives	**Volume(µL)**
nuclease-free H_2O	12,99
Buffer 10X	2
Vector DNA	2,5
Insert DNA	2,5
T4 ligase	0,01
Total	20

Incubate at room temperature for 16 hours.

Cell Lysis – Westminster – 2012

http://2012.igem.org/Team:Westminster/Protocols

We used Stratagene kit for lysing human cells and extracting DNA.

• Pellet up to a maximum of 1x 10^6 cells for 30 sec. in microfuge. If more than 1X 10^6 cells are used, the reagent volumes will have to be increased accordingly. For adherent cells, a trypsin treatment is generally used prior to this step to free the cells into suspension. Be careful to perform the trypsin step in a timely manner so that5 the cells do not remain in undiluted trypsin for a long period of time.

• Aspirate media and wash cells with 500 µl of 1x PBS.

• Pellet cells 30 sec. in microfuge at 14, 000 rpm.

• Aspirate PBS and resuspend cells in 500 µl of PBS. Repeat spin and aspiration steps.

• Resuspend cells in 100 µl PBS and 200µl sterile water.

• Lyse cells by heating to 950C for 10-15 minutes.

• Allow the cells to cool briefly by setting at room temperature for 5 min. And add 10 µl of 10 mg/ml Proteinase K to each sample. Quick vortex to mix.

• Incubate at 55°C for one hour.

• Inactivate Proteinase K by heating to 95°c for 10 minutes.

• Spin down condensation.

• Store at -20°c.

PCR Amplification – Westminster – 2012

http://2012.igem.org/Team:Westminster/Protocols

Biolabs Phusion High- Fidelity DNA polymerase was used for all PCR amplifications. The reactions and conditions are given below.

Name	50 µl reaction	10 µl reaction	Final concentration
Water (reverse osmosis)	add to make 50 µl	add to make 20 µl	
5x Phusion Buffer	10 µl	2 µl	1x
10mM dNTPs	1 µl	0.2 µl	200 µM each
Forward Primer 10 µM	x µl	x µl	0.5 µM
Reverse Primer 10 µM	x µl	x µl	0.5 µM
Template DNA	x µl	x µl	
Phusion Polymerase	0.5 µl	0.1 µl	0.02 U/µl

Conditions:

Step	Temperature	Time
Initial denaturation	98°C	30 sec
25-30 cycles	98°c 45-72°c 72°c	5-10 sec 10-30sec 15-30 sec/kb
Final extension	720c	5-10 minutes
Hold	40c	

PCR amplification with linkers required an additional 50 µM Mg^{2+} in the mastermix.

USER Amplification – Westminster – 2012

http://2012.igem.org/Team:Westminster/Protocols

Procedure

The USER mix components are mixed (Table in materials).The PCR product must be purified before used in USER cloning

● 2 μl of the USER mix is transferred to PCR tubes.

● The PCR products is added in equal amounts of each and incubated for 40 minutes at 37°C and for 30 min at 25°C.

User mix	volume
USER enzyme	1 μl
NEB (10 x diluted)	0.5 μl
BSA	0.5 μl
PCR product(s)	8 μl

Site-Directed Mutagenesis – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

• Purify template plasmid DNA from a dam+ Escherichia coli strain (to ensure that all GATC sites are methylated for later digestion with DpnI).

• Design forward and reverse primers that will bind to the region of DNA you want to mutate but that contain the modifications you wish to make. See the CAD tool PrimerX.

• Run a primer-extension reaction with a proof-reading, non-displacing polymerase such as Pfu DNA polymerase. This results in nicked circular strands of the plasmid.

• Cut up the template DNA with DpnI.

• Transform the circular nicked DNA into a highly competent strain such as XL1-Blue. These cells will repair the nicks and not restrict the unmodified product DNA.

• Select colonies with the correct DNA.

Purification of PCR Products – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

QIAquick PCR Purification Kit Protocol using a micro centrifuge was followed for purification of PCR products.

Procedure

- Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
- Place a QIAquick spin column in a provided 2 ml collection tube.
- To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
- Discard flow-through. Place the QIAquick column back into the same tube. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
- Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.
- Residual ethanol from Buffer PE is completely removed by an additional centrifugation.
- Add 30 μl of double distilled nuclease free water to elute the DNA and centrifuge again.
- Collect the flow through.

ANALYSIS OF DNA – Westminster – 2012

http://2012.igem.org/Team:Westminster/Protocols

Gel Electrophoresis

- Depending on the expected size of the fragments to be analysed, the percentage of gel is determined. For fragments more than 1.2 kb, a 1% gel and fragments less than 1.2kb 2% gel is used.
- To make a 1% gel, 0.7g of agarose is added to 70 ml of TAE buffer.

• The agarose is melted in a microwave until the solution becomes clear.

• The solution is cooled down until it can be held for 5 continuous seconds.

• 1 μl of ethidium bromide is added to the solution.

Electrophoresis Setting – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

• Ensure electrophoresis chamber is clean and dry, tape the sides (with Autoclave tape, NOT standard masking tape) to make watertight. Slot in the desired comb.

• Add gel solution to the chamber, and wait to set. The comb can then be removed from the chamber.

• Fill the electrophoresis apparatus half-full with 1x TAE buffer solution avoiding air bubbles.

• The gel is loaded with the samples, a negative sample, and the DNA ladder.

• Connect the electrodes to the apparatus. Set DC voltage at 100V and run for around 60 minutes (or until DNA separates sufficiently)

• View the gel in a gel exposer.

Gel Extraction – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

We used the QIAgen quick gel extraction kit; the protocol is adapted from the QIAquick Gel Extraction Kit Protocol.

Protocol

• The gel is exposed to blue-light to illuminate the DNA fragments (stained by ethidium bromide).

• The desired DNA band is identified and physically removed with a knife, cleaned with ethanol.(ensure to wipe the ethanol off the knife to avoid degradation of DNA)

• Add 3 volumes of buffer QG to 1 volume of gel. E.g.add 300 μl of Buffer QG to each slice of gel (volume of the slice is 100 μl, and weighs approximately 100 mg).

• Incubate at 52°C for 10 min (or until fully dissolved). To aid solvation, mix by vortexing the tube every 2–3 min during the incubation. When the gel is fully dissolved, ensure that the color of the mixture is yellow, same as that of the QG buffer.

• Add 1 gel volume of isopropanol to the sample and mix.

• Place a QIAquick spin column in a provided 2 ml collection tube.

• Apply each sample of DNA fragment to a QIAquick column, and centrifuge for 1 min at 13000 rpm.

• Discard flow-through and place QIAquick column back in the same collection tube.

• To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

• Spin the column again to get rid of any residual ethanol.

• Add 30 ml of nuclease free water at the centre of the column and keep for 1 minute.

• Place the column in a collection tube and spin for 1 minute at 13000 rpm. Collect the flow through.

TRANSFORMATION OF BACTERIAL CELLS – Westminster – 2012

http://2012.igem.org/Team:Westminster/Protocols

Preparation of Antiobiotic Resistant Plates

• 6.2 g of LB agar is dissolved in distilled water and dissolved.

• This is autoclaved and sealed for future uses.

• Agar is melted in microwave for 5 minutes, or until dissolves.

• Respective volume of required antibiotic is added to the solution after it has started cooling down.

• 20-20 ml of this is evenly poured into plates and allowed to cool.

Transformation

• DHα5 competent cells are taken out from -80°c and thawed in ice for 5 minutes.

• 1 ml of SOC is incubated at 37°C.

• 1 µl of required plasmid/part is added to the competent cells and subjected to heat shock at 42°c, in a water bath for 1 minute to enable transformation.

• Placed back in ice for 5 minutes.

• 250 µl of preheated SOC is added to this and placed in a shaker at 37°C, 200 rpm for 16 hours.

• The broth from the shaker is poured on to prepared plates with corresponding antibiotic resistance, under sterile conditions.

• 8 glass beads are added to the plates and slowly shaken to ensure even spreading.

• The glass beads are recycled.

• The plates are incubated overnight at 37°C.

E.coli Cell Culture – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

• This is the method for overnight preparation of a 10ml cell culture of antibiotic-resistant E. coli. The plasmid incorporated in the bacteria ensures that it is resistant to a corresponding antibiotic, thus the growth media will have to be provided with a suitable volume of the antibiotic.

• The plasmids we used in our lab were mostly resistant to chloramphenicol, Ampicillin, and Kanamycin.

• The media was inoculated(with a sterile loop) with a single colony of the tarnsformant, under sterile conditions and placed in an incubator for 16 hours, at 37°c.

• Before further extraction, a glycerol stock of the culture was made using 1ml of media and an appropriate volume of glycerol and stored at -80°c for viability.

Extraction of DNA – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

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We used QIAprep Spin Mini Prep Kit, the protocol is adapted from the QIAprep Mini Prep Handbook QIAprep Mini Prep Handbook.

• Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

• Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

• Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.

• Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.

• Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

• Centrifuge for 30–60 s. Discard the flow-through.

• Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity.

• Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60s.

• Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer. Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

• Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 30 μl double distilled nuclease free water to the centre of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

MAMMALIAN CELL CULTURE – Westminster – 2012

http://2012.igem.org/Team:Westminster/Protocols

Thawing

• MG63 cells were derived from banked stocks at the ATCC (American type culture collection). The epithelial breast cancer cell line MCF7 used was a gift from Dr Miriam Dwek (University of Westminster)

• Vials were quickly defrosted in a 37 ˚C water bath.

• Re-suspended in 5 ml of complete media.

• Centrifuged at 1000 rpm for 3 mins.

• Supernatant was poured off and cells were re-suspended in 10 ml complete media and seeded in a 75 cm2 tissue culture flask.

Passaging Cells – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

Upon reaching the required confluency (70-90 %) cells were passaged as follows:

- Media discarded using a sterile pipette
- Washed once with Dulbecco's phosphate buffered saline (DPBS) free from calcium and magnesium
- To detach cells from flask 2 ml of EDTA-trypsin was added and incubated at 37 ˚C for 2 minutes
- Upon cell detachment EDTA-trypsin was neutralised with 4 ml of complete media
- Centrifuged at 1000 rpm for 3 mins.
- Supernatant was discarded and cells re-suspended in complete media and seeded in to a fresh flask. Depending upon the cell line cells were passaged either at 1:3 or 1:6.

Freezing – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

- Cells trypsinised (as in passaging procedure above).
- Centrifuged at 1000 rpm for 3 mins.
- Supernatant poured off and cells re-suspended in FBS + 10 % DMSO and kept at -80 °C for 24 hours then transferred to liquid nitrogen.

Transfection of Cells (PEI Procedure) – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

All cell culture and transfection was carried in complete media Dulbecco's modified eagles media (DMEM) supplemented with 10 % foetal bovine serum (FBS). No antibiotics were used

- One day prior to transfection 250000 MCF7 cells were seeded per well of a 24 well plate.
- For each transfection reagents were prepared as follows:

a) Plasmid mixed with PEI.

b) 100 μl (10% of growth media volume) 0.15M NaCl was added to plasmid-PEI mixture

c) Transfection reagent mixture was vortexed and incubated at room temperature for 10 mins.

- Prior to transfection media from each well was replaced and PEI-plasmid-NaCl mixture was added dropwise to cells.
- Cells were incubated overnight at 37°C.
- Media replaced
- Cells allowed to recover for 48hrs then trypsinised and anlaysed as follows:

a) Media removed

b) Cells washed once with PBS

c) Trypsinised with 0.21 mM trypsin containing 4.81 mM EDTA

d) Trypsin was neutralized with complete media and cells centrifuged at 2000 rpm for 3 mins

e) Cells washed once in ice cold PBS containing 1% foetal bovine serum (FBS).

f) Cells re-suspended in 1% FBS containing 1μg/ml propidium iodide.

g) Cells analysed using CyAn™ ADP flow cytometer (DakoCytomation)

In order to distinguish between alive and dead cells propidum iodide was used and data was analysed using the summit v4.3 software. Cell lines were gated according

to an unstained sample and lasers were adjusted accordingly. Unstained cells were used to set gates for cell size and internal complexity, dead cells were removed along with doublets.

Aldehyde Dehydrogenase (ALDH) Staining – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

● Upon reaching a suitable confluency (70 - 90 %) cells were trypsinised and then neutralised using complete media (as in passaging procedure).

● Counted using a haemocytometer.

● Centrifuged at 1000 rpm for 3 mins.

● Resuspended in 0.5 ml of ALDEFLUOR buffer.

● An appropriate volume of cells is transferred to a separate eppendorf so each sample contains 5 x 105 cells (diluted in ALDEFLUOR buffer).

● 2.5 μl of the ALDH activated reagent was mixed with sample.

● 250 μl of sample was immediately removed and mixed in a separate eppendorf with 2.5 μl of the ALDH inhibitor diethylaminobenzaldehyde (DEAB) to act as a negative control.

● Samples were then incubated at 37 °C for 50 mins.

● Centrifuged at 2000 rpm for 5 mins and re-suspended in 0.5 ml ALDH buffer and analysed using a flow cytometer.

MICROSCOPY – Westminster – 2012 http://2012.igem.org/Team:Westminster/Protocols

● A 10X PBS dilution is made from PBS and MilliQ water.

● The coverslips are transferred to a 24-well plate (2 coverslip/well).

● The coverslips are washed by adding 1 ml diluted PBS to the wells. The PBS is discarded. This is done twice.

● 400 µl Formaldehyde is added to each well under the fume hood, and incubated for 12 min at RT. The liquid is transferred to the waste bin.

● The coverslips are washed 3 times with the diluted PBS.

● The coverslips are dipped in MilliQ water before laid on lint-free paper for drying.

● 4 drops each of 4 µl Vectashield is placed on a glass slide.

● When a coverslip is completely dry, it is placed on a drop of Vectashield on the glass slide. The procedure is repeated for each coverslip.

● The coverslips are fixated with transparent nail polish.

Transformation protocols

Heat Shock Protocol for bacteria transformation– Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

1. Take competent E.coli cells from –80℃ freezer.
2. Turn on water bath to 42℃.
3. Put 100 ul of competent cells in an Eppendorf tube.
4. Keep tubes on ice.
5. Add 50 ng of circular DNA into E.coli cells. Incubate on ice for 10 minutes to thaw competent cells.
6. Put tube(s) with DNA and E.coli into water bath at 42℃ for 45 seconds.
7. Put tubes back on ice for 2 minutes to reduce damage to the E.coli cells.
8. Add 1 ml of SOC Broth (with no antibiotic added). Incubate tubes for 1 hour at 37℃.

9. Spread about 100 ul of the resulting culture on LB plates (with Ampicillin added). Grow overnight.
10. Pick colonies about 12-16 hours later.

Yeast transformation – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

11. Prepare a 2 ml preculture in the selection medium of the strain to be transformed.
12. Inoculate 20 ml of YPD2% for each transformation, in order to get an OD600 = 1 next day.

$$\text{Vol. Inóculo} = \frac{OD\ final}{OD\ precultivo}\ \text{X}\ \frac{Vol.final}{2^n}$$

Where n is the number of divisions (generation time: 1´5 h for S. cerevisiae).

From now, it is not necessary to work below sterility conditions. Once reached the OD600 = 1:

13. Centrifuge at 3000 rpm 5 min.
14. Wash with sterile water.
15. Resuspend in 30 ml of LISORB.
16. Shake at ambient temperature for 30 min.
17. Centrifuge at 3000 rpm 5 min and resuspend in 1 ml of LISORB. Transfer to an eppendorf tube.
18. Centrifuge at 3000 rpm 5 min.
19. Resuspend in 100 µl of LISORB for each transformation. Transfer 100 µL aliquots in different tubes for each transformation.
20. Add 7 µl of salmon sperm DNA + 1 µl of transforming DNA.
21. Incubate 10 min at ambient temperature.
22. Add 260 µl of 40%PEG/LiAc/TE. Mix well.

23. Incubate 1 h at 30℃.
24. Add 43 µl of DMSO and give a thermal shock of 5 minutes at 42℃.
25. Centrifuge at 3000 rpm 5 min.
26. Wash with 1 ml of sterile water.
27. Centrifuge at 3000 rpm 5 min.
28. Resuspend in 0´5 ml of water and plaque:

-50 µl

-Rest (centrifuge and decant leaving 50-100 µl).

DNA extraction and purification protocols

Mini-prep – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

29. Different cultures (each one with a different construction), which are growing in a selective media (LB + Ampicillin), get centrifuged at 4500g 5 min.
30. Supernatant is removed.
31. The cells can be washed (x2) with a saline solution (PBS) in order to remove impurities.
32. The pellet is resuspended in 250 µL of Resuspension Solution (RNase A added to it previously. This solution is kept at 4ºC). Important: resuspend it completely.
33. Transfer the suspension to an eppendorf tube.
34. Add 250 µL of Lysis Solution.
35. Mix it inverting the tube 4-6 times **(DO NOT VORTEX!)** until solution gets viscous and slightly clear.

Important: Do not incubate more than 5 min.

36. Add 350 µL of Neutralization Solution.
37. Mix it inverting the tube 4-6 times. Incubate in ice for 15-30 min.

Now if it was necessary, the process could stop here keeping the eppendorf tube in ice.

38. Centrifuge 10’ (max. rpm) in order to pellet cell debris and chromosomal DNA.
39. Transfer the supernatant (≈ 800 μL) to the spin column (pipetting to avoid carrying impurities).

Important: DO NOT TRANSFER THE PRECIPITATE!

40. Centrifuge 1’.
41. Flow-though liquid is removed.
42. Add 500 μL of Wash Solution (Solution stock has to be perfectly closed, it contains ethanol!).
43. Centrifuge ≈ 1’.
44. Flow-though liquid is removed.
45. 14, 15, 16 steps are repeated.
46. Centrifuge 1’ in order to eliminate residual Wash Solution.
47. The spin column is transferred into an eppendorf tube (the collection tube is eliminated).
48. Add 50 μL of Elution Buffer to the center of spin column membrane and let it 5’ getting soaked (it increases the efficiency of process).

Important: DO NOT CONTACT THE COLUMN MEMBRANE WITH THE PIPETTE TIP!

49. Centrifuge ≈ 2’.
50. To increase the efficiency (≈ 20%) we can get the flow-though liquid and repeat the steps previously described (20 and 21).
51. The column is discarded and the solution which contains the purified plasmid can be stored in cold.

Protocol for Gel Extraction – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

52. Cut bands of interest from the agarose gel.
53. Add 300 uL of Solution L1 for each 100 mg of gel.

54. Incubate at 50ºC for 15 minutes.
55. Centrifugate in a 2 ml column at 12000xg for 1 minute.
56. Re-insert the spin column into the resaver tube and add 500 uL of Buffer L2.
57. Centrifugate 12000xg for 1 minute.
58. Discard the flow-through.
59. Centrifuge 12000xg for 1 minute.
60. Place the spin column into a new 1.5 mL microfuge tube.
61. Add 50 uL of mQ water.
62. Centrifuge 12000xg for 2 minutes.

DNA digestion and ligation protocols – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

Digestion Protocol For Plasmid Backbone Using EcoRI and PstI – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

DNA linearized plasmid Backbone (25 ng/uL)	8 uL
PstI	1 uL
EcoRI	1 uL
Buffer 10x must be a common buffer for EcoRI and PstI (e.g. buffer H in Roche system)	2.5 uL
mQ Water	x uL
TOTAL	25 uL

Mix by pipetting when both enzymes have been added. Avoid vortexing. Enzymes are kept in cooler or ice throughout all experiments.

1. The digestion mixture is kept for 3 hours at 37 ° C
2. The mixture is kept for 20 minutes at 80ºC

Digestion Protocol For Plasmid pUC57 + Construction Using EcoRI and PstI – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

Plasmid DNA	96 uL
PstI	2 uL + 2 uL
EcoRI	2 uL + 2 uL
Buffer 10x must be a common buffer for EcoRI and PstI (e.g. buffer H in Roche system)	12 uL
mQ Water	4 uL
TOTAL	120 uL

The volume used is so high because after digestion we were going to purify the different inserts from an agarose gel.
In order to optimize the digestion reaction we follow these steps

3. 2 uL of EcoRI is added and incubated for 1 hour.
4. 2 uL of EcoRI is added and incubated for 1 hour.
5. 2 uL of PstI is added and incubated for 1 hour.
6. 2 uL of PstI is added and incubated for 1 hour.

Ligation – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

Plasmid DNA*	X uL
Insert DNA*	Y uL
10X ligase buffer	1 uL
T4 ligase	1 uL
mQ Water	8-(X+Y) uL
TOTAL	10 uL

OBSERVATION: The ratio that has to exist between the number

of molecules of plasmid DNA and insert is 1:3 (the volumes depends on the concentration of DNAp and the insert).

Colony PCR – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

63. Each colony is taken from the petri dish and
    a. resuspended in 15 µl of mQ water in a eppendorf if we are working with bacteria.
    b. resuspended in 15 µl of NaOH 20 mM in a eppendorf if we are working with yeast.
64. Incubate for 15 minutes at room temperature.
65. The PCR mix is prepared as shown:

    2 ul of yeast DNA solution
    5 ul of 10X PCR buffer
    4 ul of dNTPs 2.5 mM
    2 ul of A oligo
    2 ul of B oligo
    31 ul of water

66. Once mixed, 4 ul of 10X TAQ polymerase solution is added.
67. The PCR reaction program is the next one:

    94ºC 3 minutes
    30 cycles of:
        94ºC 1 minutes
        45ºC 1 minutes 30 seconds
        72ºC 2 minutes
    72ºC 10 minutes
    4ºC Hold

Biobricks protocols

68. Digestion Protocol For Plasmid Backbone Using EcoRI and PstI
69. Digestion Protocol For Plasmid pUC57 + Construction Using EcoRI and PstI

70. Transformation Protocol Using Heat Shock
71. Ligation Protocol
72. Mini-preps. Purification protocol.
73. Protocol for Gel Extraction

Media and solutions protocols – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

LB broth for bacteria

Bacterial peptone	1% (p/v)
Yeast extract	0.5% (p/v)
NaCl	1% (p/v)

In order to obtain solid LB, add 15 g/L of bacteriologic agar.

LBA broth for bacteria

Add 1 mL of ampicilin 100mg/mL (of mQ water) in 1L of LB Broth

LB + Chloramphenicol broth for bacteria

Add 1 mL of Chloramphenicol 34mg/mL (of pure ethanol) in 1L of LB Broth

SOC broth for bacteria

Bacterial triptone	4g
Yeast extract	1g
NaCl 5M	0.4 mL
KCl 3M	0.167 mL
MgSO4	2.465 g
MgCl2	2.033 mL
Glucose	3.603 g
Distiled water	220 mL

Sterilization by filtration

YP broth for yeast

Bacterial peptone	2% (p/v)
Yeast extract	1% (p/v)

In order to obtain solid YP, add 2% of agar before sterilization.

YPD broth for yeast

Add x% (p/v) of dextrose or glucose (sterilized by filtration) in YP Broth after sterilization.

YPRE broth for yeast

Add 2% (p/v) of raffinose and 2% (v/v) of ethanol 100% in YP Broth after sterilization.

SD broth for yeast

Yeast nitrogen base w/o aminoacids and w/ amonium persulfate	0.67% (p/v)
Glucose	2% (p/v)

Aminoacids (leucine, metionine, histidine) and **nucleotides** (uracil) are added after sterilization in a final concentration of 20 mg/mL.
In order to obtain solid SD, add 2% (p/v) of agar before sterilization.

LISORB solution for yeast transformation

For 100 mL

LiAc 1M	10 mL
Sorbitol 2.4M	41.6 mL
TE 100X	1 mL

Bring to 100 ml of distilled H2O

40%PEG/LiAc/TE solution for yeast transformation

For 20 mL

LiAc 1M	2 mL
PEG 3500 50%	16 mL
TE 100X	0.2 mL

Bring to 20 ml of distilled H_2O

Yeast Induction protocol – Valencia Biocampus – 2012 http://2012.igem.org/Team:Valencia_Biocampus/Protocols

2. Colonies are picked from petri dish and suspended in suplemented SD media.
3. Incubate overnight
4. Resuspend in YPD8%
5. Incubate overnight to 5 OD.
6. Resuspend in YPRE
7. Incubate for 8 to 24 hours
8. Add a final concentration of 0.3 mM of H_2O_2
9. Measure OD and fluorescence intensity.

1. COMPETENT CELLS – UNITN Trento – 2012
http://2012.igem.org/Team:UNITN-Trento/Protocols

- Transformation Buffer: sterile 10 mM Tris-HCl, pH 7.0, 50 mM $CaCl_2$
- Grow a 50 mL culture in LB at 37 deg C from 1 colony.
- When OD ~ 0.5, collect the cells in a sterile Falcon tube and chill on ice for 10 min.
- Centrifuge at 5000 rpm for 10 min at 4 deg C. Discard supernatant.
- Resuspend cells in 15 mL of transformation buffer.
- Chill on ice for 15 min. Spin at 5000 rpm for 10 min at 4 deg C. Discard supernatant.
- Resuspend cells in 4 mL of transformation buffer.

The cells are now ready to be transformed. They can be stored in this state at 4 deg C for under a week.

Alternatively, the competent cells can be aliquoted (200µL), adding glycerol to a final conc of 15% (v/v), and the cells stored at –80 deg C.

Every time you make new competent cells you should check for possible contaminations. Plate an aliquot of the new cells in LB plates + antibiotic (i.e. ampicillin, chloramphenicol,

kanamycin). Strains such as DH5a, NEB10b, Novablue, should not grow in the presence of antibiotics.

2. TRANSFORMATION – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

- Thaw home made $CaCl_2$ competent cells on ice.
- Add 1µL of DNA to 200µL of competent cells (concentration of DNA stock should be between 50–150 ng/µL).
- Incubate on ice for 30 min.
- Heat shock at 42 deg C for exactly 2 min.
- Incubate on ice 1 min.
- Add 800µL of LB (or SOC) and shake at 37 deg C for 1 h.
- Plate the cells (use plates with the appropriate antibiotic according to your plasmid).

  You can either plate a small amount (200µL) of the cells or more.

  You should try a few conditions the first time and then choose the one that gives 30–300 separate colonies.

  If few cells are expected: spin down the cells at 2500 rpm, discard supernatant & resuspend in 150–200µL of LB and plate all the cells.

  For ligation you should increase the amount of DNA to be transformed (see cloning protocol).

- Incubate the plates O/N upside down at 37 deg C.

3. PCR – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

We have used a number of different Polymerases for amplification of our parts.

In each case we have followed the protocol suggested by the manufacturer.

Here is a list of the Polymerases that we have used.

New England Biolabs:
- Phusion
- One Taq

Kapa Biosystems:
- Kapa Hifi Polymerase

RBC:
- Find link

4. CLONING – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

Most of our parts were cloned starting from a PCR amplified product, purified and digested, which was subsequently inserted into the destination vector.

Alternatively, we used a 2A assembly strategy, where the source parts and the destination plasmid where digested separately and subsequently purified before the ligation step.

You can find here our working sheet that we used in the lab. We hope you find it helpful!

STEP 1: DIGESTION

For PCR products :

- ~ 3–4 µg of PCR product
- 2.5µL of restriction enzyme 1
- 2.5µL of restriction enzyme 2
- 10µL of buffer (i.e. NEB 4)
- 1µL of BSA 10X
- xµL of H_2O to reach 100µL

Incubate at 37C o/n. The day after add 1µL of DpnI at 37 deg C for 2 hours.
Please note that PCR product must be purified before digestion.

For plasmids:

- ~2–3 µg of vector
- 1µL of BSA
- 1.5µL of enzyme 1
- 1.5µL of enzyme 2
- 5µL buffer
- xµL of H_2O up to 50µL

The day after add 1µL of phosphatase (CIP or SAP) to the vector and incubate for 2 hours at 37 deg C.

STEP 2: PURIFICATION

Purify the digested PCR product and digested vector with a kit.

Follow the kit's protocol.

Subsequently, check the concentration by UV/VIS or electrophoresis for quantification.

STEP 3: LIGATION

Find here our ligation calculator!

Prepare your reaction and incubate at RT for 2 hours. Transform half of the reaction into 200µL of "homemade" competent cells (DH5a, NEB10b, Novablue or other appropriate strains) following a standard transformation protocol. Plate all the cells.

STEP 4: SCREENING

If your reaction worked the control plate should have none or few colonies. If you are working with psB1C3 or other RFP containing plasmid, you can screen colonies by looking at the color.

RED: no success.
WHITE: ready to screen.

Grow 6 separate colonies, each in 10 mL of LB + antibiotic overnight at 37°C.
Miniprep and quantify.

Digestion:

- DNA ~1.0 µg
- 10X buffer 2
- enzyme 1 1µL
- enzyme 2 1µL
- 10X BSA 1
- H2O up to 20µL

Incubate for 1.5 h at 37 deg C.

Run all the digested product on an agarose gel to screen colonies.

Correct constructs should have 2 bands, one corresponding to the vector and one corresponding to the insert. Send now for sequencing.

5. GIBSON ASSEMBLY – UNITN Trento – 2012
http://2012.igem.org/Team:UNITN-Trento/Protocols

We have successfully used this method to build 2 of our favorite parts (xx and XX). We did not encounter any particular difficulties.

STEP 1: PRIMER DESIGN

To this purpose you may find useful the GIBTHON tool designed by Cambridge iGEM team 2010. XX

STEP 2: PCR

If you used the Phusion method with HF buffer, to PCR out your gene, ther ei sno need of purification.

Quantify each product preferably by gel.

STEP 3: ASSEMBLY

We used the NEB Gibson kit (E2611).

- Master Mix 10µL
- Fragment 1 xµL
- Fragment 2 xµL
- H2O up to 20µL

We optimized the quantities of DNA to be used:
Big Fragments between 50–100ng.
Small Fragment s 2–3 fold excess.

If needed liophilize and resuspend to reach the right concentration.

Mix together on ice the different fragments, add the mastermix and incubate at 50C for 1 hour.

Transform 10µL of the product into competent cells (i.e. DH5a or Novablue).

STEP 4: SCREENING

Proceed as indicated in STEP 4 of the cloning protocol.

6. MUTAGENESIS (BACK TO BACK PCR) – UNITN Trento – 2012
http://2012.igem.org/Team:UNITN-Trento/Protocols

STEP 1: PRIMER DESIGN

Primers are designed back to back. The mutations should be placed in the middle of the forward primer.

For nucleotide change:

ATCGCCTCCTGCAGCGGGGGC*GGCCTGGAGACG**GGC**AACAGCGAGGAAG*

Mutation is shown in bold.
Primer forward will be the one in *italic*. Primer reverse will be the reverse complement of the underlined sequence.

For deletions:

ATCGCCTCCTGCAGCGGGG**GCGGCCTGGAGACG***GGCAACAGCGAGGAAGGCTGG*

Part that you want to delete is in **bold**.
Primer 1 will be the one in *italic* in the forward direction.
Primer 2 will be the reverse complement of the underlined sequence.

For insertions:

ATCGCCTCCTGCAGCGGGG*__GCGGCCTGGAGACG__GGCAACAGCGAGGAAGGCTGG*

Part to be inserted is shown in **bold**.
Primer forward will be the one in *italic*.
Primer reverse will be the reverse complement of the sequence underlined.

*STEP 2: PRIMER PHOSPHORYLATION – **UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols***

Make primer stocks of 100µM. Set reaction with:

- 10x PNK buffer 5µL
- 10 mM ATP 5µL
- 100µM oligo 5µL
- PNK 2µL
- H2O 33µL

Leave at 37 deg C for 45 min, up to 1 hr.
Deactivate PNK, by incubation for 20 min at 65 deg C

STEP 3: PCR – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

- Oligo 1, phosphorylated (10µM) 2.5µL
- Oligo 2, phosphorylated (10µM) 2.5µL
- dNTP (10µM) 1µL
- DNA template (50 pg- 1 ng) 1µL
- DMSO (optional) 1.5µL
- H_2O 31.5µL
- Phusion Polymerase 0.5µL

Phusion polymerase comes with 2 different buffers. Use as a default the HF buffer. If you have problems, try the GC buffer.

PCR Parameters:

1. 30 sec @ 98 deg C
2. 10 sec @ 98 deg C
3. 30 sec @72 deg C
4. 15–30 sec per kb of template @72 deg C Repeat cycles 2–4 for a total of 25 times
5. 10 min @72 deg C
6. keep at 4 deg C

We optimized the concentration of DNA template to be used to 1 ng. *Optional:* Check to see if the reaction worked by running 10µL of the reaction on an agarose gel.

STEP 4: LIGATION ***– UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols***

- PCR product 3- 5µL
- 10x Ligase Buffer 2µL
- Ligase 1µL
- H_2O up to 20µL

Incubate at room temp for 1 hour.
Transform half of the ligation product into an aliquot of competent cells. Proceed with standard transformation protocol.

STEP5: SCREENING – ***UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols***

Unfortunately for mutagenesis there is no good way to screen your correct colonies, unless you are making a big insertion/deletion.

Grow at least 6 different colonies in 10 mL LB + appropriate antibiotic O/N at 37C.

Miniprep and send for sequencing.

7. MOPS MEDIA – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

Here is our MOPS calculator!

8. CELLS GROWTH – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

- Grow one colony in 5ml of LB+antibiotic O/N at 37°C.
- In the morning make a 1:100 dilution in 30 mL of fresh LB+ antibiotic and grow the cells at 37C until you reach an 0.5 OD.
- Once you have reached an OD of 0.5 split the culture in two parts and spin down the cells for 10min at 4100rpm.
- Discard the supernatant and resuspend the each of the 2 cultures in 15ml of MOPS. One culture will be your control, the other will be the culture to be induced.
- Grow the two cultures in the thermoshaker at 37°C until the OD is 0.7.
- Induce one of the 2 cultures with 5mM arabinose or 0.1 mM IPTG (or other desired concentration).
- Take an aliquot to measure optical density every hour for 8 hours.

9. TOXICITY TEST BY SERIAL DILUTION – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

- Follow the same protocol as for the growth test.
- After induction leave the cells at 37C for 8 hours (or desired amount of time).

- After 8 hours take an aliquot (100µL) from the uninduced culture (sample A) and add it to 9.9 mL of fresh LB. Repeat the same procedure for the induced culture (sample B). These two samples will be used to make serial dilutions.

  Sample A: Prepare 3 tubes with 4.5 mL of fresh LB and label each tube. Add 500µL of cells to 4.5 mL of LB and vortex. Subsequently, remove a 500µL aliquot from this newly made dilution and add it to a fresh tube of 4.5 mL LB. Repeat the process until a 10^5 fold dilution is reached. Plate 150µL of each sample on LB + antibiotic and incubate at 37C O/N.

  Sample B: Repeat the same procedure used for sample A.

  The day after, you can compare the number of colonies in each plate for the two different samples.
  To estimate the number of cells calculate the CFU/mL using this formula:

  CFU/ml = (# colonies counted)*(dilution factor)/(mL of culture plated)

10. FLUORESCENCE MEASUREMENTS TO TEST PROTEIN EXPRESSION – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

- Follow the same protocol used for growth characterization.
- After induction leave the cells at 37C for 4 hours (or desired amount of time).
- Take a 1.5 mL aliquot of the uninduced and of the induced culture every hour to measure fluorescence intensity.
- Spin down the sample at 4000 RPM for 10 min. Discard the supernatant and resuspend in 1.5 mL of PBS. Place the sample in a cuvette and take a fluorescence spectrum using proper excitation wavelength (i.e. GFP 485 nm)
- Leave the sample at 4C O/N to allow proper folding of GFP. The day after measure again the fluorescence.

11. NINHYDRIN ASSAY – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

To test for the presence of cysteine we used a modified version of the Gaitonde test (Biochem. J., 1967, 104, 627).

- Prepare a Ninhydrin stock solution by dissolving 250mg of ninhydrin and 10ml of a solution composed by glacial acetic acid (60%) and HCl (40%). Vortex the reagent until complete solubilization. The stock solution should be made fresh each time.

- Mix 500µl of ninhydrin reagent 500µl glacial acetic acid and 500µL of the culture sample to be tested.
- Leave the solution in a 90°C bath for 10 minutes and wait for color development.
- Measure absorbance with a UV/VIS spectrophotometer between 600nm and 400nm. Maximum absorbance peak for cysteine is at 560 nm.

A standard curve for quantification is built with cysteine in a concentration range between 0 and 0.5 mM.

12. METHYLEN BLUE ASSAY FOR H2S DEVELOPMENT – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

For this test we used the protocol used by the Keasling group in Appl. Environ. Microbiol., 2000, 4497–502.

Preparation of cell lysate:

- Spin down 5 mL of cells at 17000g for 10min
- Resuspend the cells in 660µl of a 50mM Tris-HCl solution (pH 7.5)
- Add 840µl of lysis buffer (300 mM NaCl, 90 mM EDTA, 50 mM Tris-HCl , pH 7.5)
- Sonicate the suspension 3 times for 10 seconds, waiting for 1 minute between each sonication step. Keep the cells on ice during the sonication.
- Centrifuge the suspension for 20 min at 4°C.

Cysteine desulfhydrase activity assay:

- Add 0.1mM Cysteine to the cell lysate
- Incubate the mixture for 1h at 37°C
- Prepare the following stock solutions:
- Solution A: 0.02 M M N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl
- Solution B: 0.3 M FeCl3 in 1.2 M HCl
- After the incubation period at 37C add 0.1ml of solution A and 0.1 mL of solution B to the cell lysate.
- Vortex the mixture for about 1 minute.
- Allow the color to develop: the solution turns immediately to green/blue if H2S is present.

For quantification, take absorbance measurement with a UV-VIS spectrometer between 500 and 720 nm (maximum peak is at 670 nm).

13. TSI – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

We have used TSI from Sigma (cat no.44940) containing:

- agar, 12 g/L
- ferrous sulfate, 0.2 g/L
- glucose, 1 g/L
- lactose, 10 g/L
- meat extract, 3 g/L
- mixed peptone, 20 g/L
- phenol red, 0.025 g/L
- sodium chloride, 5 g/L
- sodium thiosulfate, 0.3 g/L
- sucrose, 10 g/L
- yeast extract, 3 g/L
  final pH 7.4±0.2 (25 °C)

- Autoclave
- When the medium reaches room temperature add antibiotic and IPTG (0.1 mM) and/or arabinose (5 mM).
- Make separate slant tubes for each condition to be tested.
- Once the gel is solid keep the tubes at 4 °C.
- When you decide to use the slant, with a small tip place 5-10 ul of culture in the middle of the slant tube and incubate at 37 °C for 24 hours or more.

if H_2S is developed the media will quickly blacken.

14. COPPER PRECIPITATION – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

Calibration curve:

- Make 100mM solution of Bathocuproinedisulfonic (BCS) reagent.
- Make serial dilutions of 1mM to 10pM $CuSO_4$ in LB media.
- Add 1µL of 1M ascorbate and 1µL of BCS to 100µL of each standard copper solution.
- Measure absorbance of each copper solution at 483nm.
- Use LB media without copper as blank.

Growth of the coltures:

- Grow the bacteria in 20 mL of LB + antibiotic and with 2mM CuSO4 until reach an OD of 0.6.
- Split the cells into two new sterile tubes and induce one of the two with 0.1 mM IPTG.
- For each sample measure the OD every hour up to 5 hours.
- Transfer all the media from the cuvettes to 1mL eppendorf tubes and centrifuge at 13,200 rpms for 2 minutes. Collect the supernatants.
- Add 1µl of Bathocuproinedisulfonic to 100µl of supernatant and mix.
- Repeat this step for each supernatant you collected.
- Measure absorbance of supernatant at 470nm.
- Use LB media without copper as blank.

15. CRUSTANATOR – UNITN Trento – 2012
http://2012.igem.org/Team:UNITN-Trento/Protocols

This protocol is a modified version of the protocol used by Gomez, Smith and Viles at the University of Oxford.

Materials

- 4x plexiglass panels of desired dimensions (2 for the long sides, 1 for the bottom and 1 for the top)
- 2x plexiglass panels for the short sides
- 5 m of plastic tube of 1 cm diameter
- a glass jar with a metal cap
- 3 o-rings
- silicon
- rubber sheath
- charcoal

Reagents and glassware

- beaker
- distilled water
- 100 mL of sulfurous acid (per treatment)

You will also need to connect the chamber to a compressed air source.

Instructions to build the box

- Drill a hole in one of the panels and put an O-ring on it, this will be one of the sides. Keep one of the plexiglass panels aside and assemble the box with silicon. Make sure that the box is tightly sealed.
- Once the box is assembled apply the sticky rubber sheath where the top of the box will be placed. The chamber must be hermetic at normal atmosphere pressure but should have the possibility to create a small opening when you inject compressed air.
- Drill two holes in the metal cap of the jar, and place a O-ring in each hole. Gather some charcoal from the last barbecue, and crush it with a mortar and pestle. Pour the crushed ashes inside the jar, and screw the cap to close it.
- Finally, cut the plastic tube in two pieces depending on your needs. Connect the first from the compressed air source to the jar and the second tubef rom the jar to the chamber.

Black crust formation.

- work under fume hood and wear double gloves
- Wet marble pieces with distilled water and place them into a beaker. It is preferable to leave the marble in water for 24 hours prior the experiment.
- Fill the beaker until the marble pieces is submerged roughly by half.
- Fill another beaker with sulfurous acid, and pour some on the surface of the marble piece too.
- Place the beakers inside the camber, and cover the box with the top plexiglass panel. Place a weight on it to keep it down when you will inject air inside the chamber.
- Inject compressed air for 2-3 seconds, you should be able to see ashes entering the chamber.
- Repeat this step twice, every 72 hours.
- At the ninth day open the box and collect the marble pieces. They should be covered in black crusts. A solid layer of gypsum and ashes may also form above water surface, and that can be used for tests too. Dry the marble samples.

16. APPLICATION ON THE STATUES – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

Jelly MOPS:

- Gel is freshly prepared prior to every application.
- 6 g/L agar is added to MOPS medium and heated in the microwave until dissolved.
- Cool the gel on ice.
- Add antibiotic, arabinose and/or IPTG.
- Whipp the gel using an egg beater or similar tool to incorporate air bubbles in the gel matrix.

Bacteria preparation:

- Grow bacteria in MOPS following our standard protocol.
- After the bacteria are induced (i.e. arabinose and/or IPTG).
- After 3 hours of induction, spin down the bacteria and discard the supernatant.
- Resuspend the bacteria in freshly prepared Jelly MOPS and apply them on the statues.
- Cover the gel with rice paper pre-rinsed in PBS.
- After 12 hours remove the bacteria and gently clean the surface with a cotton swab.
- If necessary apply freshly induced bacteria again.
- After the last cycle of application, rinse the surface with warm water and gently pat it with tissue paper.

TERMINATORS PROJECT – UNITN Trento – 2012

http://2012.igem.org/Team:UNITN-Trento/Protocols

17. FLUORESCENCE MEASUREMENTS – UNITN Trento – 2012

http://2012.igem.org/Team:UNITN-Trento/Protocols

- Take a 100µl aliquot of a frozen cell glycerol stock and add it to 10ml of LB with antibiotic.
- When you reach an OD of 0.4 place the samples on ice.
- Dilute the cells of each sample to an OD of 0.2 in 10 mL of LB. Make sure that you keep all the samples on ice during this step.
- Place again the samples in the 37C thermoshaker simultaneously. From now on treat all the samples equally to guarantee less variability in the results.
- Grow until 0.6 and induce with 0.5 mM IPTG.
- After 3 hours keep the cells on ice.
- Sonicate the cells 3 times for 10 seconds, with about 30 seconds lapse in between and 50% amplitude.
- Centrifuge for 1min at 4000 RPM.
- Mix 1ml of supernatant and 1ml of PBS 1X in a cuvette.
- Leave the sample O/N in the refrigerator (4°C) to allow proper protein folding of fluorescent proteins.

The day after measures fluorescence intensities with a fluorimeter.

Parameters used are:

- Voltage: 570V for the T7promoter constructs, 520V for TAC promoter.
- Excitation wavelength for A206K Venus: 485nm.

- Excitation wavelength for mCherry: 587nm.
- Emission wavelength for A206K Venus: 528 nm.
- Emission wavelength for mCherry: 615nm.

18. ETHANOL PRECIPITATION – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

- Use between 80µl and 100µl of a DNA solution already purified with a miniprep kit (concentration >100ng/µl).
- Add 0.1 volume of 3M sodium acetate and 2.5 volumes of cold ethanol.
- Mix well.
- Incubate the sample on ice for 10 min.
- Centrifuge for 30 minutes at maximum speed.
- Decant or aspirate supernatant taking care not to dislodge the pellet.
- Wash the pellet by adding enough volume of 70–80% ethanol to at least cover the pellet.
- Dislodge the pellet by vortexing.
- Finally, centrifuge for 30 minutes at maximum speed and decant or aspirate to remove the supernatant (as in previous step).
- Dry the pellet with a speed-vac, desiccator or air dry.
- *Optional*: in the beginning you can add glycogen as a carrier, useful if you have low concentration of DNA. To do so, just add 1µL of 20mg/mL glycogen before first or second step.

Note: shorter centrifugation times may decrease yields.

19. CELL FREE MEASUREMENTS – UNITN Trento – 2012 http://2012.igem.org/Team:UNITN-Trento/Protocols

We have used PurExpress in vitro protein synthesis kit from NEB (E6800S), following the protocol suggested by the manufacturer. We have used 250ng of DNA template, previously purified by ethanol precipitation.

Note: add DNA right before starting the measurements.

You nee to use a fluorimeter that is set up for kinetics measurements (i.e. PTI Quantamaster 40) and can acquire data using multiple excitation wavelengths simultaneously.

The parameters to take measures are:

	Excitation	Emission
A206K Venus	485nm	528nm
mCherry	587nm	615nm

Take a measurement every minute for 360 times. At the end of the measurement you can take also excitation and emission spectra of each protein using the same parameters.

Isolation of Plasmid DNA from E.coli _(miniprep)_ – TU Munich – 2012
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Plasmid DNA from E. coli was isolated from overnight cultures using the DNA extraction mini-prep kit (Qiagen). The principle of this method is alkaline lysis of bacterial cells followed by a selective immobilization of the plasmid DNA on a column, subsequent washing steps to remove impurities and the elution of plasmid DNA.

Isolation of Genomic DNA from S.cerevisiae – TU Munich – 2012
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Extraction of genomic DNA from *S.cerevisiae* was done using a protocol by Lõoke et. al (2011). This method is based on yeast cell lysis with a LiOAc-SDS-solution followed by DNA precipitation with ethanol. For DNA isolation, 100 µl of a stationary overnight yeast culture was pelleted and resuspended in 100 µl of 200 mM LiOAc 1 % SDS. Cells were incubated for 5 minutes at 70 °C. DNA was precipitated by adding 300 µl of 96 % ethanol and subsequent vortexing. After centrifugation for 3 minutes at 13400 rpm and discarding the supernatant, the DNA was washed with 500 µl of 70 % Ethanol an centrifuged once more. The supernatant was discarded and DNA was resuspended in 100 µl 1x TE buffer. Remaining cell debris was centrifuged down and the supernatant was transferred into a new tube. Another precipitation step with isopropanol was done to purify the genomic DNA for following PCR reactions.

Determination of DNA Concentration – TU Munich – 2012
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

DNA concentration was measured using a NanoDrop Spectrophotometer by Thermo Scientific. The concentration was calculated after determination of DNA specific absorbance at 260 nm. Furthermore, the ratio of sample absorbance at 260 and 280 nm as well as at 260 and 230 nm were measured to specify the purity of the samples. A ratio of 260/280 of ~1.8 is generally accepted as “pure” for DNA. If the ratio is appreciably lower, it may indicate contamination with proteins. The 260/230 nm ratio indicates contamination with thiocyanates and phenolate ions since these absorb at 230 nm. The value is expected to be in the range of 1.8-2.2 in case DNA is relatively pure.

Agarose Gel-Electrophoresis – TU Munich – 2012
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Agarose gel-electrophoresis was used to separate double-stranded DNA fragments by length. Ethidium bromide was applied as a nucleic acid stain (Sambrook et al., 1989).

This method was used for the restriction analysis of plasmids (analytical gel-electrophoresis) as well as for the isolation of DNA fragments (preparative gel-electrophoresis). After preparative gel-electrophoresis, the bands were cut out and purified using a Qiagen Gel extraction kit.

Polymerase Chain Reaction (PCR) – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Polymerase chain reaction (PCR) was used for the selective amplification of desired DNA fragments (for example from a plasmid). Primers were designed for the desired target sequences. The PCR reaction was divided in to three steps which were repeated up to 30 times. Firstly, the DNA template strand was heat-denatured at 95 °C to produce single-stranded DNA. Secondly, the temperature of the reaction batch was lowered to 55 – 60 °C to allow the primers to bind. Thirdly, the temperature was raised to 72 °C. This enabled the DNA polymerase to synthesize the other DNA strand. Special PCR methods that were used include colony and genomic PCR.

Colony PCR

A method to allow for higher throughput of clone screenings. Colonies were picked with a sterile toothpick or pipet tip. Some of the cells were smeared onto the wall of the PCR tube. Subsequently the toothpick was put into a cell culture tube with LB-medium and suitable antibiotic. Colony PCR was performed using OneTaq Hot Start DNA Polymerase (Qiagen) following this temperature scheme:

Initial denaturation	94 °C	10 min
30 cycles	95 °C	30 s
	59 °C	30 s
	68 °C	1 min 55 sec
Final extension	68 °C	5 min
Hold	4 °C	

Purification of PCR products

PCR products were purified using the PCR purification kit by Qiagen.

Dephosphorylation of DNA – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Dephosphorylation of digested PCR products via Shrimp Alkaline Phosphatase (Fermaentas) was done to avoid religation of the insert and enhance ligation rate. Before dephosphorylation was performed, digest solution of restriction enzymes and buffer were purified with PCR Purification Kit (Qiagen). Afterwards 1 µg sample DNA was mixed with 10 % of 10x SAP Buffer and 1 unit SAP. The mixture was incubated at 37 °C for 30 min. Inactivation occurred at 65 °C for 15 min.

***DNA Restriction Enzyme Digest* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

For the preparation of DNA fragments and the restriction analysis of plasmid DNA, DNA was cut using restriction endonucleases. Buffers and DNA concentrations were used according to the manufacturer's suggestions.

***Ligation / Cycled Ligation* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

After digestion with an restriction enzyme, plasmid fragments were inserted into vectors (which were cut with matching restriction enzymes) by ligation. The enzyme T4 ligase connected complementary overhangs of fragments by catalyzing the formation of the bond between the 5'phosphoryl group and the 3' hydroxyl group.

***Oligohybridization of Single-Stranded DNA* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Oligohybridization of oligo-nucleotides was used to create a mini MCS for cloning RFC10 compatible parts between promoter and terminator. This was achieved by using complementary oligo-nucleotides that contained the desired sequence with specific overhangs for cloning. For oligohybridization, 25 ml of 100 mM of forward and reverse oligos were put together in one tube and heated to 90 °C for 5 min. The samples were slowly cooled to room temperature in a styrofoam box overnight.

***Site-Directed Mutagenesis* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Site-Directed Mutagenesis was used to mutate specific bases of DNA sequences. Therefore, specific primers, which bind at the same site and contain a mismatch at the specific base, were required. The original base pair that had to be replaced was replaced by the mismatch. The method works just as PCR by amplifying the desired product that contains the mismatch. Afterwards, the product was digested with the restriction enzyme DpnI to destroy the plasmids strands which do not contain the desired base pair exchange. The QuikChange Site-Directed Mutagenesis Kit by Agilent Technologies was used.

***Genome Integration* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Genome integration of DNA constructs was done using an existing biobrick as an integration vector (BBa K300001 (Pavia '10)). Prior to transformation of yeast cells, the integration vector was linearized with the restriction enzyme Sbf1 (because the frequency of integration is much higher for linearized plasmids). After linearization and preparative gel extraction of the vector, it was transformed into yeast cells using the S. c. EasyComp™ Transformation Kit by Invitrogen *S. cerevisiae* and G418 antibiotic plates.

***Sequencing of Plasmid DNA* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

DNA constructs were sequenced by Eurofins mwg operon using our own sequencing primers.

***Gene Synthesis* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Some of our genetic constructs were synthesized by GeneArt and iDT. For all our syntheses, the GeneArt® GeneOptimizer® was used for codon optimization in *S.cerevisiae*.

***Protein Expression in* S. cerevisiae – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Expression was induced by transferring overnight cultures of the INVSc1 strain from glucose medium into a medium that contained galactose. Transcription in INVSc1 strains (GAL1 promoter) is repressed in the presence of glucose. Transcription may be induced by removing glucose and adding galactose as a carbon source. Transferring cells from glucose- to galactose-containing medium causes the GAL1 promoter to become de-repressed and allows transcription to be induced.

***Crude Protein Extraction from* S.cerevisiae – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

To detect the recombinant protein (for example by western blot) cell lysates from our yeast transformants were prepared. Cell lysates were produced using PMSF-containing breaking buffer and acid-washed glass beads to break the cell wall.

***SDS Polyacrylamide Gelelectrophoresis (SDS-PAGE)* – TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Firstly, glass plates were wiped with 70 % ethanol and then assembled onto a setting rig. No rubber spacers were added as they were already fixed to the glass plates. A 15 % resolving gel was made as follows:

- 5 ml protogel
- 2.5 ml 4 x Lower Tris (pH 8.8)
- 2,5 ml H_2O
- 50 µl ammonium persulphate (APS) (10 %)
- 2,5 µl N,N,N',N'-tetramethylethylenediamine (TEMED)

The tube was mixed thoroughly and added to the setting rig between the glass plates, covered with water and left to set for about 30 minutes. Then the water was poured off and a stacking gel was prepared as follows:

- 1 ml protogel
- 1.5 ml 4 x Upper Tris

- 3,5 ml H_2O
- 36 µl ammonium persulphate (APS) (10 %)
- 3 µl N,N,N',N'-tetramethylethylenediamine (TEMED)

All substances were mixed by inversion. 1 ml was applied on top of the already set resolving gel and a comb was put in. Once the gel was set, the comb was taken out and the wells were cleaned out with sterile water. The set gels were removed from the setting rig and placed in the running rig. 1x running buffer was poured into the rig ensuring the plates were covered. For protein preparation, 30 µg protein in 10 µl H20 and 2,5 µl 5 x Laemmli buffer were denatured for 5 minutes at 95 °C. Then 6 µl marker (unstained marker for coomassie-staining or prestained marker for Western Blot) was put into a well. The remaining wells were filled with 12,5 µl of the protein that was to be analyzed. The SDS-PAGE was performed at 120 V for about 1,5 h. For coomassie staining, the gel was incubated in coomassie staining dye for 20 minutes, then put into the first decolorizing solution for 20 minutes. Finally. the gel was put into a second decolorizing solution until the background color was gone. Another method that was used for protein staining was silver staining.

Western Blot **– TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

After SDS-PAGE, gels were transferred to a nitrocellulose membrane in transfer buffer (20 ml 5x SDS buffer, 20 ml methanol, 60 ml H_2O) at 500 mA for 1h. Membranes were washed 3x 15 min in PBS-T0.1 (PBS + 0,1 % v/v Tween 20) and subsequently blocked with 3 % BSA for one hour. For antibody detection, membranes were washed 3x 15 min in PBS-T0.1 and then incubated in detection solution containing the antibody straptavidin-AP (1:4000 in PBS-T0.1) for one hour. The Western blot was washed 2x 10 min in PBS-T0.1 and then 2x 10 min in PBS. Afterwards, the developing solution (15 ml alkaline phosphatase buffer, 45 µl BCIP (50 mg/ml in DMF) and 7,5 µl NBT (75 mg/ml in 70 % DMF) was added. After the appearance of bands, the blot was washed and stored in water.

Enzyme Assay and Extraction of Limonene Synthase **– TU Munich – 2012**
http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

We used an optimized protocol of Landmann et al, 2007 to test functionality of purified limonene synthase. The enzyme assay was carried out in a total volume of 500 µl containing buffer (25 mM Tris-Cl, pH 7.5, 5 % glycerol, 1 mM DTT) supplemented with cofactors (10 mM $MgCl_2$, 1 mg/ml BSA) with successive addition of 50 µM substrate (geranyl pyrophosphate, dissolved in DMSO) and 10 µg purified recombinant enzyme (extracted limonene synthase, after purification). The mixture was gently overlaid with 1 ml pentane and incubated at room temperature for 15 minutes. The reaction was stopped by vigorous mixing and centrifugation (5 min, 5000g) to separate phases. The solvent phase (upper phase) was attached to a pasteur pipette containing glass wool with sodium sulfate for drying the solvent phase. Afterwards, the combined extracts were reduced to approximately 300 µl under a stream of nitrogen. The pentane extracts were analyzed by gas chromatography-mass spectrometry to identify the enzymatically

synthesized products. An aliquot of each sample (0.5 µl) was injected into "5890 Series II GC" coupled to a "Finnigan Mat 55 S MS".

Headspace GC-MS of Limonene – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

Because Limonene is an secretory protein [Misawa, 2011] we expected an arbitrarily amount of Limonene outside the cells. To check this predication we detected Limonene via Headspace GC-MS in the yeast cell culture supernatant. Therefore the preparatory cell culture was induced with galactose after 24 hours (see '*Protein expression in S.cerevisiae*). For further 24 hours the protein expression proceeded. Afterwards the SPME needle was injected into the headspace and incubated for 30 min at 45 °C before injection into GC.

Cultivation of E.coli – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols

E.coli strain XL1-Blue was cultivated in LB-medium (lysogeny broth) and LB plates. for the preparation of 1 liter of LB, dissolve the following and autoclave:

- 10 g tryptone
- 5 g yeast extract
- 10 g NaCl

For making plates, add 15 g bacto-agar before autoclaving.

Cultivation of S.cerevisiae

S.cerevisiae strain INVSc1 was cultivated using the following media and plates:

SC-Uracil minimal Medium and plates for 10x 50 ml amino acid aliquots: Dissolve the following reagents in 500 ml deionized water:

- 1 g of adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan and
- 0.5 g of aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine.
- Autoclave at 15 psi, 121 °C for 20 minutes.
- Make 50 ml aliquots, store at -22 °C.

SC-U medium + Glucose (minimal medium) or Galactose (induction medium):

- Add 6.7 g Yeast Nitrogen Base and 850 ml deionized water to the 50 ml amino acid aliquot. Autoclave. Add 100 ml autoclaved/filter sterilized 20 % glucose (minimal medium) or 20% galactose (induction medium), store at room temperature.

SC-plates + Glucose:

- Add 6.7 g Yeast Nitrogen Base and 850 ml ELGA and 20 g agar to the 50 ml aliquot. Autoclave.Add 100 ml autoclaved/filter sterilized 20 % glucose.5. Pour plates and allow to harden. Invert the plates and store at 4 °C. Plates are stable for 6 months.

***Heat Shock Transformation of* E.coli *with Plasmid DNA* – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols**

Before transformation, $CaCl_2$ competent cells were produced after Cohen et al., 1972. For the production of competent cells, 50 ml LB medium were inoculated with an overnight culture of the used ‘’E.coli’’ strain and incubated at 37 °C, 180 rpm. After an OD $_{550}$ of 0,5 was reached, the culture was centrifuged for 4 minutes at 5000 g for 10 minutes. The pellet was then resuspended in 40 ml pre-chilled in 0,1 M $MgCl_2$ solution, centrifuged again and resuspended in 20 ml of pre-chilled 0,05 M $CaCl_2$ solution. After 30 minutes of incubation on ice, the cells were centrifuged and resuspended in 2 ml 0,05 M $CaCl_2$ solution, 15 % v/v glycerol. The competent cells were aliquoted and stored an – 80 °C. For the transformation, 100 µl competent cells and 1 ng plasmid or 5 µg of a ligation product were mixed and incubated for 30 minutes on ice. Afterwards, the cells were heat shocked at 37 °C for 5 minutes, then mixed with 2 ml LB medium and incubated at 180 rpm and 37 °C for 30-45 minutes. The transformed cells were then plated on LB medium containing an antibiotic.

***Transformation of* S.cerevisiae – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols**

Yeast cells were transformed using the S. c. EasyComp™ Transformation Kit by Invitrogen. Firstly, cells were grown in YPD medium to mid-log phase. Then, cells were pelleted and washed with a washing solution. Afterwards, cells were pelleted again and washed with Lithium cation solution in order to make the cells competent. Finally, the cells were aliquoted and stored at -80 °C. For yeast transformation, frozen competent cells were mixed with up to 5 mg of plasmid DNA and transformation solution. The transformation batch was mixed by vortexing and incubated at 30 °C to induce uptake of DNA for one hour. The transformed cells were plated on selective plates and grown for 2-4 days at 30 °C.

***Genome Integration* – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols**

Genome integration of DNA constructs was done using an existing biobrick as an integration vector (BBa K300001 (Pavia '10)). Prior to transformation of yeast cells, the integration vector was linearized with the restriction enzyme Sbf1 because the frequency of integration is much higher for linearized plasmids. After linearization and preparative gel extraction of the vector it was transformed into yeast cells using the S. c. EasyComp™ Transformation Kit by Invitrogen *S. cerevisiae* and G418 antibiotic plates.

***Phycocyanobilin (PCB) Extraction from Dried Spirulina Platensis Powder* – TU Munich – 2012 http://2012.igem.org/Team:TU_Munich/Notebook/Protocols**

Phycocyanobilin (PCB) extraction from dried Spirulina platensis powder by methanolysis was done according to a protocol by Jim Tepperman (http://openwetware.org/images/2/27/Y2H_documentation.pdf). 50 g Spirulina powder were suspended in 1.5 liter H2O (30 ml/g) in a flask covered with aluminum foil. The mixture was stirred for 10 minutes and then centrifuged (GS3) at 8000 RPM at 4 °C for

1 hr. The supernatant was decanted and 15 g TCA (final concentration of 1 % (w/v)) was added. The mixture was stirred for 1 hr in the dark (aluminum foil cover) at 4 ℃ and then centrifuged at 8000 RPM at 4 ℃ for 10 min. The supernatant was discarded. The pellets were washed on ice several times with MeOH until the formerly green supernatant turned blue. The pellets were stored at 20 ℃ overnight, wrapped in aluminum foil. Since the free chromophore is very susceptible to photobleaching, the following steps were performed under green 'safelight' conditions in a darkroom. The next day, all pellets were resuspended in a final volume of 500 ml MeOH. Then the suspension was heated in a water bath at 70– 75 ℃ with a condensing coil cooled with tap water for 5-8 hrs. The suspension was then centrifuged at 8000 RPM, GS3 rotor, 4 ℃ for 20 min and the supernatant was filtered through miracloth. The filtered suspension and the pellets were stored at 20 ℃ overnight in the dark. The next day, a second methanolysis of the pellets from the first methanolysis was done. The two supernatants were pooled, the volume of MeOH was reduced to 50 ml using a rotary evaporator and then transferred separatory funnel where it was diluted with 100 ml water. The solution was mixed with 50 ml chloroform and shook to obtain the PCB through solvent extraction. The solvent extraction with chloroform was repeated until the aqueous phase was alsmost colorless. The chloroform was evaporated with a rotary evaporator and a stream of nitrogen gas was blown over the remaining PCB to remove residues of chloroform. The dried PCB as dissolved in DMSO, aliquoted and stored at -80 ℃.

Chemo-competent cells – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Inoue buffer

Component	Volume
$MnCl_2$ * $2H_20$	9.67 g
$CaCl_2$ * $2H_20$	2.2 g
KCl	18.65 g

PIPES (0.5 M, pH 6.7)	20 ml
H_2O	ad 1 l

Sterilize through filtration (0.45 µm filter) and store at -20 °C.

Cells

1. Pick an *E. coli* colony and inoculate 25 ml SOB.
2. Let bacteria grow for 8 hours at 37 °C and 250 rpm.
3. Inoculate three 100 ml SOB volumes with 1 ml, 2 ml and 4 ml of the prepared pre-culture.
4. Incubate over night at 18 - 22 °C and 200 rpm.
5. At OD_{600} = 0.55, put culture for 10 min on ice.
6. Centrifuge cells at 2500 g for 10 min at 4 °C. Discard supernatant completely.
7. Resuspend cell pellet in 30 ml 0 °C Inoue buffer.
8. Centrifuge cells at 2500 g for 10 min at 4 °C. Discard supernatant completely.
9. Repeat the previous two steps.
10. Resuspend cells in 8 ml 0 °C Inoue buffer. Add 1.5 ml DMSO and incubate on ice for 10 min.
11. Aliquot cells à 100 µl and freeze in liquid nitrogen. Store at -80 °C.

pGEM Ligation – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Ligation for TA-cloning of PCR products

Component	**Volume**
2X Rapid Ligation Buffer	5 µl

pGEM vector	0.5 µl (25 ng)
PCR product	3.5 µl
T4 DNA ligase	1 µl (3 Weiss units)

Mix all reagents in a 0.5 ml tube. Incubate reaction at 4 ℃ over night.

Ligation – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Ligation for digested parts and vectors

Component	**Volume**
10X T4 DNA Ligase Buffer	1 µl
vector DNA	1 µl (20 - 100 ng)
insert DNA	5 µl (up to 5:1 molar ratio insert to vector)
T4 DNA ligase	1 µl (1 unit)
water	2.5 µl

Mix all reagents and incubate at 22 ℃ for 1 hour.

Chemotransformation – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Component	Volume
chemo-competent *E. coli*	100 µl
plasmid DNA	up to 10 µl (max. 1/10 of volume)

1. Add plasmid DNA to cell culture.
2. Incubate for 30 min on ice.
3. Heat shock for 90 sec at 42 ℃.
4. Add 900 µl LB.
5. Let the bacteria grow at 37 ℃ for at least 1 hour.

Restriction digest – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

control digest – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Component	Volume
Tango buffer 10x	1 µl
XbaI (RE)	0.5 µl (5 units)

SpeI (RE)	0.5 µl (5 units)
DNA	1 µl (up to 1 µg)
water	7 µl

Incubate at least for 1 hour at 37℃.

preparative double digest – Tuebingen – 2012
http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Component	Volume
Tango buffer 10x	10 µl
SpeI (RE)	5 µl (50 units)
DNA	up to 30 µg
water	ad 150 µl

1. Incubate for 8 hours at 37 ℃.
2. After 3 hours add 2 µl SpeI.
3. Add 7 µl XbaI and incubate for another 8 hours.

plasmid linearization – Tuebingen – 2012
http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Component	Volume
Tango buffer 10x	10 µl
SpeI (RE)	7 µl (70 units)
DNA	up to 30 µg
water	ad 150 µl

Incubate for at least 8 hours at 37 ℃.

PCR – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Component	Volume
Taq/Pfu buffer	5 µl
Taq/Pfu polymerase	1 µl
primer forward	0.5 µl (100 pmol/µl)
primer reverse	0.5 µl (100 pmol/µl)

dNTPs	2.5 µl (200 µM)
template DNA	1 µl
water	36 µl

PCR conditions

Step	Duration	Settings
1	2 min	94 ℃
2	45 sec	94 ℃
3	30 sec	gradient or annealing temperature
4	90 sec	72 ℃
		steps 2 - 4: 30 cycles
5	7 min	72 ℃
6	(hold)	4 ℃

Gel electrophoresis – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

TAE buffer 50x

Component	Volume
0.05 M EDTA	18.61 g
1 M acetic acid	60.05 g
2 M Tris	242.28 g
water	1 l

Adjust to pH 8.5.

Gel

Component	Volume
TAE 1x buffer	120 ml
agarose	1.2 g
ethidium bromide	1.2 µl

1. Solve agarose in TAE 1x buffer and boil until solution is clear.

2. Add ethidium bromide, when lukewarm.

Well loading

Component	Volume
PCR product or DNA	5 µl
Loading dye 6x	1 µl

Can be scaled up linearly.

LB medium

Component	Volume
Trypton	10 g
yeast extract	5 g
NaCl	5 g
water	1 l

Adjust to pH 7.0.

Agar-plates

Component	Volume
agar-agar	16 g
LB buffer	1 l

1. Solve 16 g agar-agar in 1 l LB buffer and boil until solution is clear.
2. If it is nearly cold pour it into petri dishes (approx. 25 ml per dish).

SOB medium – Tuebingen – 2012 http://2012.igem.org/Team:Tuebingen/NotebookProtocols

Component	Volume
Trypton	20 g
yeast-extract	5 g
NaCl	0.5 g
250mM KCl	10 ml
water MiliQ	1 l

1. Solve the components in 1 l water.
2. Autoclave.

3. After autoclaving add 5 ml $MgCl_2$.

Preparation of Competent Cells – Trieste – 2012
http://2012.igem.org/Team:Trieste/protocols

Work as sterile as possible at 4℃.

1. Take 100mL aliquot of frozen cells (use DH5-α cells in this case) from the -80℃ and inoculated.
2. 2. Grow the cells in the shaker at 37℃ until they reach an O.D.600nm=0,6.
3. Transfer them into sterile Falcon (50mL).
4. Centrifuge it at 4500 rpm for 10 minutes at 4℃.
5. Resuspend the bacteria pellet on ice in cold $CaCl_2$(0,1M) .
6. Keep this suspension on ice for overnight.
7. Centrifuge it at 4500 rpm for 10 minutes at 4℃.
8. Resuspend the pellet in 8mL of RF2 (MOPS 1mM, RbCl 10mM, CaCl2 75mM, glycerol 15%w/v).
9. Dispense in aliquots and freeze cells at -80℃.

Transformation - Heat Shock – Trieste – 2012
http://2012.igem.org/Team:Trieste/protocols

Use DH5-α cells in most cases.

1. Take competent *E.coli* cells from –80℃ freezer and place on ice. Allow cells to thaw.
2. Mix cells by flicking the tube gently, then remove 100µl per transformation into a sterile pre-chilled (on ice) 1,5 tube.
3. Add 7µl of DNA per 100µl cells. Quickly flick the tube several times to ensure the even distribution of DNA.
4. Immediately place tubes on ice for 30 minutes.
5. Heat shock the cells for 90 seconds in a water bath at exactly 42 ℃. Do no shake.
6. Immediately place tubes on ice for 2 minutes.
7. Add 1mL of room temperature LB media (with no antibiotic added) and incubate for 1 hour in shaker at 37℃.Can incubate tubes for 30 minutes with appropriate antibiotic added – usually Ampicillin or Kanamycin.
8. Spread about 100µL of the resulting culture on LB plates - Grow overnight (O/N). The cells may be pelleted by centrifugation at 500 x g for 5 minutes, then the cells can be resuspended and plated.
9. Pick colonies about 12-16 hours later.

Clean colony PCR – Trieste – 2012
http://2012.igem.org/Team:Trieste/protocols

1. Pick the bacterial colonies and release them in 50µL distillate/autoclaved water.
2. Boil the sample at 95℃ for 5 minutes.
3. Prepare 28µL of mix-PCR solution for each sample (6µL Buffer Taq 5x, 1,8µL MgCl2 25mM, 0,6µL dNTPs 5mM, 0,15µL per primers, 0,15µL Taq polymerase, 19,15µL H2O), blend it and then spin it.
4. Take 2mL of the sample and release into mix-PCR solution and blend it.
5. Impost the PCR machine for 30µL volume and for 30 cycles, 5 minutes at 93℃, 30 seconds at 95℃, 30 seconds at 53℃, 1 minute at 72℃, indefinitely at 4℃.

6. Insert the samples and start the PCR machine.
7. At the end of the PCR the samples are ready for electrophoresis.

E.L.I.S.A. – Trieste – 2012 http://2012.igem.org/Team:Trieste/protocols

1. Coat 96-well ELISA plate with 100µL per well of antibody I anti6HIS used 1µg/mL for selection. Coating is in 100mM sodium hydrogen carbonate, pH 9.6. Leave O/N at 25℃.
2. Rinse wells 5x with BSA-PBS 0,1%.
3. Add the bacteria transformed that express the 6HIS tag in different concentration: 10^6, 10^5, 10^4 in different wells. Then in different wells too add bacteria non-transformed in different concentration: 10^6, 10^5, 10^4.
4. Rinse wells 5x with LB media.
5. Add 200µL of LB media and possibly antibiotics. Incubate O/N at 37℃.
6. Plate and incubate at 37℃ until formation of bacterial colonies.

Western blotting – Trieste – 2012 http://2012.igem.org/Team:Trieste/protocols

Preparation

1. Inoculate bacteria in 20mL of LB media with antibiotics if required O/N.
2. Transfer 2mL of the inoculum in flask and add 18mL of LB media with antibiotics if required.
3. Grow the bacteria until the inoculum reach at O.D. 600nm the value 0,4-0,6.
4. 2mL must be recovered to form the sample “non-induced”.
5. Induce the remaining 17mL of inoculum with 17µL of IPTG.
6. Wait for 4 hours (or for the time deemed appropriate).
7. Take 2ml the induced and centrifuge it for 10 minutes at 5000 rcf.
8. Discard the supernatant and add to the pellet 200µL of Loading Buffer SDS.
9. Sonicate very strong.
10. Heat shock at 95℃ for 5 minutes.

SDS-PAGE

1. Assemblate the Western blot scaffold.
2. Seal the bottom of the Western blot scaffold using agarose-water solution.
3. Add 10mL the running gel.
4. Add immediately, before the gel get solid, 1mL of isopropanol to level off the gel surface.
5. When the running gel is solid, add stacking gel until edge.
6. Insert the comb and wait the solidification of the gel.
7. Fill the Western blot scaffold with running buffer.
8. Remove gently the comb and wash the wells with Loading buffer SDS.
9. Loading the samples into wells.
10. Run Western blot at 200V, 30mA for 2 hours.

Transfer

1. At the end of the SDS-PAGE, disassemble the Western blot scaffold and recover the gel.
2. Assemblate the sandwich with in the middle the PVDF membrane surrounded two pieces of filter paper.
3. Put the sandwich into transfer box, add transfer buffer.
4. Leave transfer proteins at 200V, 50mA O/N.

Blocking

1. Dip the PVDF membrane into PBS-milk 5%, 1 hours in shaker, to prevent non-specific binding of the antibodies, which leads to high backgrounds.
2. Discard the PBS-milk 5% solution.
3. Add Antibody I with PBS-milk 5% solution and incubate for 1 hours in shaker.
4. Discard antibody with PBS-milk 5% solution.
5. Wash three times with PBS-tween 0,1% solution shake manually.
6. Wash three times with PBS-tween 0,1% solution in shaker for 3 minutes each one.
7. Add antibody II with PBS-milk 5% solution and incubate for 1 hours in shaker.
8. Repeat step 5 and step 6.

ECL (enhanced chemiluminescence)

1. Dip the membrane in PBS.
2. Put 1mL first ECL solution + 1mL second ECL solution on film.
3. Wet the membrane in solution with the solution on film for some seconds.
4. Fix the wet membrane in obscure box.
5. Go to obscure room and lean the photograph plate on the membrane for the time deemed appropriate for to have the right exposure.
6. Dip the photograph plate into developer solution for 1 minute.
7. Wash the photograph plate with water.
8. Dip the photograph plate into fixer solution for 2 minutes.
9. Dry the photograph plate in stove at 65℃.

Stripping

1. Wash the photograph plate with PBS-Tween 0,1% 3 times shake manually.
2. Wash the photograph plate with distillate water.
3. Add Stripping solution + distillate water in a ratio of 1 to 10 and incubate 30 minutes in shaker at 25℃.
4. Repeat the point 1.
5. Blocking with PBS-milk 5% for 30 minutes in shaker at 25℃.
6. Repeat the point 1.
7. Start the protocol for ECL (enhanced chemiluminescence).
8. Should be a photograph plate without any signal.

Precipitation of Surnatant

1. Recover 1mL of the sample supernatant.
2. Add 250mL of TCA 50% (trichloroacetic acid).
3. Incubate in ice for 60 minutes.
4. Centrifuge it for 10 minutes at 3750 rfc at 4℃.
5. Discard the supernatant.
6. Add 1mL of could acetone to eliminate the TCA.
7. Speed vaac for 10 minutes (sniff the sample to control absence of acetone).
8. Resuspend the sample in sample buffer SDS 1X (the color change to yellow).
9. Add 1mL of Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) to basific the solution (the color should change to blue, if it no happen so add other Tris).

Periplasm Protein Preparation

1. Inoculate bacteria in 20mL of LB media and possibly antibiotics O/N.
2. Transfer 2mL of the inoculum in flask and add 18mL of LB media and possibly antibiotics.
3. Grow the bacteria until the inoculum reach at O.D. $_{600}$=0,4-0,6.
4. 2mL must be recovered to form the sample "non-induced".
5. Induce the remaining 17mL of inoculum with 17µL of IPTG mM.
6. Wait for 4 hours (or for the time deemed appropriate).
7. Take 2mL of the induced and centrifuge it for 10 minutes at 5000 rpm.
8. Discard the supernatant and resuspend pellet in 1/40 volume of PPB buffer (200mg/mL sucrose, 1mM EDTA, 30mM Tris-HCl pH 8.0). Keep in ice for 20 minutes.
9. Spin down cells in centrifuge at 5000 rpm for 15 minutes and collect supernatant into smaller high speed centrifuge tubes.
10. Resuspend pellet in 1/40 volume of 5mM $MgSO_4$ buffer. Incubate in ice for 20 minutes.
11. Transfer samples to small high speed centrifuge tubes.
12. Spin both peri-prep supernatant and Osmotic shock preparation at 15000 rpm for 15 minutes.
13. Collect the supernatants, the sample is ready for E.L.I.S.A.

Immunofluorescence assay

1. Inoculate bacteria in 20mL of LB media with antibiotics if required O/N.
2. Take 1mL when the O.D.$_{600}$ arrived at the bacteria concentration about $5x10^8$ units/mL.
3. Take 1mL and centrifugate 10 minutes at 6000 rpm at 4°C.
4. Wash the sample with PBS 3 times and centrifuge 10 minutes at 6000 rpm at 4°C.
5. Block with 1mL of PBS-BSA 1% and centrifuge 10 minutes at 6000 rpm at 4°C.
6. Discard the supernatant and add the primary antibody anti-HIS6.
7. Rock the sample for 3 hours at 4°C.
8. Repeat the point four
9. Discard the supernatant and add the secondary antibody anti-mouse TRICT.
10. Rock the sample for 1 hour at 4°C.
11. Repeat the point seven.
12. Discard the supernatant and add DAPI.
13. Repeat the point 7.
14. Analyze the sample with epifluorescence microscope.

Cloning **– Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage**

Plasmid DNA isolation – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

MINI PREPs for analysis and sequencing

1. A single colony was picked from a LB-agar plate or glycerol stock and inoculated in 10 mL of LB-medium with the appropriate antibiotic for selection (100 mg/L ampicillin, 50 mg/L kanamycin, 35 mg/L chloramphenicol).
2. Bacteria were grown over night at 37 °C with agitation.
3. Plasmid DNA was isolated from 6-10 mL of over-night culture with GeneJET plasmid miniprep kit according to the manufacturer's protocol.
4. Amounts ranging from 6-10 µg of plasmid DNA were obtained.
5. The purity and concentration of the isolated DNA was analysed using NanoDrop.

Fragment DNA isolation from agarose gel – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

AGAROSE ELECTROPHORESIS – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. A mixture of different sized DNA fragments was separated on an agarose gel (from 0.7 to 2% agarose in 1x TAE buffer and 0.1 µg/ml ethidium bromide) at a constant voltage of 100 V.
2. UV light (λ = 254 nm) was used to visualize DNA with intercalated ethidium bromide

FRAGMENT ISOLATION from agarose gel – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. The band with the desired DNA fragment was excised from the gel, using a clean scalpel.
2. DNA was isolated from the gel slice with GeneJet Gel Extraction Kit according to the manufacturer’s protocol.
3. Purity and amount of DNA was determined using NanoDrop.

Restriction digest – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. To digest the desired DNA restriction reactions were prepared as follows:

   for analysis of cloned DNA

   - 2µl of the appropriate restriction buffer (10X)
   - 0.5 µL restriction enzyme
   - Bring volume to 20 µL with nuclease-free water.

   or

   for isolation of specific DNA

   - 2µl of the appropriate restriction buffer (10X)
   - up to 2 µL restriction enzyme
   - Bring volume to 50 µL with nuclease-free water.
2. The sample was incubated at optimal temperature for the restriction enzymes.
3. Analysis of fragmented DNA was done by gel electrophoreses.
4. Desired DNA fragment was excised and purified using suitable DNA purification kit.

PCR reaction – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

AccuPrime and Phusion DNA polymerase were used for DNA amplification. Colony PCR was performed with Taq DNA polymerase.

1. The master mix for reactions with Phusion DNA polymerase contained:
   - DNA (1-10 ng)
   - both primers (0,4 pmol/µl)
   - 1x Phusion HF buffer
   - 0,2 µM dNTPs
   - Phusion polymerase (0,02 U/ µl) and
   - MQ up to final volume of 25 µl

2. The master mix for reactions with AccuPrime DNA polymerase contained:
   - DNA (10 ng),
   - both primers (0,4 pmol/µl),
   - 1xRnx mix,
   - enzyme (0,05 U/ µl) and
   - MQ up to final volume of 50 µl.
3. The master mix for reactions with Taq DNA polymerase contained:
   - both primers (0,4 pmol/µl),
   - 1x Taq PCR buffer II,
   - 0,2 µM dNTPs,
   - 5mM $MgSO_4$,
   - enzyme (0,125 U/ µl) and
   - MQ up to total volume of 20 µl.
   - Then the bacterial colony was added to the reaction mix.
4. All temperature profiles were optimized according to manufacturer's protocol,the melting temperature of primers, and the length of the desired PCR products. Reactions were performed in the Applied Biosystems Veriti 96 well thermal cycler.

PCR product purification. Desired PCR products were purified by GeneJet Gel Extraction Kit according to the manufacturer's protocol.

DNA concentration. An aliquot of the isolated DNA was analyzed using NanoDrop.

Gibson assembly – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

Gibson assembly master mix was prepared according to the protocol published in Gibson et al., 2009.

1. 50 ng of each PCR product was added to the Gibson assembly master mix and incubated at 50 °C 1h.
2. After incubation, the entire master mix volume was transformed into competent bacterial cells.

Ligation – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

T4 ligase ligates the 5' phosphate and the 3'-hydroxyl groups of DNA.

1. Vector and insert concentrations were estimated and insert and vector fragments joined in a molar ratio of 3:1 (100-150ng Vector DNA).
2. A ligation mixture was prepared:

   1X ligase buffer (10X)
   1 µL T4 ligase (3 U/µL)
   Bring volume to 10 or 20 µL with nuclease-free water.
   or

3. Blunt-end ligation reactions were incubated at 17 °C for 4 to 18 hours.
4. After incubation part of the ligation mixture was used for the transformation of bacterial cells (see: transformation of bacteria).

Culturing bacteria – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

For plasmid DNA propagation two bacterial strains were used: **DH5alpha** [*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*] and **TOP10** [*mcrA, Δ(mrr-hsdRMS-mcrBC), Phi80lacZ(del)M15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(SmR), endA1,nupG*].

Growth media for bacteria

Luria Broth (LB) : 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, media is supplemented with suitable antibiotics depending on the selection marker on the transfected plasmid: ampicilin 100 mg/L or kanamycin 50 mg/L.

LB agar plates: LB with 1.5% agar, media is supplemented with suitable antibiotics depending on the selection marker on the transfected plasmid.

Transformation of bacteria – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

E. coli DH5alpha and TOP10 competent cells were used for the propagation of plasmid DNA.

1. 100 µL of competent cells were thawed on ice.
2. 50 – 400 ng DNA solution was added to competent bacterial cells (depending on the concentration of the DNA solution).
3. A mixture of cells and DNA solution was incubated on ice for 30-60 minutes.
4. The mixture was heat-shocked for 3 minutes at 42 °C.
5. Cooled for 3 minutes on ice.
6. 500 µL of preheated antibiotic free LB-medium was added and incubated for one hour at 37 °C with agitation for the purpose of inducing antibiotic resistance.
7. The selection for plasmid containing and therefore antibiotic resistant bacteria was conducted by plating them on antibiotic containing LB-agar plates.

Glycerol stock for long term storage of bacteria

1. 1 mL of an overnight culture was added to 150 µL of 80% glycerol into a cryo-tube.
2. Mixed and incubated at room temperature for 30 minutes.
3. Afterwards the glycerol stock was stored at -80 °C.

Cell cultures – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

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Eucaryotic cell lines and cultivation – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

HEK293 is a human cell line derived from kidney cells and grows in a monolayer culture. Cells were grown in DMEM medium supplemented with 10% FBS.

HEK293T cell line is derived from HEK293 cells. HEK293T cells express the SV40 large T-antigen that enables episomal replication of plasmids containing the SV40 origin of replication in transfected cells. Cells were grown in DMEM medium supplemented with 10% FBS.

NK-92 is an interleukin-2 (IL-2) dependent natural killer cell line derived from peripheral blood mononuclear cells from patient with non-Hodgkin's lymphoma. The cell line is cytotoxic to a wide range of malignant cells. Cells were grown in RPMI medium supplemented with 20% FBS and 100 U/ml IL-2.

Subculturing monolayer cell cultures – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. Remove and discard culture medium from a T-75 flask containing a monolayer of HEK293 or HEK293T cells.
2. Rinse the T-75 flask with 10 ml of PBS buffer to remove all traces of growth medium (DMEM + 10% FBS) which otherwise inhibits trypsin function. Remove and discard the PBS buffer.
3. Add 2-3 ml of trypsine solution and gently tilt the flask to ensure the trypsine solution covers all the cells. Incubate the cells in trypsin for 0,5 - 3 minutes.
4. When the cells start to detach from the surface, add 7 ml of growth medium to the trypsin solution. Resuspend all remaining cells from the bottom of the T-75 flask by pipetting.
5. Transfer the cell suspension to a 15 ml centrifuge tube.
6. Centrifuge the cell suspension for 5 minutes at 1200 rpm.
7. Remove the trypsin-containing medium from the centrifuge tube.
8. Resuspend the cell pellet in fresh medium.
9. Take as much cells as you need and add fresh medium to a total volume of 10 ml.
10. Return the cells in a T-75 flask to the incubator (37 °C, 5 % CO_2).

Cell plating – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. Count cells.
2. Calculate the desired number of cells per well. Dilute cells in DMEM with 10% FBS.
3. Transfer the cells into an appropriate plate and place in a cell culture incubator.

Media and buffers

DMEM supplemented with: 1 % L-Glutamine (GlutaMax), 10 % FBS, Optionally: 1% Pen/Strep.

RPMI supplemented with: 1 % L-Glutamine (GlutaMax), 20 % FBS.

Transfection – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

TABLE: Transfection mixtures for different culture format

Culture format	jetPEI reagent per µg of DNA (µL)	Typical amount of DNA (ng)	Volume of 150 mM NaCl solution for DNA and jetPEI (µL)	Total mixtu
96-well	2	200	10	20
24-well and 8-well microscope chamber	2	500	50	10
12-well	2	1000	50	10
6-well	2	2000	100	20

Culture format	jetPEI reagent per µg of DNA (µL)	Typical amount of DNA (ng)	Volume of 150 mM NaCl solution for DNA and jetPEI (µL)	Total mixtu
10 cm	2	15000	250	50

1. Dilute plasmid DNA to desired concentration in 150 mM NaCl, vortex gently and spin down briefly.
2. Dilute jetPEI (PolyPlus) in 150mM NaCl, vortex gently and spin down briefly.
3. Add the jetPEI solution to the DNA solution.
4. Vortex the solution immediately and spin down briefly.
5. Incubate for 15 to 30 minutes at room temperature.
6. Add the jetPEI/DNA mix to the cells in and gently swirl the plate.
7. Return the plate to a cell culture incubator.

Induction systems – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

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Induction systems – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

To control our switch we needed a way to affect it from the outside. For this purpose we chose several inducible transcription systems where the controlled gene is expressed when a small molecule inducer (such as tetracycline) is present and is not expressed when the inducer is absent. We chose specific systems which do not cross react and whose inducers are orally bioavailable and safe for human use (Clackson, 2000). We decided for the systems based on tetracycline, pristinamycin, erythromycin and rapamycin analogs, as inducers. We then adapted these systems by cloning TAL regulators under their control to make them compatible with our genetic circuits.

Tetracycline, erythromycin and pristinamycin systems – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

The tetracycline, erythromycin and pristinamycin system all function in a similar manner. They are composed of a DNA binding protein (such as TetR) fused to a KRAB domain which reversibly binds a specific DNA sequence (TRE for example) and silences transcription from nearby promoters. The addition of an inducer causes the DNA binding domain to dissociate from the DNA and allows transcription to start. (Deuschle et al., 1995; Kramer et al., 2004)

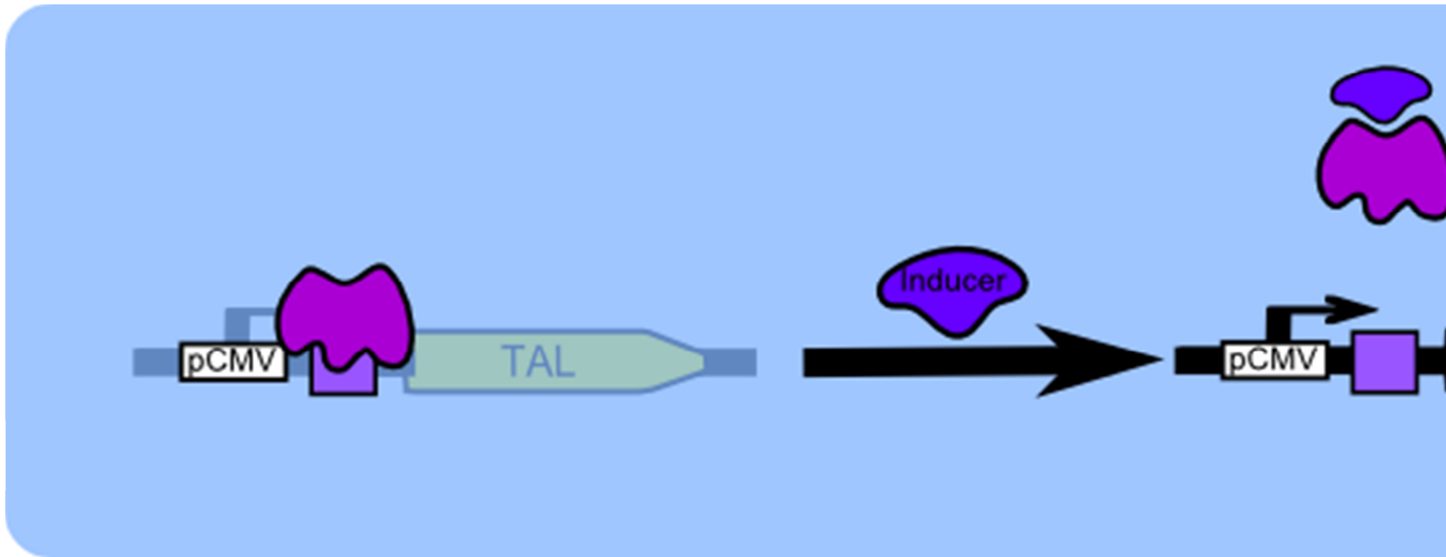

Figure 1: Induced expression of TAL.

TABLE

System	Tetracycline	Erythromycin	Pristinamycin
Regulating protein	TetR:KRAB	E:KRAB	PIP:KRAB
DNA sequence	TRE	ETR	PIR
Inducer	Tetracycline or doxycycline	Erythromycin	Pristinamyc
Constructs	pCMV_TRE_TAL-A:KRAB	pCMV_ETR_TAL-A:KRAB	pCMV_PIR
	pCMV_TRE_TAL-B:VP16	pCMV_ETR_TAL-B:VP16	pCMV_PIR

System	Tetracycline	Erythromycin	Pristinamycin
	TRE_CMV_TAL-A:KRAB	pCMV_ETR_TAL-B:KRAB	pSV40_PIR
	TRE_CMV_PEST-CL1:TAL-A:KRAB	pCMV_ETR_TAL-A:VP16	PIR_CMV_
	TRE_CMV_TAL-B:VP16	pSV40_ETR_TAL-B:KRAB	PIR_CMV_ CL1:TAL-B
	TRE_CMV_PEST-CL1:TAL-B:VP16	ETR_CMV_TAL-A:KRAB	TRE_CMV_
		ETR_CMV_PEST-CL1:TAL-A:KRAB	TRE_CMV_ CL1:TAL-A
		ETR_CMV_TAL-B:VP16	
		ETR_CMV_PEST-CL1:TAL-B:VP16	

Rapamycin system – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

In the rapamycin system the gene of interest is under the control of a minimal promoter. The gene's transcription rate is regulated by two proteins that consist of a drug binding domain and either a DNA binding domain or an activation domain. When rapamycin is added both drug binding domains bind to it, consequently joining the activation domain with the DNA binding domain, resulting in a functional transcription factor, which activates the gene of interest. Instead of rapamycin a rapamycin analogue (rapalogue), which is a 1000-fold less imunosupressive than rapamycin, but activates the inducible system like rapamycin, is usually used as the inducer. (Pollock et al., 2002)

Regulating vector	HetAct
DNA sequence	ZFHD

Regulating vector	HetAct
Constructs	ZFHD_pMIN_TAL-A:KRAB
	ZFHD_pMIN_TAL-B:VP16

Induction of cells – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. Transfect HEK293 or HEK293T cells with plasmids using JetPei transfection reagent (Polyplus transfection), following the manufacturers protocol (see cell culturing for details).
2. Two hours post transfection change media and stimulate the cells by adding dilutions of appropriate inductors to the medium in a 1:10 (v:v).

Inductor	Stock solution (solvent)	Dilution (solvent)	Concentration in cel solvent)
Rapalogue (AP21967)	1 mM (100% ethanol)	10 µM (1% ethanol)	1 µM (0,1% ethanc
Doxycyclin	1 g/L (MQ)	10 mg/L (MQ)	1 mg/L (MQ)
Pristinamycin	50 g/L (100% DMSO)	20 mg/L (1% DMSO)	2 mg/L (0,1% DMS
Erythromycin	50 g/L (100% ethanol)	20 mg/L (1% ethanol)	2 mg/L (0,1% etha

Effectors **– Slovenia – 2012**
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

Biological assay-anakinra – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells, seeded in 6-well plate, were transfected with anakinra downstream of constitutive promoter.
2. Transfected cells were incubated for 48 h.
3. To detect anakinra's effect on NF-κB signalling pathway, other HEK293T cells were transfected with plasmid coding for Renilla luciferase and plasmid reporter with NF-κB-inducible firefly luciferase expression. HEK293T cells express IL-1R, so additional transfection with a receptor gene was notneeded.
4. After 24 h, the medium was removed from cells transfected with reporter plasmids and 90 µL of anakinra-producing cells' supernatant was added to these wells.
5. After 24 h of stimulation, cells were lysed and NF-κB activation was assessed using dual luciferase assay.

Biological assay-IFN-alpha – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells transfected with eithera plasmid encoding IFN-alpha under the control of a constitutive promoter or an empty vector, and HEK293T cells transfected with the reporter vector were co-cultivated .
2. Additionally, we performed a co-transfection experiment, where HEK293T cells were transfected with both the reporter and the IFN-alpha encoding plasmids.
3. Day after transcfection cells were cultivated into 96-well plate at density 5x104 cells per well.
4. After 24 hours of incubation, dual luciferase reporter assay was performed.

ELISA for IFN-alpha – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells where plated on 6 well plates and transfected with a plasmid coding for human IFN-alpha under the control of a constitutive promoter or a control plasmid (pcDNA3).
2. Supernatants were collected after 16h and serial dilutions were measured for IFN-alpha levels by Human IFN-alpha Instant Elisa (eBioscience).

Plate reader-fluorescence(The Switch) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells were seeded in black 96-well plates and transfected with plasmids encoding the switch. Plasmids and amounts used for transfection are listed in Figure legends.
2. Two hours after transfection, media was changed and cells were stimulated with inducers. Inducers and their concentration are described in Figure legends.
3. Media supplemented with inducer or without inducer were changed after two or three days of cultivation.
4. After a maximum of 6 days after transfection, cells were lysed with 25 µL of 1x Passive lysis buffer (Promega) per well.
5. Fluorescence was measured using an automated plate reader.

Plate reader-luminescence (The Switch) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells were seeded in white 96-well plates and transfected with plasmids encoding the switch. Plasmids and amounts used for transfection are listed in Figure legends.
2. Two hours after transfection, media was changed and cells were stimulated with inducers. Inducers and their concentration are described in Figure legends.
3. Media supplemented with inducer or without inducer were changed after two to three days of cultivation.
4. After a maximum of 6 days after transfection, cells were lysed with 25 µL of 1x Passive lysis buffer (Promega).

5. Luminescence of expressed reporter firefly luciferase was measured with Orion (Berthold Technologies) using Luciferase buffer with luciferin as a substrate. For normalization Renilla luciferase activity was used. The Renilla luciferase was measured using Renilla buffer supplemented with coelenterazine.

Plate reader-absorbance (The Switch) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells were seeded in white 24-well plates and transfected with plasmids encoding the positive feedback loop switch and 10x[TALB + TALC] operator_CMV promoter_fLuciferase reporter plasmid and 10x[TALA + TALC]operator_CMV promoter_SEAP plasmid. Plasmids and amounts used for transfection are listed in Figure legends.
2. Media supplemented with inducer or without inducer were changed after two days of cultivation.
3. Two and seven days after transfection the growth medium was collected and SEAP QUANTIBlue substrate was added. After 15 minutes incubation at 37℃ the absorbance was measured at 630 nm.

Microscopy – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

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For spatial and temporal imaging of samples a Leica TCS SP5 laser scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Germany) with a 10× and 20× dry objective and an HCX plan apo 63× oil (NA 1.4) oil immersion objective was used. For image analysis we used ImageJ (Image Processing and Analysis in Java) software (http://rsbweb.nih.gov/ij/) measuring the mean grey values of each cell containing the promoter of interest.

Microscopy-cell viability with Hoechst and SytoxGreen514 (Safety mechanisms) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

Hoechst dye is a membrane permeable dye and stains all cells in a culture. On the other hand a **SytoxGreen514** dye is a membrane impermeable dye staining only dead cells. Both dyes, blue fluorescent Hoechst and green fluorescent SytoxGreen514, bind to nucleic acids causing emission of fluorescent light.

1. HEK293 cells were seeded in an 8-well microscope chamber and transfected with 200 ng CMV-mGMK_TK30 (pPCMV_mGMK:TK30).
2. Ganciclovir (GCV) in concentrations 0, 10 and 100 μg/mL was added to the cell cultures.
3. After 5 days of cultivation, a Hoechst dye (0.4 μg/mL) and a SytoxGreen514 dye (1 μM) were used to stain cells and discriminate between live and dead cells.
4. Cells were incubated for approximately 10 minutes in the dark at 37 ℃ before imaging.
5. A 405-nm diode laser was used to excite Hoechst and a 514-nm line of 25 mW multi ion argon laser was used to excite SytoxGreen514. Successive images excited at 405 nm and 514 nm were captured. Fluorescence emission was detected at 450-500 nm and 520-560 nm for Hoechst and SytoxGreen respectively.

Microscopy-cell growth (Safety mechanisms) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293 cells were seeded in an 8-well microscope chamber and transfected with 100 ng mGMK:TK30 (pPCMV_mGMK:TK30) and/or 20 ng GFP (pPCMV-GFP) (for transfection control).
2. Ganciclovir (GCV) in concentrations 0, 10 and 100 μg/mL was added to the cell cultures.
3. After 5 days of cultivation, a cell cultures were imaged.
4. A 514-nm line of 25 mW multi ion argon laser was used to excite GFP reporter protein. Fluorescence emission was detected at 520-560 nm for GFP. Bright field images were used to visualize the number of cells.

Microscopy-cell count (Safety mechanisms) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293 cells were seeded in 12-well plates and transfected with different amounts of mGMK:TK30 (pPCMV_mGMK:TK30) as indicated in Figure legend.
2. Cell cultures were treated with ganciclovir (GCV) in concentrations as indicated in the Figure legend.
3. After incubation the cells were resuspended by pipetting.
4. Cells suspensions were then mixed with trypan blue.
5. Viable cell number was determined by counting the cells under a light microscope using a Bürker-Türk counting chamber.

Microscopy-detection of reporter proteins (The Switch) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

Fluorescent proteins were used as reporters in "The switch experiments". The fluorescent proteins used were blue (tagBFP), yellow (mCitrine), orange (mCherry) and red (mNeptun) fluorescent proteins. mCherry was used as transfection control while the others were used as reporters of "the switch".

1. HEK293T cells were seeded in an 8-well microscope chamber or 12-well plate and transfected with plasmids encoding the switch. Plasmids and amounts used for transfection are listed in Figure legends.
2. Two hours after transfection, media was changed and cells were stimulated with inducers. Inducers and their concentration are described in Figure legends.
3. Media supplemented with inducer or without inducer were changed after two or three days of cultivation.
4. Images of cells expressing reporters were taken two days after transfection and then each day for 5 days.
5. A 405-nm diode laser was used to excite tagBFP, a 514-nm line of 25 mW multi-ion argon laser was used for mCitirne, a 543-nm HeNe laser was used for mCherry and a 633-nm HeNe laser was used to excite mNeptune. Successive images excited at 405, 514, 543 and

633 nm were captured. All intensities of laser and photomultipliers were kept unchanged during one set of experiments to enable comparison of images. Fluorescence emission was detected at 450-500 nm, 520-560 nm, 560-600 nm and 640-700 nm for tagBFP, mCitrine, mCherry and mNeptune, SytoxGreen respectively.

Microscopy-alginate degradation (Microencapsulation) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

To observe the degradation of alginate beads, 2000 kDa FITC-dexstran (Sigma) was added to 200 µL of culture medium containing alginate beads with immobilized HEK 293T cells. Because FITC-dexstran cannot penetrate the alginate beads, we can easily observe bead degradation upon addition of alginate lyase from Sphingobacterium multivorum (Sigma).

1. Alginate beads suspended in culture medium were seeded in an 8-well microscope chamber (200 µL).
2. 20 µL of 1 mg/mL FITC-dextran were added into well.
3. After the dye was evenly distributed throughout the suspension, 8 µL of Sphingobacterium multivorum alginate lyase were added.
4. The microscope was set to capture images every 20 seconds.
5. Screenshots were collected for at least 15 minutes.
6. A 488-nm line of 25 mW multi-ion argon laser was used for FITC. Fluorescence emission was detected at 520-560 nm. At the same time a bright field image was taken.

Microscopy-secreted alginate lyase enzymatic activity (Microencapsulation) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK293T cells were seeded 1×10^6 on 10 cm cell culture dish and grown in DMEM medium supplemented with 10 % FBS.
2. After reaching 50 – 70 % confluency, cells were transfected with 15 µg of DNA per culture dish with jetPEI transfection reagent (Polyplus Transfection).

3. Protein production lasted for 72 hours.
4. Cell supernatants were collected and concentrated 50-times using Sartorius Vivaspin 6 concentrators (5 kDa MWCO PES).
5. Alginate beads were produced with Büchi BIOTECH Encapsulator (see Microencapsulation: Encapsulation procedure 1.-6.).
6. Beads were incubated with concentrated supernatants for 72 hours in an 8-well microscope chamber.
7. 20 µL of 1 mg/mL FITC-dextran were added into wells.
8. A 488-nm line of 25 mW multi-ion argon laser was used for FITC detection. Fluorescence emission was detected at 520-560 nm.
9. Beads' diameters were assessed using Leica LAS Image Analysis software.

Microscopy-encapsulated cell viability (Microencapsulation) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

To observe encapsulated cells' viability, HEK 293T cells were stained with Hoechst and 7-aminoactinomycin D (7-AAD) viability stains. Hoechst stains both live and dead cells, while 7-AAD stains dead cells only.

1. Encapsulated cells were grown in DMEM culture medium supplemented with 10% FBS.
2. 200 µL of the microcapsule suspension was collected and alginate-PLL capsules were seeded into an 8-well microscope chamber.
3. 5 µL of 7-AAD and 1 µL of Hoechst stain were added to one well.
4. Encapsulated cells were protected from direct light and stained for 30 minutes at 37 °C.
5. A 405-nm diode laser was used to excite Hoechst and a 543-nm line of HeNe laser was used to excite 7-AAD.
6. Fluorescence emission was detected at 450-500 nm and 600-700 nm for Hoechst and 7-AAD respectively.

Flow cytometry – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

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Flow cytometry is a laser based technology employed in cell counting and biomarker detection. It allows simultaneous multiparametric analysis of the physical as well as biochemical and biological characteristics of particles. We used a CyFlow Space (Partec) flow cytometer equipped with three lasers (405, 488 and 633 nm). The CyFlow detects forward scatter and side scatter signals and up to 6 colors of fluorescence.

Flow cytometry - the annexin assay (Safety mechanisms) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

To determine the percentage of cells undergoing apoptosis as a result of herpes simplex virus thymidine kinase (HSV-TK) (pCMV-mGMK_TK30) transfection and ganciclovir treatment we labeled cells with Annexin V conjugated with phycoerythrin (PE). Annexin V is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for the phospholipid phosphatidylserine and therefore binds to apoptotic cells with phosphatidylserine exposed on their surface.

1. HEK293 cells were seeded in 12-well plates.
2. Cells were transfected with pCMV-mGMK_TK30 and treated with ganciclovir. Concentrations of ganciclovir and plasmids are indicated in Figure legends.
3. After incubation the cells were washed with PBS buffer and resuspended by pipetting.
4. Cells were pelleted with centrifugation at 1200 rpm.
5. The cell pellet was washed in 1x Annexin Binding Buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4).
6. The pellet was then resuspended in 1x Annexin Binding Buffer and PE-Annexin V (5 µl per 100 µl cell suspension) was added.
7. Samples were incubated for 20 minutes in the dark at room temperature and then immediately analyzed with a flow cytometer.
8. Along with site and forward scatter, the signal in the FL2 channel (540-580 nm) was also recorded.

Flow cytometry - the propidium iodide assay (Safety mechanisms) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

To determine the percentage of dead cells due to cytotoxic activity of natural killer cells against HEK293T cells expressing MICA protein, cells were stained with propidium iodide dye, which intercalates into DNA and stains only dead cells, because it is a membrane impermeant fluorescent molecule.

1. HEK293T cells seeded in 12-well plates were transfected with plasmids expressing MICA (pPCMV-MICA_pcDNA3).
2. Two days after transfection with CMV-MICA_pcDNA3, cells were resuspended in PBS at final concentration $1x10^6$ cells/mL, stained with CFSE (0,6µM) and incubated for 10 minutes at 37°C. Staining was quenched by the addition of 5 volumes of ice-cold culture media (RPMI+ 20% FBS) to the cells. After 5 minutes incubation on ice, cells were pelleted by centrifugation and then washed by resuspending the pellet in fresh media (RPMI+ 20% FBS) a further two times for a total of three washes. CFSE was used to discriminate between HEK293T and NK-92 cells or between NK target cells K562, which were used as a positive control, and NK-92 cells.
3. HEK293T cells or K562 cells were mixed with NK-92 cells in different ratios (1:1, 1:5, 1:10) and incubated for 4 hours at 37 °C in culture medium consisting of RPMI, 20% FBS and hIL-2 (100 U/ml).
4. After incubation of HEK293T or K562 cells with NK-92 cells, cells were treated with propidium iodide.
5. Along with site and forward scatter the signal in the FL1 channel (530-580 nm) was also recorded.

Flow cytometry - detection of reporter proteins (The Switch) – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

Reporters such as fluorescent proteins were used to detect the expression of effectors in "The switch experiments". As reporters we used blue (tagBFP) and yellow (mCitrine) fluorescent proteins.

1. HEK293T cells were seeded in a 12 or 24-well plate and transfected with plasmids encoding the switch.
2. Two hours after transfection, media was changed and cells were stimulated with inducers.
3. Medium with inducer or without the inducer was changed after two days of cultivation.
4. Cells were collected at different time points (2 days after induction and then 3 days after the second media change).
5. Cells were washed and resuspended in PBS buffer.
6. A 405 nm diode laser was used to excite tagBFP and a 488-nm diode laser was used for mCitirne.
7. Along with site and forward scatter signals in the FL1 (540-580 nm) channel (mCitrine) and the FL5 (450-480 nm) channel (tagBFP) were also recorded.

Microencapsulation – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

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Cell preparation for encapsulation – Slovenia – 2012 http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. HEK 293T cells were seeded 5x10^5 per 10 cm cell culture dish (3 per encapsulation) and grown in DMEM medium supplemented with 10 % FBS.
2. After reaching 50 – 70 % confluency, cells were transfected with 15 µg of DNA per culture dish with jetPEI transfection reagent (Polyplus Transfection).
3. The medium was removed the next day.. Transfected cells were detached using 3 mL of trypsin solution and centrifuged after the addition of fresh medium to inactivate the trypsin.
4. Supernatant was removed and cells were resuspended in 15 mL DMEM with 10% FBS.
5. Cells were counted using Countess automated cell counter (Invitrogen).
6. HEK 293T cells were again centrifuged and supernatant was removed.
7. Cells were resuspended in 2 mL of pre-warmed MOPS buffer.
8. 10 mL of pre-warmed alginate solution (1,5%) was added to cell suspension.

Encapsulation – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. The encapsulator was equipped with a 200-µm nozzle.
2. The reactor vessel was filled with 225 mL 100 mM CaCl2.
3. Cell-alginate mixture was transferred to a 20 mL syringe with a Luer lock.
4. The syringe was connected to the bead producing unit (BPU).
5. Microcapsules were produced at a flow rate of 12-14 units, vibration frequency 1030-1100 Hz and voltage for bead dispersion 900-1300 V.
6. Polymerization lasted for 10 minutes.
7. The polymerization solution was drained and 75 mL of 0,05% poly-L-lysine (PLL) solution was added.
8. Beads were incubated in PLL solution for 10 minutes.
9. The PLL solution was removed and beads were washed twice (for 1 and for 5 minutes) with 150 mL of MOPS buffer.
10. 100 mL of 0,03% alginate was added and beads were incubated for 10 minutes.
11. Alginate solution was drained and beads were washed once with 150 mL of MOPS buffer for 1 minute.
12. 150 mL of depolymerization solution was added for 10 minutes.
13. Depolymerization solution was removed and capsules were resuspended in 150 mL MOPS and collected in a bead collection flask.
14. MOPS was removed and microcapsules were transferred to T-75 with 10 mL DMEM, 10% FBS media supplemented with penicillin and streptomycin.

Buffers and solutions

10 mM MOPS buffer (pH = 7,2)
MOPS buffer with NaCl (pH = 7,2): 10 mM MOPS, 0,85% NaCl
Polymerisation solution (pH = 7,2): 10 mM MOPS, 100 mM CaCl2
Depolymerisation solution (pH = 7,2): 10 mM MOPS, 50 mM Na3-citrate, 0,45% NaCl
0,05% poly-L-lysine (15-30 kDa) in MOPS buffer (pH = 7,3)
0,03% alginate in MOPS buffer (pH = 7,2)
1,5% alginate (low viscosity) in MOPS buffer (pH = 7,2)

Protein detection – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

SDS-PAGE – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. Samples were loaded on a 12% acrylamide gel and ran at a constant voltage (200 V) for 1 h.
2. Proteins were then blotted on a nitrocellulose membrane at a constant current (350 mA) for 1 h.
3. The membrane was washed with MQ and PBS and blocked for 1,5 h by incubation in I-Block blocking reagent at room temperature.

Immunodetection – Slovenia – 2012
http://2012.igem.org/Team:Slovenia/Notebook#topofthepage

1. All proteins to be detected had a Myc tag at the C-terminus.
2. The membrane was incubated with primary antibodies (rabbit anti-Myc diluted 1:500) overnight at 4 °C and 150 rpm. Membrane was washed.
3. Washing in wash buffer three times for 5 minutes each.
4. Membrane was incubated with secondary antibodies (anti-rabbit secondary antibodies, conjugated with HRP, diluted 1:3000) for 45 minutes at room temperature and 150 rpm.
5. HRP activity was detected by addition of SuperSignal West Femto or Pico Substrate (Thermo Scientific). Images were captured with Syngene G:Box chemiluminescent imaging system.

Buffers and solutions

Wash buffer: 1x PBS, 0,01% (v/v) Tween 20

Fluorescent protein purification – Potsdam – 2012
http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

1. Prepare 50ml of LB medium with appropriate antibiotics, inoculate with glycerol stock of the strain of interest (E.coli XL 1 Blue with CFP/YFP)
2. Nurture overnight at 37℃, 200rpm.
3. Inoculate 10ml of the overnight culture into 2x 500ml LB medium with antibiotics
4. When OD600 is 0,3-0,5 add IPTG to the final concentration of 500 mM.
5. Nurture for another 6 hours and centrifuge (Beckman centrifuge: 5000rpm, 4℃, 45min)
6. Freeze in -80℃ freezer
7. Resuspend the pellet in 20ml HEPES buffer
8. Transfer to 50ml Falcon tubes
9. Sonication (3x2min) on ice – 6mm tip, cycle 50%, output 5-6, timer HOLD.
10. Centrifugation: 30 min, 19000rpm, 4℃
11. Transfer the supernatant to new Falcons
12. Sonicate for 1min
13. Filter through 0,45µm filter
14. Store on ice before loading at the column
15. Equilibrate the column: 30 times column size with HEPES buffer
16. Load the column with the sample
17. Wash with washing buffer
18. Elute with elution buffer
19. Load the protein gel

HEPES buffer: – Potsdam – 2012
http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

10mM HEPES

500mM NaCl

Washing buffer:

10mM HEPES

500mM NaCl

30mM Imidazol

Elution buffer:

10mM HEPES

500mM NaCl

50mM Imidazol

Phage amplification and clean up: – Potsdam – 2012
http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

1.Overnight culture of 20ml LB+Tet+ER2738 stock solution

2. Main culture on the next day (200ml LB+tet+overnight culture)

3. Addition of phage when OD600 0,3-0,5

4. After addition of phage: 15min in 37℃ (no shaking)

5. Move to 28℃ incubator

6. After 45min add Kana

7. After 4h (divide into 4x50ml Falcon tubes)

8. centrifuge 5000g, 15min, 4℃

9. Supernatant into new tube

10. centrifuge 5000g, 15min, 4℃

11. 4x40ml of supernatant + 4x8ml PEG-NaCl solution

12. Incubate on ice overnight in the fridge

13. Centrifuge: 5000g, 45min, 4℃

14. Discard supernatant

15. Centrifuge 5000g, 5min, 4℃

16. Remove supernatant with pipette

17. Resuspend pellet in 4x1ml TBS (pH 7,5)

18. Transfer to 4x 1,5ml Eppie

19. Centrifuge 21000g, 10min, 4℃

20. 4x900µl of supernatant mix thoroughly with 4x180µl of PEG-NaCl solution

21. Incubate on ice for 1h

22. Centrifuge 21000g, 10min, 4℃

23. Remove supernatant with pipette

24. Resuspend pellet in 0,3ml TBS (pH 7,5)

25. Centrifuge 21000g, 10min, 4℃

26. transfer 280µl of supernatant into new 1,5ml Eppie

PEG-NaCl (100ml):

20% w/v polyethyleneglycol 6000-20g

2,5M NaCl in water

TBS:

50mM Tris

150mM NaCl

pH 7,5 HCl/NaOH

RNA-Extraction with Phenol/Chloroform and LiCl-Precipitation from eukayrotic cells – Potsdam – 2012
http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

2x Lysis Buffer

1 M Tris-Cl, pH 7.5 2 mL

5 M NaCl 1,2 mL

0,5 M EDTA pH 8 1,2 mL

H2O 7,2 mL

10% SDS 8,0 mL*

- Note

Add in order given, otherwise SDS will precipitate. Don't put on Ice afterwards, otherwise SDS will precipitate.

Procedure

1) Use bottles or tubes that are set aside for RNA work. Harvest 15ml cells by centrifugation for approx. 2 minutes @ 2500g. Cells should make a nice pellet.

2) Discard the supernatant and take up the all liquid with a pipette.

3) Resuspend cells in 600 µl 1x Lysis Buffer to cell suspension and shake slowly for 20 minutes at 4℃ in coldroom.

4) Set up 2ml tubes for phenol/CHCl3 extractions

5) Extract cell suspension with 600µl ml Phenol/CHCl3/Isoamyl Alcohol (25:24:1, e.g. Roti© from ROTH) and shake well for 1 minute

6) Centrifuge @ 12000g for 5 minutes.

7) Collect upper, aqueous phase (avoid interphase material) and repeat steps 5-7. (Total of 4 Phenol/CHCl3 extractions. Do not worry if phase is pink.)

8) After second extraction add 6µl of 4mg/ml proteinase K solution (Roche) and incubate for 10 min at 65℃.

9) Repeat the steps 6-8 twice.

10) Transfer the upper, aqueous phase to a new tube.

11) Extract the upper with 400µl CHCl3/Isoamyl Alcohol to reduce phenol concentration.

12) Centrifuge 2,5 minutes @ 12000g.

13) Take upper, aqueous phase and transfer to a new 1,5ml tube.

14) Add same volume LiCl (5M) to a total concentration of 2,5M.

15) Incubate for 1h at -20℃.

16) Centrifuge @ 16000g for 20min at 4℃.

17) Discard supernatant, be carefull with the pellet.

18) Perform ethanol-precipitation: Add 500µl of 100% Ethanol to the pellet an resolve it.

19) Incubate the tube for 30 min at -20℃.

20) Centrifuge @ 16000g for 20 min at 4℃.

21) Wash pellet in 70% cold ethanol and dry in speed vac.

22) Resuspend pellet in 40 µl H20. Store at -80℃.

Glycerol Stocks of transformed Cells – Potsdam – 2012
http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

- before minipreping

take 500 µl E.coli culture add 500 µl 99.8% Glycerol

freeze at -80℃

Recipes for stock solutions **– Potsdam – 2012**

http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Ampicillin (Amp) – Potsdam – 2012

http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Stock solution: 100 mg/ml

- Filter in H2O (Milipore) !

Working concentration: 20-100 µg/ml (50 µg/ml)

Chloramphenicol (Cm) – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Stock solution: 25 mg/ml

- In 70% EtOH

Working concentration: 25-170 µg/ml (100 µg/ml)

Kanamycin (Kan) – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Stock solution: 50 mg/ml

- Filter in H2O (Milipore) !

Working concentration: 10-50 µg/ml (30 µg/ml)

Tetracycline (Tet) – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Stock solution: 25 mg/ml

- In 70% EtOH

Working concentration: 10-50 µg/ml (10 µg/ml in liquid culture; 12,5 µg/ml in Plates)

- Be careful: Light sensitive, take aluminium foil and wrap tube!

TAE (50x) – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

1L TAE (50x)

242 g tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (or 18.612 g EDTA Disodiumsalt dihydrate M=372.24)

ad 1 L

CaCl2 – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

1 M CaCl2

- 110,98 g CaCl2
- 1 l H2O milipore

1l

$CaCl_2 \times 2 H_2O$: M= 147,02 g/mol

- weight 147,02 g
- fill up to 1000ml with Milipore H_2O
- steril filtration (Big Filtration)

DYT – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

DYT growing medium

5 Liter:

80g Bactotrypton

50g Bacto yeast

25g NaCl

- add 2l Multipore –H_2O
- add the magnetic stir bar
- ad 5l Multipore –H_2O
- fill into a jar
- autoclave

LB-Medium + Agar – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Preparation of LB-Medium + Agar:

Only start to prepare the LB Medium if it will be autoclaved on the same day (8:00 am, 10:00 am, 12:00 am, 14:00 pm).

Always work with a measuring cylinder !

Take a 1L flask and fill in:

- 10 g Bactotryptone
- 10 g NaCl
- 5 g Bactoyeast
- Add 500 ml Millipore H_2O and dissolve the three substances.

- While dissolving, prepare 19 or 20 100 ml flasks and fill in 0,75 g Agar (0,015 g Agar/ml).
- Label it with your name, the date and LB+Agar.
- As soon as the substances are dissolved in 500 ml millipore H2O add another 500 ml.
- Then fill 50 ml LB-medium in each 100 ml flask and autoclave it.

Competent *E.coli* cells – Potsdam – 2012

http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

Production of competent E-coli-cells

Work always sterile and cold and speedy!

- All volumes deal with the common cell line!
- The cooling-centrifuge is in the tool shed , cool down to 4°C early enough, close the lid correctly!
- Prepare early enough min. 100 Eppis (1,5µl) (per cellline) and cool down to -80°C before using
- Use Milipore-filter for sterile CaCl2 , keep cool!
- prepare 15ml LB-Medium (or DYT) with the specific antibiotic (XL1-blue Tet, BL21 none!), inoculate and incubate over night
- prepare 200ml LB-Medium (or DYT) with the specific antibiotic, inoculate with 2ml of the over-night-culture. Nurture the culture until OD600 at 0,35 (0,2-0,5) (if the OD is too high, the cell won't be competent)
- keep cell suspension in sterile falcons (50ml) 20 min on ice, then centrifuge for 20min; 4°C; 2500g
- discard supernatant, carefully resuspend on ice with 10ml cold CaCl2-solution (put a little of the 10ml solution in every falcon before!), pool every resuspended aliquot of one cell line and add 10ml CaCl2-solution (total volume 20ml). Keep 30 min on ice, then centrifuge for 20min; 4°C; 2500g
- discard supernatant, carefully resuspend pellet in 5,5ml CaCl2(80mM)/Glycerol (4:1), aliquot in Eppis á 60µl and store immediately at - 80 °C

Glycerol – Potsdam – 2012

http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

- fill Glycerol into a flask
- autoclave

IPTG – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

MW= 238,3 g/mol

1M Stock solution: 238,3g in 1l H2O

- 20ml: 4,766g in 20ml H2O
- filtern (“sterilize by filtration)
- this solution is stable for 2-4 months

TE Buffer (1×) – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

100ml

10 mM Tris-HCl (pH 7.5) -> M= 121,14 g/m

1 mM EDTA -> M=egal; c=0,5M

Autoclave

- 1,2114g Tris + 0,2 ml EDTA (0,5M)
- add 70 ml H2O (Milipore)
- titrate with HCl to pH 7,50
- ad 100 ml H2O (Milipore)
- filter

Check for competent Cells – Potsdam – 2012 http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

- PUC18 50pg/µl -> 2 µl to 1 Eppi of cells
- Trafo
- Amp
- 1Cana +1Amp –additional plate with untransfected cells for control

Make DNA Ladder Mix (Gene Ruler, Thermo Scientific) ready to use – Potsdam – 2012
http://2012.igem.org/Team:Potsdam_Bioware/Lab/Protocols

- if you work with Gelred DON`T follow the instructions from Thermo Scientific! DNA-Ladder must be diluted 10x stronger because Gelred is very sensitiv. In the end the DNA Ladder must be diluted 1:60 in 1x Loading Dye Solution.

Example: Mix 10 µl DNA Ladder, 120 µl 5x Loading Dye Solution and 470 µl deionized wather --> 600 µl Ladder ready to use

Transformation – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Official iGEM transformation protocol: http://partsregistry.org/Help:Protocols/Transformation

We use this protocol with the following modifications:

- 45 s heat shock in stead of 60 s.
- LB medium in stead of SOC.

The cells used are Subcloning Efficiency™ DH5α™ Competent Cells from Life Technologies/Invitrogen

Inoculation after transformation – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Using a sterile toothpick, pick/scratch a single colony from the transformants. Drop the toothpick into a plastic tube with 3 mL sterile liquid medium with the appropriate antibiotic(s). Close the tube but leave the cap slightly open to allow oxygen to enter, and incuate at 37 C with shaking.

DNA Isolation – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

We use the Promega Wizard Plus SV Minipreps DNA Purification System A1460 with the sentrifugation version of the protocol supplied by the vendor.

DNA Concentration measurements – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Concentrations of DNA after isolation was measured with the NanoDrop ND-1000 Spectrophotometer.

Restriction digest – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

We are using the single reaction protocol from partsregistry.org : [1]

- 250 ng of DNA is added together with the appropriate amount of dH2O, for a total volume of 16 uL.
- Add 2,5 uL of the appropriate NEBuffer
- Add 0,5 uL og BSA
- Add 0,5 uL of Enzyme 1
- Add 0,5 uL of Enzyme 2
- The total volume should now be 20 uL. Mix well and spin down.
- Incubate the restriction digest at 37C for 1h
- Run a portion of the digest on a gel (8ul, 100ng), to check that both plasmid backbone and part length are accurate.

Ligation – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Ligation protocol from partsregistry.org [2]:

- Add 11 uL of dH2O
- Add 2 uL of each sample to be ligated (insert and backbone)
- Add 2ul of T4 DNA Ligase Reaction Buffer
- Add 1ul of T4 DNA Ligase
- Mix well, and spin down
- Incubate for 30min at 16C and 20min at 80C to heat kill
- Use 2ul of ligation to transform into competent cells

Remember to do religation of backbone!!!!!!!!

Linearized plasmids – Trondheim – 2012 http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Official iGEM protocol

Gel electrophoresis – Trondheim – 2012 http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Agarose with Gel Green is used when molding the gel. Choose an appropriate ladder as a referance to size of the fragments and put 2 µl of ladder in wells on either side of the samples. When applying the samples, add 20% loading dye to the sample. For instance, if the initial sample is 10 µl, add 2 µl loading dye. Apply the samples to individual wells in the gel.

Let the gel run for 45 min at 90 V, and if the bands are poorly separated, run a little longer. Beware that if the gel runs for a very long time, the smaller bands may move out of the gel.

Gel purification – Trondheim – 2012 http://2012.igem.org/Team:NTNU_Trondheim/Protocols

We are using the QIAquick Gel Extraction kit, following the protocol from www.qiagen.com. [3]

Preparation of samples for RNA isolation – Trondheim – 2012 http://2012.igem.org/Team:NTNU_Trondheim/Protocols

- Grow overnight cultures
- Mix 1 ml cell culture with 2 ml RNA-protect (QIAGEN)
- Vortex for 5 seconds
- Incubate for 5 minutes in room temperature
- Spin down at 6000 g for 10 minutes in 4 °C

RNA isolation – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

(RNAqueous kit from Ambion)

- Add 100 µl Lysozyme/TE-mix to each sample
- Incubate for 5 minutes in room temperature
- Add 300 µl lysis/binding solution
- Vortex to make sure everything is solved
- Add 400 µl water for 64 % ethanol
- Turn the tubes 4 times, and transfer to filtertubes
- Centrifuge for 1 minute at 13000 g
- Add 700 µl wash solution 1
- Centrifuge for 1 minute at 13000 g
- Add 500 µl wash solution 2/3
- Centrifuge for 1 minute at 13000 g
- Add 500 µl wash solution 2/3
- Centrifuge for 1 minute at 13000 g
- Centrifuge for an additional minute at 13000 g
- Add 40 µl preheated elution buffer
- Centrifuge for 1 minute at 13000 g
- Add 20 µl preheated elution buffer
- Centrifuge for 1 minute at 13000 g
- Measure concentrations on NanoDrop

DNAse reaction – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

- Calculate the volume of RNA solution necessary to obtain a total of 3000 ng RNA
- Add SIV to 25 µl
- Add 2.7 µl DNAse buffer and 1 µl DNAseI
- Incubate for 30 minutes at 37 °C
- Add 5 µl inactivation mixture and flip the tubes
- Incubate for 2 minutes in room temperature
- Centrifuge for 2 minutes at 13000 g
- Transfer 2 µl of the supernatant to a new tube and add 18 µl SIV
- Incubate for 10 minutes at 65 °C

cDNA reaction – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

- Prepare bulk mixture:
  - 5 µl bulk reaction mixture
  - 1 µl RNA primers
  - 1 µl DTT
- Mix 4 µl sample with 3.5 µl bulk mixture
- Incubate for 1 hour at 37 °C
- If qPCR is not to be performed immediately, the cDNA samples should be frozen down at -80 °C

OD measurements – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Unless otherwise noted, all OD measurements are made at 600 nm with a PerkinElmer Lambda 35 spectrometer, with un-inoculated medium as reference.

Fluorescence measurements – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Fluorescence were measured with a Tecan Infinite M200 Pro microplate reader using Nunclon flat bottom black polystyrol 96 well plates.

Recipes

Growth media – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

LB-medium (LB-Lennox):

Antibiotics – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Ampicillin stock solutions: 100 mg/mL dissolved in MQ water
Kanamycin stock solutions: 100 mg/mL dissolved in MQ water
Chloramphenicol: 34 mg/mL dissolved in 100% ethanol

Store at -20 C after preparation and between use.

Ampicillin media concentration: 100 ug/mL = 1 mL stock solution/L medium
Kanamycin media concentration: 100 ug/mL = 1 mL stock solution/L medium Chloramphenicol media concentration: 24 ug/mL = 0.7 mL stock solution/L medium

When transforming low copy number plasmids, it may be necessary to reduce the antibiotic to allow for the reduced antibiotic resistance of the tranformant.

Glycerol stocks – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Prepare a solution of 60 % glycerol in water. Add 400 uL glycerol solution and 800 uL of the culture to be stored in a cryogenic tube. Place in 5 C refrigerator for 30 min, then move to -80 C freezer.

Cake – Trondheim – 2012

http://2012.igem.org/Team:NTNU_Trondheim/Protocols

Batter is prepared following the protocol by Kakemonsen (1). Sliced pieces of *Malus domestica* is inserted into the batter, while sucrose and ground *Cinnamomum verum* bark is mixed 2:1 by volume and distributed evenly on top of the batter, which is placed in a 26 cm diameter open-top vessel. The mixture is incubated in a dry air oven at ~180 C for 45 min.

Agar media (Plates) – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

1) To a mixture of NaCl (5g), tryptone (5g), agar (7.5g) and yeast extract (2.5g), 250ml of distilled water was added and mixed in well.

2) An additional 250ml of distilled water was added.

3) This was heated till boiling.

4) Autoclaved at 121ºC, 15psi for 10mins.

5) Into warm media (~50℃) antibiotic at the required concentration was added.

6) The media was then poured into labelled plates.

7) After drying plates were stored in the fridge until.

Cell Counting – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1. Cells were suspended in media in wells and diluted (1:1) in 25ul of trypan blue (detects dead cells). Prepare Hemacytometer and carefully squirt samples into chambers.

2. These were then placed under microscope and count cells in central chamber (5x5 grid). Cells on border are counted too. Dead blue cells are not counted.

X (cell count) x 2 (dilution factor) x 104 = cells / ml

e.g. 42 x 2 x 104 = 840,000 cells per ml

. To calculate volume with desired number of cells.

No cells needed/cells per ml e.g. 200,000 / 840,000 x 1000 (ul) = 238ul/well

Flow Cytometry – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) Grow transformed Ecoli competent cells in an overnight culture containing 0, 1 or 10mM of Potassium Nitrate.

2) Pellet the cells then fix them in 4% PFA (500µL)

3) Spin down and resuspend in PBS (500µL)

4) Analyse samples using a Flow Cytometer:

BM-RFP transformed Ecoli were analysed using a BD Acuri C6 flow cytometer. Laser 488nm / Filter FL2-585

MB-CFP samples were analysed using a BD FACSAria II Cell Sorter. Laser 405nm. Detect V/450/40-A

Fluorometry Testing – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) Samples selected for testing were grown overnight at 37 degrees Celsius.

2) Samples of 5ml were pelletted in an eppendorf tube.

3) The pellets were resuspended using Tris buffer (1mL).

4) The cells were lysed using sonification. The sonifier was cleaned using ethanol. The samples were put in ice for 1 minute between each sonification. Each sample was sonified 5 times. Each time for 10 secs. Note: Be careful that the eppendorf does not touch the sonifier as it may shatter the eppendorf.

5) These were then centrifuged at a cold temperature for 10mins.

6) Following centrifugation the clear liquid was pipette off into fresh eppendorfs.

7) These samples were then diluted with Tris buffer (1500µL of Tris to 500µL sample).

8) Depending upon the reporter protein attached, the emission and excitation spectrums were different. For CFP, an excitation of 410nm and an emission wavelength scan of of 440nm to 500nm was used. For RFP an excitation of 560nm and an emission spectrum of 600-650nm was measured.

9) The fluorometer was zeroed using Tris buffer.

Gel Electrophoresis – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) An agarose gel (1% w/v) is made by dissolving 1g of agarosse mixed in with 100ml of 1X TAE buffer which is heated before the addition of 5µL of ethidium bromide when hand holdable warm. (Ensure this is done in a fume cupboard with the relevant safety equipment). Depending on the fragment sizes expected different densities of gel were made. The smaller the fragments, the denser the gels.

2) Tray is assembled through taping the ends. After addition of agarose, put in the comb to create wells. The agarose mixture was poured into this.

3) When dry this was put into the electrophoresis machine and the wells filled.

4) Electrophoresis was run for 1 hour to 1 and a half hours at 120V.

Hydrating Dry DNA (Synthesised DNA) – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) The dry form synthesised DNA was centrifuged for 1min at 14500 rpm.

2) To these tubes, 20 µL of sterilised water was added.

3) These tubes were vortexed briefly (30secs) and centrifuged briefly (20secs).

4) These were then placed in ice.

Inoculations – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1. To 5ml samples of sterilised LB broth antibiotics were added. Ampicillin was added at 500 µl and chlorophenicol was added at 3.75ml.

2. To each of these broths, E.coli colonies growing on plates was inoculated.

Isolating DNA (Bioline) – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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(Using Bioline ISOLATE Plasmid DNA Mini Kit and following the given instructions for low copy plasmids)

1) The culture was centrifuged at 14500rpm for 1 min to obtain a cell pellet.

2) The supernatant was discarded and the pellet resuspended with 500 µL of Resuspension Buffer using a vortex mixer or pipetting.

3) To the sample, 500 µL of Lysis Buffer P was added and mixed by inversion 5 times.

4) An aquilot of 600 µL of Neutralisation Buffer was added and the sample inverted 5 times.

5) To obtain a cell debris pellet the solution was spun at 14500rpm for 10 mins and the supernatant transferred to a collection tube.

6) After 1 min of centrifugation at 12000rpm the filtrate was discarded and 500 µL of Wash Buffer AP was added.

7) Another round of centrifugation at 12000rpm for 1 min was followed by the discard of the filtrate and the addition of 700 µL Wash Buffer solution.

8) The sample was centrifuged at 12000rpm for 2 mins and the spin column placed in an elution tube.

9) To the tube 100 µL of Elution Buffer was added and the sample incubated at room temperature for 1 min.

10) The isolated DNA was stored in eppendorf tubes and refrigerated at 4 ºC for later use.

LB Media – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) To a mixture of NaCl (5g), tryptone (5g) and yeast extract (2.5g), 250ml of distilled water was added and mixed in well.

2) An additional 250ml of distilled water was added.

3) This was heated till boiling.

4) The media was then transferred into culture flasks (5.5ml each) with the lids partially closed.

5) These were autoclaved at 121°C, 15psi for 10mins.

Ligation – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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Digest vector and insert with relevant enzymes.

Use the equation to calculate ng of insert required for different ratios:
3 insert: 1 vector
1 insert: 1 vector
1 insert: 3 vector

$$\frac{\text{Ng vector x bp size of insert}}{\text{Bp size of vector}} \times \frac{\text{ratio of insert (e.g 3)}}{\text{ratio of vector (e.g 1)}} = \text{ng insert}$$

For a 20µl total reaction:
2µl T4 DNA ligase
2µl T4 DNA ligase buffer
16µl insert : vector ratio

Leave at room temperature overnight

Potassium nitrate solution (1M Stock Solution) – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) 20.2g of potassium nitrate was dissolved in distilled water.

2) The dissolved solution was made up to 200ml.

3) This was autoclaved at 121°C, 15psi for 10mins.

Purification by Centrifugation – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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Using Promega Wizard SV Gel and PCR Clean-Up System kit

1) Using 10µL of membrane binding solution per 10mg of gel slice ratio, the gel slice was dissolved.

2) The dissolved solution was added to an SV minicolumn inside a collection tube.

3) This was left for 1min at room temperature.

4) This was centrifuged for 1 min at 14 000 rpm.

5) The overflow was discarded.

6) Membrane Wash Solution (700µL)containing ethanol was washed through and centrifuged for 1 min at 14 000 rpm.

7) The overflow was discarded and Membrane Wash Solution (500µL)was washed through and centrifuged for 5mins at 14 000 rpm.

8) The overflow was discarded and recentrifuged with the lid off for 1min at 14 000 rpm.

9) Nuclease Free Water (50µL) was added to the SV column which had been transferred from a collection tube to an eppendorf tub. This was left for 1 min at room temperature before centrifugation for 1min at 14000rpm.

10) This was stored in a fridge.

Restriction Digest – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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For multiple samples which need to be digested with the same enzymes, a master mix was created, which involved assembly of buffer, BSA and enzymes at a higher amount than that needed. This saved time decreased the inaccuracy of pipetting very small quantities.

Note: The buffer is chosen through maximising the efficiency of the enzyme or enzymes.

Single Digest for validation – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) A mastermix of a multiplication of the following amounts were set up in an eppendorf and mixed through pipetting up and down then briefly centrifugation: buffer (2µL), acetylated BSA at concentration of 10µg/µL (0.2µL) and restriction enzyme (0.5µL) were assembled in an ependorf. Direct assembly was made if only one sample was needed.

2) Into another eppendorf, DNA (1µL) and sterilised and deionised water (16.3µL) was added.

3) From the master mix, 2.7µL was pipetted into the DNA and water mixture.

4) The mixture was mixed through pipetting up and down with restriction enzymes.

5) The tube was centrifuged briefly.

6) This was incubated at 37°C for 2-3 hrs.

Double Digest for isolation of a fragment – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) A mastermix of a multiplication of the following amounts were set up in an eppendorf and mixed through pipetting up and down then briefly centrifugation: buffer (2µL), acetylated BSA at concentration of 10µg/µL (0.2µL) and restriction enzymes (0.5µL of each) were assembled in an ependorf. Direct assembly was made if only one sample was needed.

2) From the master mix, 3.2µL was pipetted into separate eppendorfs.

3) Into these tubes, 1µg of DNA and water that makes each sample up to 20µL is added. The water is added first.

4) The mixture was mixed through pipetting up and down with restriction enzymes.

5) The tube was centrifuged briefly.

6) This was incubated at 37°C for 5 hours to overnight.

Transfection – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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Mammalian Transfection MB-CFP

Media:

. DMEM (Invitrogen #21885) without serum (SFM-DMEM) or with serum DMEM #21885 + 10% Fetal Calf Serum.

. DNA is Mammalian-Bacterial – Cyan Fluorescent Protein, Hybrid NO sensor MB-CFP.

. LipoD293 Transfection reagent: (SignaGen #SL100668)

. Nitric Oxide donor: SNAP S-Nitroso-N-Acetylpenicillamine (SNAP), 25 mg (Molecular Probes® #N-7892)

. SNAP (Stock 25mg/ml DMSO) 500µM final conc.

. Mammalian cells (MCF7-Human cancer cell line)

1. Cells were seeded into 6 channel slide (Ibidi µ-Slide VI0.4 #80606) at 30µl of 3x105 cells/ml dilution.

2. On day of transfection make Tube 1 (50ul SFM + 3µl LipoD) Tube 2 (50ul SFM + 0.5µg DNA).

3. Scrape tubes / vortex to mix well.

4. Add Tube 1 (LipoD) to Tube 2 (DNA).

5. Scrape tubes / vortex to mix well and leave for ~15min.

6. Meanwhile remove media from cells, wash 100µl SFM DMEM.

7. Add 50µl Transfection mixture to relevant channel of Ibidi 6-channel slide. (Each channel is transfected with 250ng of DNA.)

8. Remove media 6 hours post transfection and replace with 150µl DMEM+FCS.

9. The following day SNAP was added (500µM final concentration).

10. ~24h post transfection cells imaged on CCD2 inverted

Transformation – UEA Norwich – 2012 http://2012.igem.org/Team:NRP-UEA-Norwich/Protocol

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1) Competent cells were thawed on ice for 15mins

2) Into pre-chilled 2ml tubes, 50 µL competent thawed cells and 2µL of re-suspended DNA were added and mixed through flicking.

3) These were put back into ice and left for 30mins.

4) From the ice, the tubes were taken and immersed in a 42 ºC water bath for 1 min.

5) After heat shocking, cells were incubated on ice for 5mins.

6) To these cells, 200 µL of pre-warmed LB broth (37 ºC) was added.

7) Cells were then incubated at 37 ºC for 2 hours and shaken every 5mins

8) From each tube two inoculations were made. 1) 20 µL of cells plus 180 µL of LB on chloramphenical LB agar plates, 2) 200 µL of cells. Agar plates contain chloramphenicol at a concentration of 50µg/ml.

9) These were incubated for 17 hours at 37 ºC.

1.PCR: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

1.1.Colony PCR: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

Prepare mastermix according to your sample amount (except Taq and template)

(For 1x sample preparation)

- dNTP 1 µL
- Buffer 5 µL
- MgCl2 3 µL
- **Forward Primer 0,5 µL
- **Reverse Primer 0,5 µL
- Template -
- Taq Polymerase 0,2 µL
- dH2O 39,8 µL
- TOTAL 50 µL

* You should add ingredients from largest amount to smallest amount.

* Before addition of primers and template you can do vortex.

** First you should pour ddH20 onto dried primers according to the amount written in the primer sheet then you should dilute (1:10) it in to new eppendorf. (10 ml primer+ 90 ml ddH20)

Pour your samples into PCR tubes and add template that you pick from the petri with toothpick or tip and finally add taq polymerase.

Then place your samples into the PCR machine and do regular PCR.

1.2 PCR: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

For a 25ul rxn:

- Use 1ul of 60ng/ul or 100ng/ul DNA

- Use 1ul of each primer at 3.2pmole/ul concentration or 1.25ul of each primer at 100ng/ul concentration
- 2.5ul 10x PCR Buffer w/ Mg (1.5mM)
- 0.5ul 25mM MgCl2
- 0.5ul dNTP
- 0.125ul Taq
- 18.375ul sterile water to equal a 25ul rxn

(*if not making master mix, dilute Taq so that you can add 1ul of Taq and 17.5ul sterile water to equal a 25ul rxn)

For a 50ul rxn:

Use 2ul of 60ng/ul or 100ng/ul DNA

Use 2ul of each primer at 3.2pmole/ul concentration or 2.5ul of each primer at 100ng/ul concentration

5ul 10x PCR Buffer w/ Mg

1ul 25mM MgCl2

1ul dNTP

0.25ul Taq

36.75ul sterile water to equal a 50ul rxn

(*if not making a master mix, dilute Taq so that you can add 1ul of Taq and 36ul sterile water to equal a 50ul rxn)

Keep the reagents on ice.

Add the Taq last, and keep it in the freezer until you are ready to add it.

Vortex briefly and quick spin.

Cycle:

- 95°C for 1-5minutes (usually 4min)

- 95°C for 1min
- 55°C for 1min Cycle 30 times
- 72°C for 1.5 to 2min (usually 2min)

- 72°C for 10min
- 4°C hold

2.CULTURES: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

2.1 Liquid Culture: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- 5 ml LB is mixed with appropriate antibiotic and a culture tube is prepared with certain labels.
- A pipette tip is used to pick up a colony from the plate and release it into the LB by stirring and pipetting up and down
- Incubate the culture at 37°C for 13-15 hours. Do not let the cells become old to not release their plasmids
- If the culture becomes saturated: you can reinoculate 30µL into a new 3mL LB tube

2.2 Glycerol Stock: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- Put 500µL of a mid-log culture into a 1.5mL tube with 500µL 80% glycerol
- Store at -80C

2.3 Spreading Plates: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- Put 50 µL and 150 µL of whether LB culture or transformed cells.
- Spread the cells into the plate until there is no seen liquid.
- Wait until the plates dried.
- After 14-16 hours colonies will show up.

2.4 Streaking Plates: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- Put a drop your culture on the plate
- Using a pipette tip, spread the drop out into a zigzag. Then use one edge of the zigzag to draw out another zigzag. Repeat to have about 3 zigzags (this makes the culture get spread out more and more with each streak)
- Wait until the plates dried.
- After 14-16 hours colonies will show up.

3.GELS: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

3.1 Gel Preparation (%1 gel) - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- Add 0,5 gr agarose to 50 ml TAE buffer.
- Until agorose being melted, heat it in microwave and do not forget to stir it often.
- After melting, it should be cool enough to handle it.
- Add 5 ml EtBr.
- Then, pour it to container and pay attention not to form bubbles in gel.

3.2 Gel Photo Imaging: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- Adjust zoom and position using visible light
- Before turning on UV load your settings file which has the following parameters:
- Preview tab, all three options checked
- Active image
- Dynamic integration, auto exposure, 10 frames
- 50/50 brightness/contrast
- Maximize brightness with camera knob (counterclockwise)
- Turn on UV light
- Lower brightness from camera knob if necessary

4.GETTING THE DNA PARTS FROM KIT PLATE: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- 10 ul ddHO is added into the kit plate target part.
- Wait 5 min.and get the part by well pipetting.

5.TRANSFORMATION: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

Materials:

- Resuspended DNA (2 ul)
- Competent cells (50ul per transformation)
- Ice
- 2ml tube (1 per a transformation')
- 42ºC water bath
- Petri dishes with LB agar and appropriate antibiotic (2 or 3 per transformation)
- Glass beads or spreader
- 37ºC incubator

Preparation:

- Start thawing the competent cells on ice.
- Add 50 µL of thawed competent cells into pre-chilled 2ml tube.
- Add 1 - 2 µL of the resuspended DNA to the 2ml tube. Pipet up and down a few times, gently. Make sure to keep the competent cells on ice.
- Close tube and incubate the cells on ice for 30 minutes.
- Heat shock the cells by immersion in a pre-heated water bath at 42ºC for 60 seconds.
- Incubate the cells on ice for 5 minutes.
- Add 400 µl of LB media (make sure that the broth does not contain antibiotics and is not contaminated)
- Incubate the cells at 37ºC for 2 hours while the tubes are rotating or shaking. Important: 2 hour recovery time helps in transformation efficiency, especially for plasmid backbones with antibiotic resistance other than ampicillin.
- Label two petri dishes with LB agar and the appropriate antibiotic(s) with the part number, plasmid backbone, and antibiotic resistance. Plate 20 µl and 200 µl of the transformation onto the dishes, and spread. This helps ensure that you will be able to pick out a single colony.
- Incubate the plate at 37ºC for 12-14 hours, making sure the agar side of the plate is up. If incubated for too long the antibiotics start to break down and un-transformed cells will begin to grow. This is especially true for ampicillin - because the resistance enzyme is excreted by the bacteria, and inactivates the antibiotic outside of the bacteria.
- You can pick a single colony, make a glycerol stock, grow up a cell culture.

6.RESTRICTION DIGESTION: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

NEB Buffer 5 µl

BSA 0.5 µl

Enzyme 1 0.5 µl

Enzyme 2 0.5 µl

Plasmid* à 1000ng/ml

To complete to 50 µl, add ddH2O.

1- Keep it in waterbath for 2.5 hours at 37°C.

2- Run the samples on the gel.

7.LIGATION: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

- 10X T4 ligase buffer: 2.0 µL
- 6:1 molar ratio of insert to vector (~10ng vector)
- Add (8.5 - vector and insert volume)µl ddH2O
- T4 Ligase: 1 µL
- Incubation at room temperature 1 hour and 15 min at 65°C for enzyme inactivation.
- Alternatively: Incubation at +4°C overnight.

Calculating Insert Amount:

8.MATERIALS AND CHEMICALS: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

8.1 TAE Buffer: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

For 1 L of 50 x TAE buffer you need:

242.48 g Tris

41.02 g Sodiumacetate

18.612 g EDTA

Adjust pH to 7.8

Solve in dH2O

20 mL of the stock is diluted in 1 L dH2O for the gel electrophoresis.

8.2 DNA Loading Buffer

50 % (v/v) glycerol

1 mM EDTA

0.1 % (w/v) bromphenol blue

Solve in ddH20

8.3 LB Medium

For 1 L of LB medium you need:

10 g Trypton

5 g yeast extract

10 g NaCl

12 g Agar-Agar (for plates)

Adjust pH to 7.0

8.4 LB Agar

For 1 L of LB Agar you need

10 g Trypton

5 g yeast extract

10 g NaCl

12 g Agar-Agar (for plates)

Adjust pH to 7.0

9.QIAGEN Kits - METU – 2012 http://2012.igem.org/Team:METU/Protocols

9.1 Plasmid Isolation: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

Usually there is a protocol in the kit. First, you should read it.

- Take 2 ml cell in to the eppendorf (2ml).
- Spin down the cells at 10000g for 1 min.
- Discard supernatant.
- Add 2 ml cell in to the eppendorf again, totally 4 ml will be pelleted.
- Spin down the cells at 10000g for 1 min.
- Discard supernatant.
- Resuspend the cells with 250 µL p1* buffer and vortex.
- Add 250 µL P2 buffer and make sure the blue color is equally spread. Gently inverse the eppendorf 4-5 times. DON’T VORTEX!!!
- Wait 5 min at room temperature.
- Add 350 µL N3 buffer (cold +4°C) inverse gently and immediately.
- Incubate on ice for 15 min.
- Spin down the cells at 10000g for 15 min at +4°C.
- Place the supernatant into the tube that is placed into the kit.
- Spin down the cells at 10000g for 1 min.
- Remove the below part of the tube and discard supernatant.
- Add 500 µL PB buffer.
- Add 750 µL PE buffer and wait 1 min near the flame.
- Spin down the cells at 10000g for 1 min.
- Spin down the cells at 13000g for 1 min again.

- Wait 5 min near the flame.
- Put the filter parts onto the 1,5 ml eppendorfs.
- Add 50 µL elution buffer.
- Wait 1 min near the flame.
- Spin down the cells at 13000g for 1 min.
- Store your plasmids at+4°C for 1 hour before further experiments.

9.2 Gel Extraction: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

We used QIAGEN kit do with the following changes:

http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAquickSpin_EN.pdf

Changes in that protocol:

At step 0: Wear disposable lab coat or long sleeves to prevent UV-burn. Use a razor blade/scalpel to excise the band.

At step 4: Add 10 ul for 3M NaOAc no matter if the color is yellow or not. This will correct the pH and should turn the QG back to yellow.

At step 7: Combine samples of identical DNA in the same column (spin multiple times if necessary).

At step 13: Elute in 30 ul rather than 50 ul to really maximize yield.

9.3 PCR Purification - METU – 2012 http://2012.igem.org/Team:METU/Protocols

We used the protocol provided with the QIAGEN kit.

- 3 volumes of Buffer QG is added to 1 volume of the PCR sample and mixed by vortexing.
- 1 volume of the INITIAL sample volume of isopropanol is added to the sample and mix.
- Place a QIAquick spin column in a provided 2 ml collection tube. Apply the sample to the QIAquick column with the pipette and centrifuge for 30–60 s.Discard flow-through. Place the QIAquick column back into the same tube

- Add 0.75 ml Buffer PE to the QIAquick column with the pipette and centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back in the same tube.
- Centrifuge the column for an additional 1 min.
- Label the top of clean 1.7 ml microcentrifuge tube(s) with the name of your sample(s). Transfer QIAquick column(s) to the tube(s).
- To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane. Let it stand for 1 min and centrifuge the column for 1 min.

10.Competent Cells: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

Materials:

- Detergent-free, sterile glassware and plasticware
- Table-top OD600nm spectrophotometer
- 1 ml DH5 alpha cells
- LB
- 0,1M CaCI2

Preparation:

- Take 1ml from the DH5 alpha cells which were grown one day ago and put it in to 100ml LB medium. Keep it at 370C for 2 hours and measure it by spectrophotometer at 600 nm wavelength until absorbance reaches OD of 0.375.
- Divide 100 ml sample into 2 falcon and centrifuge for 10 min at 5000 rpm at +4°C.
- Discard the supernatant.
- Add 10 ml 0.1 M cold CaCl2 into supernatant and dissolve the pellet. Put it on ice for 10 min.
- Centrifuge at 5000 rpm for 5 min at +40C. Then, discard the supernatant.
- Add 10 ml CaCl2 and dissolve it by shaking it up and down.
- Put the sample on ice for half an hour.
- Centrifuge at 5000 rpm for 5 min at +4°C .Then, discard the supernatant.
- Put 2 ml CaCl2 and dissolve the pellet.

· Put it on ice for 5 min and keep it at +4°C.

11. 3A Assembly Kit: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

We used the linear plasmids and the procedure that the iGEM provides us;

11.1 Digestion: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

Enzyme Master Mix for Plasmid Backbone (25ul total, for 6 rxns)

5 ul NEB Buffer 2

0.5 ul BSA

0.5 ul EcoRI-HF

0.5 ul PstI

0.5 ul (Used to digest any template DNA from production)

18 ul dH20

Digest Plasmid Backbone

Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)

Add 4 ul of Enzyme Master Mix

Digest 37C/30 min, heat kill 80C/20 min

11.2 Ligation: - METU – 2012 http://2012.igem.org/Team:METU/Protocols

Add 2ul of digested plasmid backbone (25 ng)

Add equimolar amount of EcoRI-HF SpeI digested fragment (< 3 ul)

Add equimolar amount of XbaI PstI digested fragment (< 3 ul)

Add 1 ul T4 DNA ligase buffer.

Note: Do not use quick ligase

Add 0.5 ul T4 DNA ligase

Add water to 10 ul

Ligate 16°C/30 min, heat kill 80°C/20 min

Transform with 1-2 ul of product

Agarose Gel Electrophoresis – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

For the analysis of PCR-amplified products, agarose gel electrophoresis is the method of choice. This method takes advantage of the separation of DNA in dependance of the charge-mass ratio. The separation is based on the electric attraction of the negative charged DNA which is guided towards the positive charged anode upon application of a current. The PCR samples are run on agarose gels with different percentages according to the product sizes: small products run faster than bigger products. Later on, these fragments within the gel are made visible by examination under the UV light to ensure the correct DNA fragment length synthesized in the PCR reaction. Prior to UV analysis, a staining method of the DNA, here using ethidium bromide (EtBr), is obligatory. EtBr is an intercalating agent which embeddes itself within the DNA helix. Thus, the absorption spectrum is biased so that it is suitable for DNA detection. The determination of separated molecule sizes is done accodrding to a common DNA size standard.

Pouring the Gel

1 % agarose gels are standard to separate DNA. The percentages and thus the degree of polymerisation of the gel influences the degree of separation, i.e. PCR products of similar size can be distinguished by applying a percentage lower than 1 %. To pour a 1 % agarose gel weight 1 g of Ultra Pure Agarose for every 100 ml of 1x TAE buffer. For preparation of 1x TAE buffer fill 10 mL of 50x TAE buffer stock ad 500 mL ddH_2O. Ensure that the 1 % agarose in 1x TAE buffer is boiled throughly and dissolved completely without streaks. Incomplete boiling will bias the separation results. The liquid is poured in a gel tray with the wanted comb which are assembled in a holder. After solification the gel is placed in a gel chamber and is fully covered with 1x TAE buffer post removal of the comb.

Loading the Gel

Prior to loading, the DNA samples are homogenized with 6x Loading Dye (LD) resulting in a 1x final concentration of the LD (4 µL 6x LD ad 20 µL PCR reaction). Be aware of the fact, that PCR samples should not be mixed with

LD if further experiments with the samples are needed to be done. If this is the case, mix the PCR samples with LD on parafilm. Meanwhile, the PCR samples are kept on ice. The 1 kb DNA ladder is stored in the 4 °C fridge and is suitable for larger PCR products ranging from 250 bp to 10000 bp (GeneRuler 1 kb DNA Ladder (Fermentas)). Load the gel with x µL of LD-mixed samples next to the first well with 5 µL marker. The amount loaded for PCR samples depends on the concentration and the size of the products. Note that PCR tubes should be closed to prevent drying up exposed to air.

Running the Gel

When all samples have been loaded, connect the power supply. Ensure that the plus pol is at the opposite site of the loaded wells. Run big gels applying 100 V for a more accurate separation for about 1 h. The time may be adjusted according to the loading front.

Staining the Gel

To make the separated DNA bands visible under the UV light, the samples were stained in a EtBr bath for about 10 min. Then, the gel was rinsed in water for approximately 5 min. The EtBr stained gel was exposed to UV light under a gel documentation station, a gel photo was taken and saved in the iGEM2012 folder. The file should be labelled with the date, group number and short description of what was analyzed. A printed version should be pasted into the group notebook.

Cloning Protocols – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

Cloning Protocols: Restriction (double digest)

- choose the correct restriction enzymes depending on your backbone and insert (e.g. backbone: *SpeI* and *PstI*; insert: *XbaI* and*PstI*)
- calculate the appropriate amount of enzyme which is necessary for a 5 fold over-digestion. Therefore fill in all parameters of the following equation:

$$\frac{\lambda DNA(48500\ bp) \times restriction\ sites}{backbone\ or\ insert\ size\ (bp) \times \lambda{-}restriction\ sites} = \mathrm{U}\ /\ 1\ \mu\mathrm{g\ DNA}$$

- Keep in mind that you have to calculate the enzyme amounts separately for backbone **AND** insert (if the differ in size). The result

of th the equation must be divided by 10 because most enzymes have a concentration of 10 U / µL. Finally, you have to multiply the value by factor 5 for 5 fold over-digestion in order to guarantee a complete digestion of the parts.

- choose the buffer by using the following tool: Fermantas double digest
- prepare reaction mix: 1 µg template, buffer, enzyme 1 and enzyme 2 (dependent on the concentration it is often necessary to add purified water)
- Incubation for 1.5 h at 37 °C

Cloning Protocols: Ligation

- Combination of 50 ng of vector with a 2-fold molar excess of insert.
- Addition of an appropriate volume of T4 Buffer.
- Addition of 0.5-1 µL T4 ligase.
- Adjustment of volume to 20 µL with ddH_2O
- Incubation over night at 16 °C
- Deactivation of ligase for 10 min at 65 °C

Cloning Protocols: Chemical Transformation

- Thawing of competent cells on ice
- Addition of 1 µL DNA
- Incubation on ice for 20 min
- Heat shock for 1 min at 42 °C
- Incubation on ice for 5 min
- Addition of 270 µL LB
- Incubation in thermoblock for 45 min (incubation time dependent on used vector!) at 37 °C and 300 rpm
- Smearing of cells on plates and incubation at 37 °C (centrifuge it, discard supernatant, resuspend cells in rest-medium, 20 µL on plate)

Competent Cells – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

Chemical Competent Cells

Preparation of $CaCl_2$ buffer for competent cells! Before you start make sure that the $CaCl_2$ buffer is ice-cold when needed and the centrifuge is cooled to 4 °C.

- Inoculate LB-liquid medium with 1 ml of overnight culture.
- Let cells grow to OD_{600} 0.4-0.6.
- Transfer them to 50 mL Falcons and centrifuge for 5 min at 1500 g and 4 °C.
- Resuspend pellet in 5-10 mL $CaCl_2$ buffer; here 7 mL (prechilled).
- Centrifuge 5 min 1500 g, 4 °C
- Resuspend pellet in 5-10 mL $CaCl_2$ buffer; here 7 mL (prechilled).
- Incubate cells 30-60 min on ice; here 30 min; Eppis prechilled in 5-10 mL on dry ice (or in liquid nitrogen)
- Storage at -80 °C

Electrocompetent Cells

- Cells grow to OD_{600} 0.5
- 2x washing with 10 mL % 15 Glycerol
- Pellets are resuspend in backwash, pooled to about 1mL
- centrifuge at 4800 rpm at 4°C for 5 min
- remove uper clear part
- electroporation (100µL per cuvette)

Transmission Electron Microscopy, negative staining with 2 % PTA – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

The TEM and the sample preparation was conducted by Dr. Michael Hoppert.

Strains

- MG1655
- BL21 J61002_rfp
- BL21 pSB1C3_QC_fliC_18C
- BL21 pSB1C3_QC_flhDC_18C

Media and buffers

- LB-broth
- LB-agar
- 2 % PTA (phosphotungstic acid)

Execution Inoculate the strains of interest in 5 mL L-broth with necessary antibiotics respectively

- Incubate them at 37 °C with 100 rpm (to minimize the chance of the flagella breaking of) over night
- Inoculate fresh 5 mL LB with necessary antibiotics with the overnight culture
  - For electron microscopy of flagella it is necessary to reach a high cell density without reaching a growth state where the flagella are discarded
    - The first time electron microscopy was conducted the new cultures were inoculated with 200 and 500 µL of the overnight culture, but for the cell density was not high enough the one ml of the cultures was spun down
  - One possibility to reach a high OD is to pour LB-agar (with antibiotics) in the glass test tubes and cover it with LB-broth (with antibiotics) and inoculate it, nutrients can freely diffuse through the agar as well as the broth but the cells are constricted to the liquid phase and thus a higher density can be reached
    - The second time electron microscopy was conducted the method described above was applied
- Let grow until a suitable OD_{600} is reached and check the motility of the cells with the light microscope
- Prepare the samples
  - Place a drop of the culture, a drop of ddH_2O and a drop of 2 % PTA on a slice of parafilm
  - Pick a TEM grid (the microscopic slide in TEM) with forceps and place it on the drop of the culture for a suitable amout of time
  - Pick the grid up and let the cell suspension dry for a suitable amount of time
  - Place the grid on the 2 % PTA for a suitable amount of time
  - Let the grid dry for a suitable amount of time

- Wash the grid through placing it on the ddH_2O drop for a suitable amount of time and let it dry afterwards
- Place the prepared grid in the electron microscope

Library Selection – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

The library containing vectors with the mutagenized *tar*-gene was transformed into the *E. coli* strain BL21. In order to determine certain receptor derivates that enables chemotaxis to a certain molecule a "Library Selection" protocol was determined. For hints and advice in general for swimming assays view the method "swimming/chemotaxis assay".

Attractants

- Coffein: stock solution x mM
- 2-Ethyl-1-Hexanol
- Geraniol
- Sodium-Cyclamat
- D-aspartic acid
- L-aspartic acid 4-benzylester
- Vanillin

Media

- LB-broth
- 0.3 % tryptone-swimming agar(1 % tryptone, 0.5 % NaCl, 0.3 % agar)
- LB agar

Execution

First round of selection

- Thaw one 1 ml cryostock of the library in BL21 and pour into a 200 mL flask filled with LB-media with chloramphenicol
- Inoculate the BL21 strain with the parent plasmid in 5 mL LB broth with chloramphenicol
- Grow the cultures over night at 37 °C with approx. 180 rpm

- Fill 7 + 1 control (whatmanpaper with H2O) 12 cm petridishes with 0.3 % tryptone-swimming agar with chloramphenicol
- Apply 100 µL of the attractant to a steril 2x2cm whatmanpaper respectivly and position it in the center of a petridish
- Spin down at least 1.5 mL of the culture containing the library and at least 1.5 mL of the culture containing the BL21 strain with the parent plasmid with 1.5 X g for 10 minutes
- Discard the supernatant and resuspend the pellet in the remaining medium
- Drop 3 times 5 µL of the library and once 5 µl of the reference strain (BL21 with the parent plasmid) respectively, on each plate
- Let the drops dry for at least 20 minutes until inverting the plates and placing in the incubator at 33 °C over night

Second round of selection

- Determine the drop with the fastes and most directed swimming behaviour on each plate
- In order to select the fastest cells the cells containing agar is cut out:
  - Cut the yellow eppendorf tips of to the first mark (approx 1 cm)
  - The first cut out is shortly befor the swimming front --> I
  - The second cut out is on the swimming front --> II
  - The third cut out is shortly behind the swimming front --> III
  - Place each cut out either with or without the tip in at least 0.5 ml LB media in an test tube or an E-cup
- Incubate the cultures for at least 1 h at 37 °C with approx. 180 rpm
- Meanwhile fill 7 + 1 control (whatmanpaper with H_2O) 12 cm petridishes with 0.3 % tryptone-swimming agar with chloramphenicol
- Apply 100 µL of the attractant to a steril 2x2 cm whatmanpaper respectivly and position it in the center of a petridish
- Transfer the culturesinto an E-cup and spin them down with 1.5 X g for 10 minutes
- Discard the supernatant and the resuspend the pellet in the remaining medium
- Drop 5 µl of the 3 different library cut outs and 5 µl of the reference strain (BL21 with the parent plasmid) respectively on each plate
- Let the drops dry for at least 20 minutes before inverting the plates and placing them in the incubator at 33 °C over night

Third round of selection

- See second round of selection

Plating of the selected clones

- The plates of the third round of selection are treated as described before, but the cultures are not spun down
- 100 µl of a 10^{-2} dilution is plated on LB-plates containing chloramphenicol respectively
- Incubate the plates in an incubator over night at 33 °C

Minipreparation and sequencing of plasmid DNA

- A suitable amount of clones are selected from each plate and used to inoculate 5 mL LB media with chloramphenicol respectively
- Incubate the cultures over night at 37 °C with approx. 180 rpm
- Isolate the plasmid DNA according to the instructions of the the peqlab kit
- Sequence the plasmid DNAas described

Retransformation of the plasmid DNA

- In order to determine wether the observed chemotaxis is dependent on the cells themselfes or on the inserted vector the isolated plasmid DNA is transformed into fresh BL21 cells according to the described protocol

Determination of the swimming behaviour of the freshly transformed BL21 cells

- Colonies of the freshly transformed BL21 cells (Retrafo) as well as of the selected BL21 clones (Trafo) are used to inoculate 5 mL LB-media with chloramphenicol, respectively and grown over night at 37 °C with approx. 180 rpm
- Pour 7 x 2 x 3 0.3 % tryptone-swimming agar plates
  - Each attractant has 2 additional controls: one time the whatmanpaper is soaked with H_2O_{dest}. and the other time with aspartate
  - The whole approach is conducted for the "Trafos" as well as for the "Retrafos"
- Treat and drop the cultures as described
- Let the drops dry for at least 20 minutes before inverting the plates and placing them in the incubator at 33 °C over night

Overlap PCR for removal of restriction sites – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

The DH10B *fliC* gene contained three *Pst*I sites and one *Spe*I site that had to be removed. In order to achieve this in a relatively short time we applied overlap PCR. If you are not familiar with this method you can find a detailed description here.

Separation Assay – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

One of our goals was to be able to separate two strains that swim with a different speed from each other when they are in a mixed culture. Only when they are in a mixed culture they are incubated at the exactly same conditions and thus the true effect of an attractant can be determined. In this case we utilized different resistance markers that were inserted into two different strains. This version of the separation assay is the final version determined through many tests with the strains Δtar+pSB1C3 (ampicillin resistance, rfp) and Δ*tar*+pSB1C3_tar_QC_18C (chloramphenicol resistance). Specifications for this strain combination are marked in navy, variations in purple. For hints and advice in general for swimming assays view the method "swimming/chemotaxis assay".

Strains

Two strains containing plasmids with different resistance markers for example the biobrick vectors pSB1C3 (chloramphenicol resistance) and J61002 (ampicillin resistance) with different inserts.

Media

- LB broth
- LB agar with two different resistance markers respectively
- 0.3 % tryptone swimming agar (1 % tryptone, 0.5 % NaCl, 0.3 % agar)

Execution

Applying the mixed culture to the 0.3 % tryptone swimming agar plates

- Inoculate the two strains of interest in 5 mL LB broth with the neccessary antibiotic respectively
- Incubate cultures over night at 37 °C with approximately 180 rpm
- Fill a suitable number of 12 cm petridishes with 0.3 % tryptone-swimming agar without antibiotic and let the agar solidify
  - Alternatively M9 agar can be used
- Apply 100 µL of the to be tested attractant to a steril 2x2 cm whatmanpaper respectivly and position it in the center of the petridish
  - When the strains Δtar+pSB1C3 and Δ*tar*+pSB1C3_tar_QC_18C were tested aspartate was used as a attractant
- Measure the OD_{600} of the over night cultures
- calculate the neccessary amout you have to take from each culture to gain a cell ratio of 1:1
- Mix the cultures
- Spin down the mixed culture and 1 ml of the not mixed ultures repectively with 1.5 X g for 10 minutes
  - No change in the results were observed when this step was not conducted
- Remove the supernatant completly and add 100 µL of fresh LB broth
  - Discard the supernatant and resuspend the pellet in the remaining medium, this can only be applied, when the cultures are dropped on the plated immediatly
- Drop two times 5 µL of the mixed culture and once 5 µL of each of the not mixed strains (references)
- Let the drops dry for at least 20 minutes until inverting the plates and placing them in the incubator at 33 °C over night

Separation of the different strains

- Determine the drop of the mixed culture with the fastes and most directed swimming behaviour on each plate
- In order to determine the faster strain and to separate them the agar is cut out at three different positions:
  - Cut the yellow eppendorf tips of to the first mark (approx 1 cm)
  - The first cut out is at the swimming front --> I
  - The second cut out is between the swimming front and the center od the original drop -->II

    - The third cut out is in the center of the original drop --> III
    - Place each cut out either with or without the tip in at least 0.5 mL LB media in an test tube or an E-cup
- Incubate the cultures for at least 1 h at 37 °C with approx. 180 rpm
- Meanwhile prepare the same amout of LB agar plates (9 cm) with the different selection makers
    - When the strains Δ*tar*+pSB1C3 and Δtar+pSB1C3_tar_QC_18C were tested LB agar plates containing either chloramphenicol or ampicillin were poured
- Prepare a dilution series from 10^{-1} to 10^{-5} of each of the three cultures
- Plate out 100 µL of the dilutions 10^{-3}, 10^{-4} and 10^{-5} on LB agar plates with the two different selection markers, respectively
    - Alternatively: Plate out the 10^{-2} and the 10^{-4} dilution. Dependent on the incubation time a bacterial lawn will be observed on the 10^{-2} plates
- Incubate the plates in an incubator over night at 33 °C
- Count out the colonies

Sequencing – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

Sequencing is used to identify the nucleotide arrangement of a given DNA or RNA template. For example we used sequencing to check if our new designed plasmid-constructs include the correct insert. But before we could sequence our samples or better before we could give our samples to the sequence laboratory, we had to prepare the samples. First of all, the plasmids have to be isolated from cells. Therefore we used the PeqGOLD MiniPrep Kit I from Peqlab. After isolation of the plasmids the concentration has to be determined via Nanodrop. For sequencing we needed a concentration of 250-300 ng in a maximal volume of 4 µl purified water. Finally, 1 µL of the sequence primer (concentration of 5 pmol) was added. In most cases we used the standard Biobrick primer VF2 and VR.

Standard PCR – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

The polymerase chain reaction (PCR) is a method for *in vitro*-amplification of DNA sequences. For the amplification of a DNA fragment the heat resistent enzyme DNA polymerase is responsible. There are several types of DNA polymerases purchaseable, e.g. some of which are very fast or are not error-prone due to proof-reading activity. In order to choose the appropriate DNA polymerase, this link might be of interest: [http://barricklab.org/twiki/bin/view/Lab/ProtocolsTaq; 06/30/2012.](http://barricklab.org/twiki/bin/view/Lab/ProtocolsTaq)

To allow binding of the DNA polymerase primer are required. Thus, only the flanking sites for the sequence of interest needs to be known to synthesize specific primer. Each primer has a specific annealing temperature according to it GC-content. Primers used here were ordered by Sigma®.

A single PCR cycle encompasses three steps: denaturation, annealing and elongation. In the first step the DNA double strands are separated at about 95 °C. Next hybridization of the primers at their annealing temperature happens and finally the DNA seqthesis takes place at the optimal working temperature of the chosen DNA polymerase. This amplification cycle is normally conducted between 25-35 cycles depending on the amount of PCR product one wants to synthesize. In one PCR reaction following components are mixed: DNA template, forward and reverse primer, DNA polymerase with appropriate buffer and deoxynucleotides. Note that an increasing cycle number is prone to incorporate errors in the amplified DNA fragment. This is due to the fact that the dNTPs will be depleted and the saturation phase is reached.

-> 50 µl / reaction:

- 5 µL 10x Buffer
- 5 µL dNTPs (0.2 mM each)
- 2.5 µL Primer (0.5 µM) (2x forward and reverse)
- 0.5 µL Template (ca. 20-100 ng)
- 0.5 µL Pfu-Turbo (1.25 U)
- ad 34 µL ddH_2O

Program:

- 1 min 95 °C (initial denaturation)
- 30 s 95 °C
- 30 s 58 °C
- 2 min/kb 72 °C
- 10 min/kb 72 °C (final elongation)
- 30 Cycles

Swimming/Chemotaxis Assay – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

Another one of our aims was to invent a protocol whith enables us to observe and quantify swimming and chemotaxis of different *E. coli* strains. Here we use different agar combination dependent on the desired effect and soaked 2x2 cm Whatmanpapers as attractants for chemotaxis.

Strains

In general any strain can be used but some like for examle BL21 are more suitablte for chemotaxis assays, for they exibit the ability to swim.

Media

- LB broth
- 0.3 % tryptone swimming agar
- alternatively: M9 swimming agar
  - this agar is more suitable to observe chemotaxis for the nutrient content is lower
  - keep in mind that some *E. coli* strains are not able to grow on M9 media without additional supplements e.g. the strain DH10B requires the aminoacid leucin

Execution

- Inoculate the strains of interest in 5 mL LB broth with the neccessary antibiotic respectively
- Incubate cultures over night at 37 °C with approximately 180 rpm
- Fill a suitable number of 12 cm petridishes with 0.3 % tryptone-swimming agar or M9 agar with antibiotics or additional supplements when necessary
  - in some cases the swimming ability could be enhanced through the addition L-methionine to the M9 agar
  - pour the plates fresh before use but let them dry with a slightly open lid under the hood until the condensation water is evaporated to reduce the risk of smearing of the drops

- Apply 100 µL of the test-attractant to a steril 2x2 cm whatmanpaper and position it in the center of the petridish
- Spin down 1 mL of the cultures with 1.5 X g for 10 minutes
  - No change in the results were observed when this step was not conducted, but for the cultures have a higher viscosity the risk of spillage and smearing is lower
- Remove the supernatant completly and add the amount of fresh LB broth neccessary for the number of desired drops
  - Alternatively: Discard the supernatant and resuspend the pellet in the remaining medium
- Drop 5 µL of the cultures on the petridish, at maximum 4 drops per dish
  - The best results were observed when the drop was placed in a distance of 2-2.5 cm from the whatmanpaper
- Let the drops dry for at least 20 minutes until carefully inverting the plates and placing them in the incubator at 33 °C over night

QuikChange Protocol

To remove disturbing restriction sites within the gene for the successful usage of BioBrick system, the QuikChange reaction is used.

-> 20 µL / reaction:

- 2 µL 10x Buffer
- 0.4 µL dNTPs (10 mM each)
- 0.8 µL Primer (10 µM) (2x or as premix)
- 0.4 µL Template (ca. 20-100 ng)
- 0.4 µL Pfu-Turbo
- ad 20 µL ddH_2O

Program: -> 1 min 96 °C

- 20 s 96 °C
- 20 s 58 °C
- 1.5 min/kb 72 °C
- 15 Cycles

-> 5 min 72 °C -> Store at 4-8 °C

After PCR add 1 µL *Dpn*I directly into PCR tube. Incubate reaction 1-2 h at 37 °C. Transform 5 µL into 50 µL competent cells.

Quantitative Real-Time PCR – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

Quantitative real-time PCR is a powerful tool for quantitation of nucleic acid. The quantitative rtPCR was performed by using SYBR® green 1 (5′ Prime). All real-time PCR systems detect a fluorescent dye and correlate this fluorescence signal to the amount of PCR product in the reaction. There are several methods that quantify the mRNA amount via fluorescence including the method SYBR® green 1 that is used in our experiments. SYBR® green 1 is a nonsequence-specific DNA binding dye that intercalates into dsDNA (it does not bind to single stranded DNA). It emits a strong fluorescent signal upon binding to dsDNA.

Experimental design

- 5 mL of LB-media with 50 µg/mL chloramphenicol were inoculated with *E. coli* strain BL21DE3 haboring the vector pSB1C3 with following combinations of promoters and Tar receptor constructs:
- Constitutive promoter constructs expressed in E. coli:
  - Tar receptor under the control of constitutive promoter J23100
  - Tar receptor under the control of constitutive promoter J23104
  - Tar receptor under the control of constitutive promoter J23105
  - Tar receptor under the control of constitutive promoter J23106
  - Tar receptor under the control of constitutive promoter J23109
  - Tar receptor under the control of constitutive promoter J23112
  - Tar receptor under the control of constitutive promoter J23113
  - Tar receptor under the control of constitutive promoter J23114
  - (For more details click Parts Submitted)

- Cultures were incubated overnight at 37 °C, shaking.
- For quantitative real-time PCR, RNA was isolated from *E. coli* BL21DE3 by using the RNA isolation Kit "Nucleus Spin II (Machery and Nagel)". Cultures are inoculated over night at 37 °C to 10^9 cells. The isolated RNA is then converted to cDNA using the "Reverse Transcription Kit from Qiagen"
- Each primer (forward or reverse) concentration in the mixture was 5 pmol/µl
- The housekeeping gene *rrsD* (ribosomal RNA, 16S) was used as reference

- The experiment was set up by following the PCR reaction mix illustrated in the table
- All experimental set ups were prepared in triplets for calculating the average
- In addition: to avoid contaminations, a non-template control for our PCR assays was always carried with the samples.
- The quantitative real-time PCR was performed by using ”LightCycler Software Version 4.05 (Roche)”

Primer for qrtPCR:

- Primers for amplification of reference cDNA *rrsD*
  - rrsD_rev_transcript_fw:
    - cgtcagctcgtgttgtgaaatg
  - rrsD_rev_transcript_rev:
    - cgtgtgtagccctggtcgtaag
- Primers for amplification of promoter constructs cDNAs: Primer Set 1
  - query_L1_fw:
    - tgacgtcaacctgggattta
  - query_R1_rev:
    - ggaggaatccatcatcatcc

20 µl / reaction:

- 9 µL 2.5x real master mix/ 20x SYBR
- 2 µL sense primer (5 pmol)
- 2 µL antisense prime (5 pmol)
- 1 µL cDNA (diluted 1:10 in H2O)
- 6 µL water

Program:

- 3 min 95 °C (initial denaturation)
- 30 s 95 °C (denaturation)
- 30 s 61 °C (annealing for our experiment)
- 2 min/kb 72 °C (elongation)
- 42 Cycles

Analysis via the $2^{-\Delta\Delta CT}$ (Livak) Method

First, we normalized the CT of the target gene to that of the reference (ref) gene, for both the test sample and the calibrator sample (calibrator sample was the weakest expressed promotor construct in our case):

- ΔCT(test) = CT(target, test) − CT(ref, test)
- ΔCT(calibrator) = CT(target, calibrator) − CT(ref, calibrator)

- Second, we normalized the ΔCT of the test sample to the ΔCT of the calibrator:
  - ΔΔCT = ΔCT(test) − ΔCT(calibrator)
- Finally, we calculated the expression ratio:
  - $2^{-\Delta\Delta CT}$ = Normalized expression ratio

SDS-PAGE – Goettingen – 2012 http://2012.igem.org/Team:Goettingen/Project/Methods

Discontinuous SDS-PAGE (sodiumdodecylsulfate-polyacrylamide-gelelectrophoreses) serves to separate proteins in an electric field corresponding to their molecular weight. Two compounds of Laemmli-Buffer β-Mercaptoehanol and SDS are necessary for the separation: β-Mercaptoethanol is a reducing agent of disulfide bridges of proteins. SDS, a strong anionic detergent, binds to hydrophobic amino acid residues, which lead the proteins become negatively charged relative to their mass (~1.4 g SDS/g protein in a 1% SDS-solution). SDS also denaturizes proteins. The SDS-compensated proteins move from a cathode to an anode through a porous polyacrylamide gel. Depended on the size of the porous, which is due to the concentration of polyacrylamide, the proteins are separated. Here a 5% stacking- and a 12% separation-gel was prepared for separating proteins in a range of 30 – 200 kDa. Protein samples were mixed 1:1 (v/v) with 2x Leammli-Buffer (20 mM Tris/HCl pH 6.5, 4% SDS, 10% β-Mercaptoehanol, 40% Glycerol, 0.002% Brompheolblue) and loaded on the gel. As size standard an unstained protein molecular weight marker (#26610, Thermo Scientific) was used. The Gel was developed at 35 mA for approximately one hour in 1x SDS-running buffer (25 mM Tris (10x), 192 mM Glycerin, 0.1% SDS) and afterwards stained with a 1% Coomassie Brilliant Blue solution (0.002% Coomassie Brilliant BlueR-250, 10% Ethanol, 5% Acetic acid) that fixes the proteins.

Plasmid Preparation from *E.coli* (Mini Preparation) – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

1. transfer 1,5 ml of an overnight culture in a reaction tube
2. centrifuge at 8000 rpm at RT for 5 min
3. resuspend the pellet in 100 µl solution 1
4. addition of 200 µl solution 2
5. mix till the solution is clear
6. incubate at RT for 5 min
7. addition of 150 µl cold solution 3, mix till protein clumps are build
8. incubate 10 min on ice, centrifuge 15 min with 10000 rpm
9. transfer the supernatant in a clean reaction tube
10. fill the reaction tube with 96 % EtOH, mix and let it precipitate at -20 °C for 10 min
11. centrifuge 10 min at 10000 rpm
12. wash the pellet with 70 % EtOH
13. dry the pellet and resuspend it with 30 µl water or TE buffer

Solution 1

- 50 mM glucose
- 10 mM EDTA
- 25 mM Tris-HCl pH8

Solution 2

- 0,2 M NaOH
- 1 % SDS

Solution 3

- 3 M KaAc pH 5,5

Plasmid Preparation from *Saccharomyces cerevisia* – Frankfurt – 2012

http://2012.igem.org/Team:Frankfurt/Protocol

1. overnight culture in 5 ml
2. centrifuge 2 ml of the cells for 1-2 min
3. wash the cells with water
4. resuspend the pellet in 400 µl buffer 1 with RNase
5. addition of 400 µl buffer 2 and mix carefully
6. addition of 2/3 volume of glass beads
7. cell destruction: vibrax the cellsin a 2 ml reaction tube for 5 min at 4°C
8. transfer 500 µl of the supernatant in a clean reaction tube
9. addition of 250 µl buffer 3, mix, incubate it for 10 min on ice
10. centrifuge at 10000 rpm, 15 min
11. transfer the supernatant in a clean reaction tube, fill with isopropanol and mix
12. centrifuge at 13000 rpm, 30 min
13. wash the pellet with cold 70 % EtOH and let it dry
14. resuspend the DNA in 30 µl water or TE buffer

P1

- 50 mM Tris/HCl pH 8
- 10 mM EDTA
- 100 µg/ml RNase A

P2

- 0,2 M NaOH
- 1 % SDS

P3

- 3 M KAc

Transformation – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

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Yeast Transformation – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

1. Inoculate the synthetic complete medium (SC) with the strain
2. Incubate with shaking overnight at 30°C
3. Harvest the cells at a OD600 0,5-0,6 by centrifugation (3000x g, 5 min, RT)
4. Wash the cells with 0,5 vol of sterile water (resuspend by shaking, centrifugate with 3000x g, 5 min, RT)
5. Resuspend the cells in 0,01 vol of sterile water, transfer the suspension to a reaction tube and pellet the cells (3000x g, 5 min, RT)
6. Resuspend the pellet in 0,01 vol of sterile filtrated FCC (frozen competent cell) solution
7. Aliquot 50 µl of the solution into the reaction tubes
8. Store the cells at -80°C for at least one night (up to one year)
9. Mastermix for the FCC transformation mixture:

Substance	Volume [µl]
PEG 3350 (50% (w/v))	260
LiAcetat 1.0 M	36
Single-stranded carrier DNA (10 mg/ml)	10
Total volume	306

1. Prepare DNA aliquots: Solute enough DNA (e.g. 100 ng plasmid) in 54 µl of water
2. Unfreeze the cells in a 37°C block for 15-30 sec
3. Centrifuge the solution at 13000x g for 2 minutes
4. Remove the supernatant
5. Add 306 µl of FCC transformation mixture to the cells
6. Add 54 µl of the DNA to the solution and vortex shortly

7. When all the reaction tubes are prepared, vortex the samples well until all pellets are completely resoluted
8. Incubate the samples for 40 minutes at 42°C in a heating block
9. Centrifuge the cells at 13000x g for 30 sec and pour off the supernatant
10. Resuspend the cells in sterile water by vortexing
11. Spread onto the appropriate medium
12. Let the cells grow at 30°C

Yeast transformation and gap repair (example)

DNA Fragment	**Size[bp]**	**Concentration[ng/µl]**	**Equimolar Quantities°[ng]**	**Used Volume[µl]**
HMG-CoA	1600	224	222	1
tHXT7	360	51,6	50	1
pPFK1	600	54	83	1,5
ERG20	1100	139	152	1
tPFK2	360	47,4	50	1
pPGK1	600	49	83	1,7
GGPPS	900	14,5	125	8,6
	5520		765	+ 19,8 µl linear p426 + 18,4 µl water

° determination of 50 ng for 360 bp
use of a vector-insert relation of 1:1
samples: linear p426 with 7 inserts

positive control (p426)

negative control (linear p426)

plating the samples on SCD-ura and incubation at 30° C

General procedure of a transformation via gap repair – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

1. Amplification of the required DNA fragment with homologue ends of the plasmid (in our case homologue ends of the gene/promoter/terminator beside the DNA fragment) via PCR and an agarose gel to review the correct size
2. Linearization of the plasmid and an agarose gel for review
3. Yeast transformation with the PCR products and the linear plasmid (homologue recombination)
4. Selection on an agar plate without the metabolite, which is on the plasmid for yeast selection (in our case it was uracil on p426 and histidin on p423)
5. Inoculation of clones and isolation of the plasmids from yeast
6. Transformation of a plasmid in *E.coli* and selection on LB medium with ampicillin (the plasmids have an ampicillin resistance)
7. Isolation of the plasmids from *E.coli*
8. Diagnostic restriction of the plasmid in order to find the correct plasmid with all inserts
9. Transformation of the correct plasmid in yeast to do further experiments

E.coli Transformation – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

a) Production of competent cells

1. Inoculate 400 ml LB medium (pre-warmed to 37°C) in a 1 l flask with 100 µl of a fresh over night culture

2. Harvest the cells at a OD600 0,60-0,65 (in a room with 37°C, sterile)

3. Aliquot the solution in 8 x 50 ml falcons (sterile, pre-cooled on ice)

4. Incubate for 30 min on ice

5. Centrifuge for 12,5 min with 4000x g, 4°C

6. Resuspend the pellets in each 10 ml sterile water (4°C)

7. Centrifuge for 10 min with 4000x g, 4°C

8. Repeat steps 6 and 7 two times

9. Resuspend the cells in 10 ml 10% glycerine (+millipor, sterile, 4°C)

10. Centrifuge for 15 min with 4000x g, 4°C

11. Add 800 µl of 10% glycerine (sterile, 4°C) to the cells

12. Aliquot 40 µl of the solution in each reaction tube (pre-cooled) and freeze them in liquid nitrogen

13. Store them up to 6 months at -70°C

b) Transformation (on ice)

1. Add DNA (e.g. 1 µl of extracted yeast plasmids) to the frozen cells

2. Unfreeze the cells on ice

3. Fill the cells into cuvettes for electroporation

4. Electroporation (*for 2 mm cuvettes:* voltage I = 2,5 kV; resistance R = 2000 ohm; electrical current A = 25 µF; *for 1 mm cuvettes:* I = 2,2 kV; resistance R = 2000 ohm; electrical current A = 25 µF)

5. Fill the cells with 1 ml of SOC medium back in a reaction tube

6. Shake them for 1 hour at 37°C

7. Centrifuge for 1 min 8000x g

8. Decant the supernatant

9. Spread onto the appropriate medium

PCR – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

PCR protocol

Components	Final Concentration	Volume of Example PCR
Polymerase-buffer (e.g. for Phusion Polymerase)	1x	10 µl (5x)
dNTPs	200 µM	5 µl (2 mM)
Primer fw/rev	respectively 0.2 µM	respectively 1 µl (10 µM]
Template	0.2 ng/µl	1 µl (10 ng/µl)
DNA-Polymerase (e.g. Phusion Polymerase)	0.02 U/µl	0.5 µl (2 U/µl)
Water	add to 50 µl solution	31 µl
Magnesiumchlorid	10 mM	0.5 µl (1M)
Total Volume	50 µl	50 µl

Add all components with the right concentrations to a PCR tube. Pay attention to defrost phusion-polymerase on ice and add it at the very end. Put the PCR tube, containing a 50 µl reaction mixture, into a PCR cycler. Make the following settings for running the PCR programm:

PCR programm for one PCR run

Steps	Temperature	Time

First Denaturation	98 °C	30 sec
Denaturation	98 °C	10 sec
Annealing	Depending on Primer	20-30 sec
Polymerization	72 °C	1 min/kbp for Tay-Polymerase; 20 sec/kbp for Phusion Polymerase
Final Polymerization	72 °C	5 min

Let the PCR-Programm run for about 35 cycles.

Restriction Digestion for Linearization of a Plasmid – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

Digest the choosen plasmid with one suitable restriction enzyme.

Example for digestion reaction:

Componen	Concentration
Shuffle Plasmid	1 µl (3-5 µg/µl)
Restriction Enzyme	respectively 1µl

Suitable Buffer	5 µl (10x)
Water	add to 50 µl
Total Volume	50 µl

Incubate the digestion mix 2 hours at 37 °C. AFter that purify the solution with e.g. a PCR purification kit.

Culture Media – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

Full Medium (YEPD) for Yeast	
Yeast Extract	1 % (weight/volume)
Pepton	2 % (w/v)
Glucose	2 % (w/v)

Synthetic Complete Medium (SC) for Yeast	
Yeast Nitrogen Base	0.17 % (w/v)
Ammoniumsulfate	0.5 % (w/v)
Glucose	2 % (w/v)

Amino Acid Mix°	50 ml/l
Histidin**	0.25 mM
Tryptophan**	0.19 mM
Leucin**	0.35 mM
Uracil**	0.44 mM

pH has to be regulated with KOH to pH=6.3
° contains no His, Leu, Trp and Uracil
** addition of this components depents on the respective selection medium

SOC-Medium for Regeneration of transformed *Escherichia coli`s* after Electroporation	
Trypton	2 % (w/v)
Yeast Extract	0.5 % (w/v)
NaCl	10 mM
KCl	2,5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

pH has to be regulated to pH=6.8-7.0

Full Medium (LB) for *E.coli*	
Yeast Extract	0.5 % (w/v)
Trypton	1 % (w/v)
NaCl	0.5 % (w/v)

pH has to be regulated with NaOH to pH=7.5

Every cluture medium has to be autoclaved to be sterile.

Agar Plate – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

$LB_{ampicilline}$-Agar

Add 2 % agar to LB-medium. After autoclaving and cooling-down to 60 °C steril ampicillin is added. Plates were poured.

$LB_{chloramphenicol}$-Agar

Add 2 % agar to LB-medium. After autoclaving and cooling-down to 60 °C ethanolic chlorampenicol solvation is added.

SCD-Agar

Add 2 % agar to SCD-medium. After autoclaving and cooling-down steril amino acid solution is added. Dependent on the respective selective medium Histidin (0.25 mM), Trypthophan (0.19 mM), Uracil (0.44 mM) or Leucin (0.35 mM) are added. Plates were poured.

$YEPD_{G418}$-Agar

Add 2 % agar to YEPD-medium. After autoclaving and cooling-down sterile G418 (final concentration 2g/l) is added. Plates were poured.

Gel Electrophoresis – Frankfurt – 2012 http://2012.igem.org/Team:Frankfurt/Protocol

Agarose Gel (1x)	
TAE puffer	1x
Agarose	1 % (w/v)

Solve agarose in TAE by boiling it. After cooling-down to 55-60 °C gel is poured.

TAE Puffer (50x) for Gel Electrophoresis	
EDTA	18,6 g
Tris	242g
Glacial Acetic Acid	57,2 ml
Purified Water	1000ml

pH has to be regulated with glacial acetic acid to pH=8.

Gels were run with a tension of 80-140 V.

Ligation – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Ligation is performed with a ration of 1:5 plasmid backbone : Insert . The Ligation mix is incubated for at least 10 min at room temperature or for at least 1 hour at 16℃ before the ligase is heat inactivated at 65℃ for 20 min.

Ligation mix:

DNA Mix	x µL
Ligase Buffer 10x	2.5 µL
Ligase	0.5 µL
H_2O	to 25 µL

Transformation – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- Allow competent cells to thaw on ice
- Add ligation-mixture or 3-5 µl DNA to 50 µL cells
- Incubate on ice for 30 min
- Heatshock 45 sec at 42 ℃
- Add 900 µL LB medium and incubate in a shaker at 37 ℃ 60 min.
- Spin cells down and remove supernatant
- Resuspend cells in 50 µL medium and spread them onto a agar plate

Glycerol Stocks – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

For longtime storrage 0.6 mL of cells (overnight culture) are mixed with 0.4 mL 100 % Glycerol and stored at -80℃.

PCR – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Template	1 ng
Phusion buffer (5x)	10 µL
Forward Primer (10µM)	2.5 µL
Reverse Primer (10µM)	2.5 µL
Phusion polymerase	0.2 µL

H_2O	to 50 µL

For colony PCR the colony is resuspended in 10 µL water and the PCR mix is adjusted with respect to the additional 10 µL. The initial denaturation step is extended up to 5 min. PCR protocol:

- Initial denaturation at 98℃ for 30 sec
- 25-35 cycles:
  - Denaturation at 98℃ for 5 sec
  - Annealing for 20 sec (Temp. depends on Primer)
  - Extension at 72℃ for 30 sec per kb
- Final extension at 72 ℃ for 5 min

SDS-Page – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Compound	12% Running gel	5% Stacking gel
H_2O	6.67 mL	3.54 mL
1.5 M Tris-HCl, pH 8.8	5 mL	
0.5M Tris-HCl, pH 6.8		3 mL
10% (w/v) SDS	200 µL	80µL
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	8 mL	1.32 mL
10%(w/v) APS	100 µL	50 µL
TEMED	30 µL	15 µL
Total 4 gels:	20 mL	8mL

As a marker for the SDS-Page “PageRuler plus prestained protein marker” is used. Gel is stained with Coomassie Blue.

Native-page acrylamide gels were made with the same composition, just lacking SDS and stacking layer. Also, native gels contained an acrylamide top to bottom gradient of 6 - 20 %.

Cell Lysis – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Lysis Buffer (Tris 50 mM, NaCl 150 mM, EDTA 5 mM, pH=7.4)

- Spin down 1 mL of liquid culture
- Resuspend in 1 mL of lysis buffer

- Add lysozyme to a concentration of 1 mg/mL
- Freeze cells in dry ice for 30 min
- Thaw the cells and centrifuge at 4℃
- Use supernatant for testing

Miller Assay – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Z Buffer (NaH_2PO_4 40 mM, Na_2HPO_4 60 mM, KCl 10 mM, pH=7)

Add 4mg/mL of ortho-Nitrophenyl-β-Glactoside (ONPG) just before useage.

20 µL cell lysate is dissolved in 180 µL of Z-Buffer with ONPG. ONP activity is measured in a 96 wellplate at 420 nm every minute over a time period of 10min. β-Galactosidase activity determined based on the slope of the measurements.

TECAN plate reader Sample preparation – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

5 mL of fresh LB containing necessary antibiotic resistances was inoculated with 50 µL of overnight culture and grown at +37 ℃ while shaking vigorously. After cell density reaches OD_{600} ~ 0.1-0.2, 1 mL was withdrawn and mixed with IPTG. Then, three 200 µL samples were taken and transferred into sterile 96 well plate (Thermofisher scientific, Denmark) and used for data analysis as a triplicate. Cells were later grown in TECAN Infinite 200Pro plate reader (Switzerland) at 37 ℃, while shaking in orbital mode with 5 mm amplitude. Each 15 min fluorescence (excitation at 488±4.5 nm, emission at 530±10 nm; 25 flashes and integration over 20 µs) and cell density (absorbance at 600±4.5 nm; 15 flashes) measurements were taken.

Single Cell analysis using flow cytometry – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

For sample preparation the OD600 was measured to gauge the volume for sample-taking ensuring similar cell-concentrations for each measure. Cells are harvested and resuspended in an appropriate amount of Phosphate buffered saline (PBS). It is possible that the cells has to be diluted later because the flow is too high due to high concentration.

Measurements were performed in the BD LSRFortessa™ using following lasers and filters:

- SSC and FSC: 488 nm - 488 nm/10 nm
- GFP: 488 - 530/30
    - excitation: 488 nm
    - emission: 530 ± 15 nm
- mRFP and mCherry: 561 - 610/20
- YFP: 488 - 542/27

- eCFP: 445 - 473/10

High-performance liquid chromatography (HPLC) – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

The HPLC was performed with a ... column. Sample preparation: Cells were lysed in acteonitril incubated for 15 min on ice and then centrifuged for 30 min at top speed. The supernatant was applied onto the column.

HPLC Method:

- Column (preheated up to 40 °C) was equilibrated with 92% A and 8% B
- 50 µl per sample were applied to the column
- The proportion B was increased linearly to 50% in 7 min. The last 3 min it is increased to 100%.

Buffers:

A: Water , 0.1% formic acid

B: Methanol, 0.1% formic acid

Purification of $TetR_{DBD}$-UVR8-HisTag – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Cells, expresing $TetR_{DBD}$-dUVR8 with 7xHis tag at the C-terminus under P_{tac} promoter, were grown overnight in the 100 mL of LB media at 37 °C without IPTG induction. Culture were then centrifuged at 4000 rpm for 20 min at 4°, supernatant discarded and cells were resuspended at 10 mL of Lysis buffer (50 mM $K\text{-}PO_4$, 500 mM NaCl, 10 mM Imidazol) and lysed with 1 mg/mL lysozyme at room temperature followed with rapid freezing in dry ice and kept frozen for 1h. Cells then were thawed and incubated for another hour with DNase and frozen as before. Cells debris were removed by centrifugation and supernatant was loaded on Ni ion affinity chromatography column, washed with lysis buffer containing 50 mM imidazol and eluted with 50 mM $K\text{-}PO_4$, 500 mM NaCl, 300 mM imidazol. Elute was concentrated and diluted 1:1 with glycerol and stored at -20 °C.

Mediums – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Agar plates – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- 1 % gels in TAE buffer

LB medium (1L) – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- 10 g Bacto-tryptone
- 5 g yeast extract
- 10g NaCl

LB agar (1L) – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- LB medium
- 15 g Agar

Chemicals – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

Antibiotic Stock Solutions (1000x) – ETH Zurich – 2012
http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- Kanamycin: 50 mg/mL
- Ampicillin: 100 mg/mL
- Chloramphenicol: 34 mg/mL

X-Gal – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- 20 mg/mL in DMSO

IPTG – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- 100 mM in water

aTc – ETH Zurich – 2012 http://2012.igem.org/Team:ETH_Zurich/MaterialMethods

- 1 mg/mL in ethanol

Cultivation of tranformed cells: – Copenhagen – 2012 http://2012.igem.org/Team:Copenhagen/Protocols

Materials:

- LB Media
- Antibiotics (Matching the Resistance marker gene)
- Inoculation needle

Procedure:

1. A colony is chosen from the LB-plate, and the colony is transferred to a small tube with 20 µl water.
2. 5 µl of each colony is transferred to 5 ml LB + 5 µl antibiotics (in our case: chloramphenicol) with a pipet tip.
3. Incubate over night at 37℃ in the shaking incubator.

Plating on LB plates: – Copenhagen – 2012 http://2012.igem.org/Team:Copenhagen/Protocols

Materials

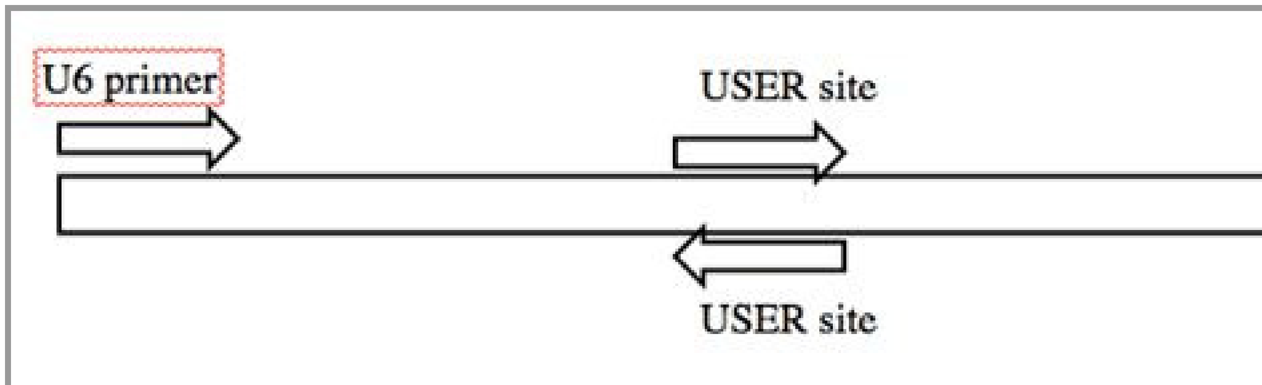
- LB plates (with antibiotics)
- Transformed *E. coli* cells
- Sterilized Gridalzky spatula

Procedure

- The transformed cells is transferred to an LB plate containing antibiotics and dispersed with the Drigalski spatula.
- The transformed cells are incubated over night at 37℃.
- Next day; the plates are checked for visual colonies. These are cultivated.

Primer design for point mutation in Lux Casette – Copenhagen – 2012 http://2012.igem.org/Team:Copenhagen/Protocols

- The Lux cassette of 5798 bp had a XbaI restriction site that was removed by designing to primers of 25-30 base pairs with overlapping sequences. This sequence was designed as a USER site that covered the XbaI restriction site. The chosen USER site sequence should be identical to the template strand, except for the single base pair, which is to be mutated.
- Two primers to each end of the Lux cassette were also designed.
- Two PCR reactions were carried out with two sets of primers. One set each containing a primer annealing to the 3' and a primer containing the new USER site sequence. And an other set containing a primer annealing the 5' end respectively together with the complementary primer encoding the new USER site sequence.



PCR reaction for amplification of backbone pSB1C3 – Copenhagen – 2012 http://2012.igem.org/Team:Copenhagen/Protocols

The following protocol is used to amplify the pSB1C3 backbone vector.
To each PCR tube the following is added:

- 2.5 µl of each primer
- 4 µl template DNA
- 41 µl MasterMix

Master Mix	
10 mM	X7 Buffer
10 mM	dNTP's
2 mM	X7 DNA polymerase (Produced in our lab)

The PCR programme used:			
Step	Temperature/℃	Time	Cycles
Int. Denaturation	95°	120 sec	
Denaturation	95°	30 sec	32
Tm	55°	12 sec	32
Elongation	68°	1.25 min	32
Final extension	68°	10 min	
Hold	10°	∞	

Colony PCR – Copenhagen – 2012
http://2012.igem.org/Team:Copenhagen/Protocols

20 µl H_2O is added to each PCR tube. Colonies are chosen from the plates and resuspended in the PCR tubes. This serves as the template solution.
To each PCR tube the following is added:

- 0.5 µl of each primer
- 3 µl template DNA
- 5 µl MangoMix™ (5 mM) (Bioline)

MangoMix™
MangoTaq DNA polymerase
Orange reference dye
$MgCl_2$
dNTP

The PCR programme used:			
Step	Temperature/℃	Time	Cycles
Int. Denaturation	96°	3 min	
Denaturation	96°	20 sec	25
Tm	60°	20 sec	25
Elongation	72°	* sec	25
Final extension	72°	7 min	
Hold	10°	∞	

*= elongation time for MangoTaq DNA polymerase should be calculated so it is compatible with the size of the template. Taq writes 1kb/60sec.

PCR reactions for individual genes – Copenhagen – 2012
http://2012.igem.org/Team:Copenhagen/Protocols

The following protocol is used to amplify the individual genes.
To each PCR tube the following is added:

- 2.5 µl of each primer
- 0.5 µl template DNA
- 44.5 µl MasterMix

Master Mix	
10 mM	X7 Buffer
10 mM	dNTP's
2 mM	X7 DNA polymerase (Produced in our lab)

The PCR programme used:			
Step	Temperature/℃	Time/Sec	Cycles
Int. Denaturation	98°	30 sec	
Denaturation	98°	10 sec	32
Tm	60°	20 sec	32
Elongation	72°	* sec	32
Final extension	72°	7 min	
Hold	10°	∞	

*= elongation time for X7 DNA polymerase should be calculated so it is compatible with the size of the template. X7 polymerase writes 1kb/30-45sec.

USER Cloning – Copenhagen – 2012
http://2012.igem.org/Team:Copenhagen/Protocols

Procedure:

1. The PCR product must be purified from a gel before (GenEluteTM HP plasmid MiniPrep kit (Sigma-Aldrich)) used in USER cloning.
2. The PCR products is added to Eppendorf tubes to give a total volume of 8 µl in each tube (Thus, in case of two PCR products, add 4 µl and 4 µl or 3 µl and 5 µl etc.)
3. The USER mix components are mixed: (in each tube)

USER Mix	1x USER Mix
NEBuffer 4 (10x diluted)	0.5 µL
BSA	0.5 µL
Dpn1	1 µL

4. The USER mix is transferred to each Eppendorf tube and incubated for 2 hours at 37℃.

5. 1 µL USER enzyme is added pr. tube and the mixture is incubated for 40 minutes at 37℃ and for 2 hours min at 25℃.
6. Transformation: See transformation protocol.

USER enzyme: USER™ (Uracil-Specific Excision Reagent) Enzyme generates a single nucleotide gap at the location of a uracil. USER Enzyme is a mixture of Uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase Endonuclease VIII (New England biolabs® Inc.).

Transformation in *E. coli* DH5α or E. Cloni – Copenhagen – 2012 http://2012.igem.org/Team:Copenhagen/Protocols

The preparations for the transformation can preferably be done, while the USER cloning is incubating.

Materials

- *E. coli* DH5α (Bioline) or E. cloni-5α (Lucigen) competent cells
- USER reaction

Procedure:

1. LB plates are taken out of the refrigerator and marked. Remember to use LB plates with the right antibiotics.
2. 50 µL competent *E. coli* DH5α cells per USER reaction is taken from the -80C° freezer and place on ice. Additionally, 1,5 ml tubes are placed on ice.
3. 5 µL USER reaction mix is added to the 50 µL competent *E. coli* DH5α cells. Mix well by pipetting.
4. The cells are placed on ice for 30 min.
5. The hot plate is set on 42 ℃, and each transformation is heat shocked for 1 min. The cells are put directly on ice for 2 min afterwards.
6. 200 µL recovery medium is added pr. cell tube.
7. The cells are placed on hot plate on 300 rpm for 30 minutes.
8. The hot plate is increased to 1000 rpm, and the cells are placed hereon for additional 1 hour.

Control of Lux cassette – Copenhagen – 2012 http://2012.igem.org/Team:Copenhagen/Protocols

Procedure:

1. One colony is chosen from the LB-plate, and transferred to a small plating tube with 2.5 ml LB-medium with antibiotics.
2. Incubate over night at 37℃ in the shaking incubator.
3. The over night culture is transferred to an Erlenmeyer flask with 100 ml LB-medium and regularly checked with OD
4. When OD600 has reached 0.4-0.6 100 µl IPTG is added
5. The flask is taking into a dark room to see the immediate effect.

1 General protocols - XMU-China - 2012 http://2012.igem.org/Team:XMU-China/protocols

1.1 Stock solution

50 mg/mL Kanamycin

- 0.5 g Kan, 10 mL water, filter sterilize with millipore express membrane, freeze in aliquots

100 mg/mL Ampicillin

-1 g Amp, 10 mL water, filter sterilize with millipore express membrane, freeze in aliquots.

50 mmol/L Arabinose

- 0.1876 g Arabinose, 25 mL water, filter sterilize with millipore express membrane.

0.25 mg/mL Anhydrotetracycline

- 0.1 mg Anhydrotetracycline, 0.4 mL PBS, filter sterilize with millipore express membrane.

1.2 Preparation of Competent BL21

Thaw an aliquot of cells (without any plasmid in them) on ice

- To 50 mL of sterile LB, add 100μL aliquot of the thawed cells: remember, this LB does not have any antibiotic in it, so work as aseptically as possible (i.e. autoclave all solutions and use sterile pipettes).
- Grow cells in the shaker at 37 ℃ and 200 rpm, until they reach an OD_{600}= 0.3-0.4. This usually takes 1.5-2 hours.
- Ice down the LB with growing cells for 10 min.
- Aliquot into sterile 1.5 mL tubes and spin down at 6000rpm for 10 min at 4 ℃; discard supernatant.
- Ice down sterile 100 mM $CaCl_2$ and 100mM $MgCl_2$ solutions during centrifugation.
- Gently resuspend each pellet with 400 μL 0.1 M $MgCl_2$ and 100 μL 0.1 M $CaCl_2$.

- Centrifuge 6000 rpm for 10 min and discard supernatant.
- Resuspend each pellet on ice in 100 µL 0.1 M ice cold $CaCl_2$ and combine into one tube

1.3 Transformation

- Add 10 µL of DNA. Swirl gently with pipette.
- Incubate tubes on ice for 20 min
- Heat pulse tubes in 42 ℃ water bath for 30 seconds.
- Incubate on ice for 2 min
- Add 790 µL of LB broth to each tube and incubate for an hour at 37 ℃ with shaking.
- Spread 100 µL and 50µL of each culture on an LB agar plate containing the appropriate antibiotics and incubate overnight at 37 ℃ (spread using beads).

1.4 Plasmid Purification

- Centrifuge sample in eppendorf tube approximately 1.5 mL at a time, draining off supernatant after each spin and adding more cell solution
-Resuspend the pelleted cells in 250 µL of the resuspension Solution (mixture with Solution I and RNasa A). The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
- Add 250 µL of the Lysis Solution (Solution II) and mix thoroughly and gently by inverting the tube 5-6 times, letting it stand for 1-2 min at room temperature until the solution becomes viscous and slightly clear.
- Add 350 µL of the Neutralization Solution (Solution III) and mix immediately and thoroughly by inverting the tube 5-6 times.
- Centrifuge for 10 min at 12,000 rpm to pellet cell debris.
- Apply the supernatant to the supplied spin column by decanting. Avoid disturbing or applying the white precipitate.
- Centrifuge for 1 min at 12,000 rpm. Discard flow-through and place the column back into the same collection tube.
- Add 500 µL of the Wash Buffer PB to the spin column. Centrifuge for 1min at 12,000 rpm and discard flow-through. Place the column back into the same collection tube.
- Add 500 µL of the Wash Buffer W to the spin column. Centrifuge for 1min at 12,000 rpm and discard the flow-through. Place the column back into the same collection tube.

- Repeat the step 9 again.

- Discard flow-through and centrifuge for an additional 3 min to remove residual Wash Solution.

- Place the spin column in a clean 1.5 mL centrifuge tube, and pipet 20 μL Elution Buffer TE (prewarm to 60 ℃) directly to the center of the column without touching the membrane. Let it stand for 2 min at room temperature and centrifuge for 1 min at 12,000 rpm.

- Discard the column and store the purified plasmid DNA at -20 °C.

1.5 Reaction system of restriction endonuclease

Table 1. The digestion system

	Prdfix Insertion		Suffix Insertion		Restriction analysis	
Components	EcoRI-XbaI	EcoRI-SpeI	PstI-XbaI	PstI-SpeI	Double digestion	Single digesti
DNA Sample/μL	80	80	80	80	16	16
10×M buffer/μL	10	—	10	—	—	—
10×H buffer/μL	—	10	—	10	2.0	2.0
PstI/μL	—	—	5.0	5.0	1.0	—
XbaI/μL	5.0	—	5.0	—	—	—
SpeI/μL	—	5.0	—	5.0	—	—
EcoRI/μL	5.0	5.0	—	—	1.0	2.0
Total/μL	100	100	100	100	20	20

- System1、2、3 and 4 are used for Standard BioBrick Assembly .

- System 5 and 6 are used for restriction analysis. Digestion of sample: at least 500 ng DNA / 10 μL volume. Digest for 1 h at 37 °C, afterwards inactivated by adding 10× loading buffer and standing for 10 min at room temperature.

1.6 Standard BioBrick Assembly

- Digestion of insert: 2 μg~5 μg DNA / 100 μL volume, 10× H buffer, *Eco*R I, *Spe* I. Digestion

and inactivation. Clean up the insert via gel electrophoresis. When cutting the insert out of the gel, try avoiding staining or exposure to ultraviolet light of the insert.

- Digestion of vector: 2 μg~5 μg DNA / 100 μL volume, 10× M buffer, *Eco*R I, *Xba* I. Digestion and inactivation. Clean up the insert via gel electrophoresis. When cutting the insert out of the gel, try to avoid staining or exposure to ultraviolet light of the insert.

1.7 Suffix Insertion

- Digestion of insert: 2 μg~5 μg DNA / 100 μL volume, 10× M buffer, *Xba* I, *Pst* I. Digestion and inactivation. Clean up the insert.
- Digestion of vector : 2 μg~5 μg DNA / 100 μL volume, 10× H buffer, *Spe* I, *Pst* I. Digestion and inactivation. Clean up the vector.

1.8 Ligation

- After digestion and clean-up, the next step is ligation. ligation at 16 °C for 4 h or at 4 °C for 16 h. Table 2 is the system of ligation.

Table 2 Ligation system

Components	Volume/μL
Digested vector	7
Digested insert	1
10× T4 ligation buffer	1
T4 ligase	1
Total	10

1.9 Restriction analysis

- Pick one colony with a sterile tip and cultivation in 20 mL LB for overnight at 37 °C
- Isolation of Plasmid
- Digest BioBrick, the system of Restriction analysis refer to table1

- Gel electrophoresis : add 2.2 μL loading buffer to digestion mixture. An agarose concentration is 1 %.

1.10 Gel Extraction

- Weigh a 1.5 mL centrifuge tube for each DNA fragment to be isolated and record the weight.
- Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close as possible to the DNA to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 mL tube and weigh. Record the weight of the gel slice.
- Add Bing Buffer BD at a ratio of 100 μL of solution per 100 mg of agarose gel slices.
- Incubate the gel mixture at 55-65 ℃ for 7-10 min or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved.
- After the dissolved gel mixture cool down, transfer it to the Spin Columns assembly and incubate for 2 min at room temperature.
- Centrifuge the Spin Columns assembly in a microcentrifuge at 12,000 rpm for 1 min, and discard the flow-through.
- Wash the columns by adding 500 μL of Wash Buffer PE to the Columns. Centrifuge the columns assembly for 1 min at 12,000 rpm, and discard the flow-through.
- Repeat step 7 again.
- Centrifuge the Columns for an additional 3 min to completely remove residual wash buffer.
- Empty the Collection Tube and recentrifuge the column assembly for 1 min with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
- Place the spin column in a clean 1.5 mL microcentrifuge tube, and pipet 20 μL deionized water (pH is 8.0-8.5 and prewarm to 60 ℃)directly to the center of the column without touching the membrane. Incubate at room temperature for 2 min.
- Centrifuge for 1 min at 12,000 rpm. Discard the columns and store the microcentrifuge tube containing the eluted DNA at−20 ℃ .

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2 Characterization - XMU-China - 2012 http://2012.igem.org/Team:XMU-China/protocols

2.1 Fluorescence Measurements

- The samples to be tested are cultured from plates in 20 mL of the Basal Minimal Medium with appropriate antibiotics and incubated overnight at 37 ℃ at 200 rpm.
- The culture is checked for OD_{600} next day and then subculture by the same medium with antibiotics at 37 ℃ shaking for 2 hours.
- Add corresponding inducer at concentration gradients into the above-mentioned culture and keep on incubating. During the time incubating, every 15 min, take 1 mL bacteria liquid, then centrifuge the cells(6000 rpm, 10 min) and resuspend them in 1 mL PBS. At last, pipette to a 96 well plate.
- The plate reader made by Molecular Device then read.
- The program does the following:
- In endpoint reads, following measurements are taken in a time interval of 15 min: absorbance (600 nm filter) and fluorescence (485 nm and 520 nm for GFP).
- The results then transfer to excel sheet and interpret.

2.2 Protein electrophoresis

2.2.1 Preparation of Samples

- The samples to be tested are cultured in the Basal Medium with appropriate antibiotics, and take 200 μL bacteria liquid to determine its OD_{600} at appropriate time.
- Dilute or concentrate the next 200 μL bacteria liquid in order to let the OD_{600} equals to 4.0 while the computational formula is the actual $OD_{600} * 200 = 2.0 * X$, and X presents the total volume of the bacteria liquid after being diluted or concentrated while its unit is μL as well.
- Add 30 μL diluted or concentrated liquid into corresponding 1.5 mL centrifugal tubes, then mix up them with 10 μL loading buffer.
- Put these centrifugal tubes into metal bath and heat them in 100 ℃ in around 5 to 8 min, then centrifuge them at the speed of 13000 rpm for 5 min, the supernatant is what we need.

2.2.2 Manufacture Albumen Gel

- Prepare a clear centrifuge tube in the capacity of 50 mL, and make running gel, high concentration one, following the formula below. Then mix up them and pour the mixture into a glass pane.

Running Gel	
Final Gel Concentration (5 mL; 1 ea ;1.0 mm thick; 10%)	
ddH_2O	1.18 mL
4 × Running Gel Buffer (pH 8.8,1.5 M Tris-HCl)	1.25 mL
Monomer Solution	2.48 mL
10% SDS	50 mL
10% Ammonium Persulfate	50 mL
TEMED	5 mL

- Add some absolute alcohol to planish the top of gel. There will be approximately 60 min for its solidification.
- After solidification, pour out the alcohol and make stacking gel following the formula below. Then mix them up, add the solution onto the running gel in the glass panes until it being filled up with the gel. Insert a clean comb into stacking gel. Wait for about 40 min for stacking gel solidification.

Stacking Gel	
Stacking Gel Concentration (1 ea ;1.0 mm thick; 10%)	
ddH_2O	1.35 mL
4 × Running Gel Buffer (pH 6.8,1.5 M Tris-HCl)	0.58 mL
Monomer Solution	0.3 mL
10% SDS	25 mL
10% Ammonium Persulfate	25 mL
TEMED	5 mL

2.2.3 Electrophoresis

- Take out the glass pane with finished gel and then fasten it in an electrophoresis tank. Add some 1× Tank Buffer to detect whether liquid leak or not.
- Take out the comb slowly and use pipette to add approximately 10 to 20 μL processed samples into the wells in stacking gel.
- Add 1× Tank Buffer until the liquid level is above the platinum line in the electrophoresis tank.
- Cover up the electrophoresis tank and connect it with the electrophoresis device. Set the program 120 V- 60 min and start it up.
- When the green marker band run to the bottom of running gel, stop the device.

2.2.4 Dyeing (Colloidal Coomassie Brilliant Blue)

- Take out the gel and put it into a clean petri dish. And add appropriate Commassie Blue Staining Solution. Please make sure that the solution can cover all the gel.
- Put the petri dish onto the orbital shaker and dye for approximate 30 min.
- Pour out the staining solution then add enough destaining solution. Destain about 30 min.
- Renew the destaining solution for about 2 or 3 times until the blue background of gel being taken off.
- Pour out the destaining solution and add appropriate water to clear it.

2.2.5 Scanning

Scan the processed gel and save the picture for analysis.

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3 Immobilization - XMU-China - 2012 http://2012.igem.org/Team:XMU-China/protocols

3.1 Prepare Sodiumcellu-losesulfate (NaCS)

- Deep freeze H_2SO_4 and absolute ethanol at -20 ℃ for at least 2 hours;
- Prepare a sulphuric acid and ethanol solution at the proportion of 1.51:1(120 mL H_2SO_4 and 80 mL alcohol), maintaining it at -18 °C for at least 2 hours;
- Put the sulphuric acid and ethanol solution and 500mL industrial alcohol in an ice box, maintaining them at 0 °C for at least 1 hour;
- Immerse 4 g dry absorbent cotton in the solution in ice-bath for 66 min. Then squeezed out the solution and rinsed the reacted linters with 0℃ industrial alcohol in draught cupboard;

- Squeeze out the alcohol, then put the linters in 400 mL deionized water and regulated pH to about 3. Stir and dissolve it for 10 min, then filtrate it;
- Collected the filtrate and regulated pH to 9.3 accurately;
- Add industrial alcohol gradually to the solution until there appears the largest volume of white retiary floccule on the top of the solution;
- Centrifuge the floccule for 5 min at 5,000 rpm and collect it;
- 65 ℃ drying for at least 24 hours until it is completely dry, then collect the final production.

3.2 Prepare microcapsules

- Centrifuge 10 mL bacteria sample for 3 min at 6000 rpm and collect the deposit;
- Add 10 mL NaCS solution and mix it completely with the cells;
- Put a 6% PDMDAAC solution on a magnetic stirrer and stir it at a certain speed, maintaining a small eddy in the center of liquid surface;
- Drop the mixture into the fringe of the eddy by a 1 mL injector until it form a spheroidic membrane. It takes 10 min to react completely and form microcapsules;
- Tip all microcapsules to a strainer and rinse it with sterile water. Then transfer all microcapsules into LB medium with Ampicillin, 37 ℃ shaker incubate at 100 rpm.

3.3 Immobilize cells into calcium alginate beads:

Solutions	Preparation
3% w/v sodium alginate solution	3 g sodium alginate+100 mL deionized water
0.05 mol/L $CaCl_2$ solution	5.55 g $CaCl_2$+1000 mL deionized water（121 ℃ autoclaving for 20 min）

- Centrifuge 10 mL sample at a time for 1 min at 6,000 rpm, drain off supernatant after each spin. Repeat the procedure until 0.2~1.0 g deposits are collected.
- Resuspend the deposits with sterile water and centrifuge for 1min at 6000 rpm, drain off supernatant.
- Mix the deposits with sterile water in a mass ratio of 1:5.
- Add equivalent volume of sodium alginate solution, mixing thoroughly.

- Draw the mixture in a 1 mL injector and then drip it one by one through the pinhead into 100 mL stirring $CaCl_2$ solution. Set it aside and let the beads fully harden for 2 hours.
- Drain off the $CaCl_2$ solution and rinsed the calcium alginate beads with sterile water for 1~2 times. After that, add 100mL $CaCl_2$ solution to the beads and set it overnight.
- Separate the calcium alginate beads and $CaCl_2$ solution by a colander. The immobilization of cell sample is finished.

3.4 Immobilize cells into intra-hollow Ca-alginate capsules:

Solutions	Preparation
1% w/v sodium alginate solution	1.5 g sodium alginate powder+ 100 mL sterile distilled water（121 ℃ autoclaving for 15 min）
Cationic Mixture	1.2 g CMC powder（121 ℃ autoclaving for 15 min） 50 mL 4% w/v $CaCl_2$ solution（121 ℃ autoclaving for 15 min） Under sterile condition, add 50mL of sterile distilled water into the CMC powder. Dissolve the CMC and mix it with the 50 mL 4% w/v $CaCl_2$ solution.
3% CaCl2 Solution	12 g $CaCl_2$ powder+ 400 mL distilled water.（121 ℃ autoclaving for 20 min）

- Place 3 mL cationic mixture with 0.6mL cell suspension into a 4 mL centrifuge tube, mixing thoroughly.
- Put a magnetic stirrer at the bottom of a beaker, agitate a 100 mL sodium alginate solution at a constant rate, which maintains a small eddy in the center of liquid surface.
- Draw the mixture ($CaCl_2$/CMC/*E.* coli) with a 1 mL syringe, and then drip it one by one from a height of 10 cm into the sodium alginate solution. Keep stirring for 5 min.
- Before sifting out the capsules, dilute the gelation reaction system by adding 100 mL aseptic

distilled water. Then rinse the capsules with aseptic distilled water on a colander.

- Transfer the capsules to a 3% w/v $CaCl_2$ solution and stir for 20 min.

- Finally, rinse the capsules with distilled water to remove excess $CaCl_2$.

- The immobilization of cell sample is finished.

3.5 Immobilize cells into NaCS-PDMDAAC microcapsules

Solutions:

200 mL sulfuric acid and ethanol solution(1.51:1) maintained at 0 ℃ ;

1000 mL industrial alcohol maintained at 0 ℃;

Deionized water;

NaOH solution.

All of the above procedures are carried out at room temperature and all steps are performed under aseptic condition.

Digestion – UT-Tokyo – 2012 http://2012.igem.org/Team:UT-Tokyo/LabWork/RegularMethods

Materials

- Plasmid
- 10x buffer
- 100x Acet BSA
- Emzyme (EcoR I, Xba I, Spe I, Pst I)

Protocol

1. Add plasmid

- MilliQ up to 20uL
- 2uL 10x H or M buffer
- 0.2uL BSA
- 0.5uL emzyme I
- 0.5uL emzyme II

2. Incubation at 37 ℃ for more than two hour.

Ligation – UT-Tokyo – 2012 http://2012.igem.org/Team:UT-Tokyo/LabWork/RegularMethods

Materials

- Vector DNA
- Insert DNA
- 2x Ligation Mix

Protocol

1. Make reaction liquid

- MilliQ up to 20uL
- 10uL 2x Ligation Mix
- Vector DNA
- Insert DNA

2. Incubation at 16 ℃ for 15-30 min.

Transformation

Materials

- BioBrick parts / ligation products
- SOC or LB (No antibiotic) 500uL
- TE 15uL
- plates
- competent cells

Protocol

to thaw out igem parts

1. With a pipette tip, punch a hole in the foil

2. Add 15uL of TE (MilliQ),and pipetting

3. Pipette 1uL of the resuspended DNA Transformation into your desired competent cells

4. Hold on ice for 30 min.

5. Heat shock at 42℃ for 45 seconds (and on ice after it)

6. Add 300uL of LBborth in each epp

7. Wait for 10 mins

8. Hold at 37℃ for 30 min.

(this step can be skipped with ampicillin selection)

9. Plate out

10. Incubate at 37℃

Miniprep – UT-Tokyo – 2012 http://2012.igem.org/Team:UT-Tokyo/LabWork/RegularMethods

Material

- kit of Promega (SVMinipreps)
- incubative tube
- 1.5mL epp tube
- MilliQ

Protocol

1. pour contents out of the incubative tube into the 1.5mL tube as you can

2. centrifuge for 10min (15,000rpm)

(you can centrifuge incubative tube directly when it can endure up to 6,000g)

3. throw supernatant fluid away not to damage the precipitation

(you should decant by using yellow tip first / remove culture medium as you can / throw waste water away in bio hazard!)

4.add 250uL cell resuspension solution (red label)、suspend completely

(incomplete suspending decreases yields / you should use epp stand like a washboard)

5. add 250uL Cell lysis solution（green label）

6. turn the tube upside down four times slowly not to bubble

7. add 10uL Alkalin　Protease　Sol. （small bottle）

8. turn the tube upside down four times slowly not to bubble

9. wait for 5min　（Be careful not to exceed 5min! colon bacillus will disintegrate too much!）

10. add 350uL Neutralization Sol. (blue label）

11. turn the tube upside down four times slowly not to bubble

12. centrifuge for 10min (15,000rpm)

13. put the supernatant fluid to column (germ's wreckage is adhering below)

14. centrifuge for 1min (15,000rpm)

15. throw flow through (the liquid in the tube below) away

16. add 750uL Wash Sol. to column and centrifuge for 1min (15,000rpm)

17. throw flow through away, put 250μL Wash Sol. to column and centrifuge for 1min (15,000rpm)

18. change the column into 1.5ml tube and centrifuge for 2min (15,000rpm)

19. change the tube into new one and add 50uL MilliQ

(use Nucleas-Free Water in the kit instead of MilliQ)

20. centrifuge for 1min (15,000rpm) after waiting for 1min

21. take 1 to 1.5uL and determine the concentration by NanoDrop (Don't dilute)

22. label them

Gel extraction, PCR clean-up – UT-Tokyo – 2012

http://2012.igem.org/Team:UT-Tokyo/LabWork/RegularMethods

Material

- kit of Promega (SVMinipreps)
- Gel

Protocol

1. Gel Slice and PCR Product Preparation

Dissolving the Gel Slice *Cut out gel with wanted band and put it in a tube. *Add 3 parts Mem. binding sol. to 1 part Gel volume.

Processing PCR Amplifications *Add an equal volume of Membrane Binding Solution to the PCR amplification.

2. Shake & Incubate at 50-65 degrees C until gel is completly dissolved.

3. Put in the column.

4. Centrifuge at 15,000 rpm for 1 minute

5. Empty collection tube and add 700 ul Mem. Wash sol, centrifuge for 1 minute.

6. Empty collection tube and add 500 ul Mem. Wash sol, centrifuge for 1 minute.

7. Change the column to a new 1.5 ml Eppendorf and centrifuge at 15,000 rpm for 1 minute.

8. Change the old tube to a new 1.5 ml Eppendorf, add 30ul Nuclease-Free Water, incubate at RT for 1 minutes, then centrifuge at 15,000 rpm for 1 minute.

9. Measure concentration, label the Eppendorf.

Gel Extraction Procedure – Tianjin – 2012

http://2012.igem.org/Team:Tianjin/Protocol

Excising and Dissolving the Gel

1. Excise a minimal area of gel (up to 400 mg) containing the DNA fragment.
2. Add Gel Solubilization Buffer (L1) to the excised gel in the tube size as indicated:
3. Place the tube with the gel slice and Buffer L1 into a 50°C water bath or heat block. Incubate the tube at 50°C for 15 minutes. Invert the tube every 3 minutes to mix.
4. Purify DNA using Centrifugation as described below.

Purifying DNA using Centrifugation

1. **Load.** Place a column into a 2-mL Receiver Tube. Pipet the dissolved gel piece onto the column. Centrifuge the column at >12,000 × g for 1 minute. Discard the flow-through and place the column into the 2-mL Receiver Tube.
2. **Wash.** Add 500 μL Wash Buffer (L2) containing ethanol to the column. Centrifuge the column at >12,000 × g for 1 minute. Discard the flow-through and place the column into the Receiver Tube. Centrifuge the column at maximum speed for 1 minute.
3. **Elute.** Place the column into a clean 1.5 mL microcentrifuge tube. Add 50 μL of TE Buffer to the column. Incubate the tube for 1 minute at room temperature. Centrifuge the tube at >12,000 × g for 2 minutes.
4. **Store.** The elution tube contains the purified DNA. Store the purified DNA at −4°C for immediate use or at−20°C for long-term storage.

Agarose Gel Electrophoresis – Tianjin – 2012

http://2012.igem.org/Team:Tianjin/Protocol

General Procedure

- Cast a gel
- Place it in gel box in running buffer
- Load samples
- Run the gel
- Image the gel

Casting Gels

The amount of agarose to use in your gel depends on the DNA in question. Use the following table as a rough guide:

1. Measure out the appropriate mass of agarose into a beaker with the appropriate volume of buffer (see the documentation for your gelbox -- 50mL makes a good, thick gel for a 7x10cm gelbox).
2. Microwave until the agarose is fully melted. This depends strongly on your microwave, but a 90 seconds at full power or 3 minutes at half power seem to provide decent results. As long as you do not burn the agarose and nothing bubbles over, this step is robust.
3. Let the agarose cool on your bench until touching the bottom of the beaker with your bare hand doesn't burn you (~5 minutes for a 50mL gel).
4. At this point add your DNA stain, e.g., ethidium bromide. The beaker will cool unevenly (surface first), so you must be careful not to cause ripples and bubbles.
5. While the solution is cooling, seal the open edges of your gel box with one long piece of masking tape on each side. Make sure it is sealed well or the gel will leak.
6. Pour the agarose solution into the taped gelbox. Carefully pop or shove to the side any bubbles, put in the comb, and let it cool for about 30 minutes, until the gel is solid.
7. If your gel is at all purple, and you are using ethidium bromide as the DNA stain, you need to decrease your concentration by at least a factor of ten.

According toOpenWetWare.org

T4 Ligase Ligation – Tianjin – 2012

http://2012.igem.org/Team:Tianjin/Protocol

Materials

- T4 DNA Ligase
- 10x T4 DNA Ligase Buffer
- Deionized, sterile H_2O
- Purified, linearized vector (likely in H_2O or EB)
- Purified, linearized insert (likely in H_2O or EB)

Procedure

10µl Ligation Mix

1. 1.0 µL 10X T4 ligase buffer (use 10µl aliquots in -20 freezer; repeated freeze-thaw cycles can degrade the ATP in the buffer that's critical for the ligation rxn)
2. 6:1 Molar ratio of insert to vector (~10ng vector). Gradients are used sometimes.
3. Add (8.5 - vector and insert volume)µL ddH2O
4. 0.5 µL T4 Ligase

Method

1. Add appropriate amount of deionized H2O to sterile PCR tube
2. Add 1 µL ligation buffer to the tube.
3. Pipette buffer up and down before pipetting to ensure that it is well-mixed.
4. Add 0.5 µL T4 ligase. PIPETTE half the volume of the mixture UP AND DOWN to ENSURE MIXING OF THE ENZYME.
5. Also, the ligase, like most enzymes, is in some percentage of glycerol which tends to stick to the sides of your tip. Just touch your tip to the surface of the liquid when pipetting to ensure accurate volume transfer.
6. Let the 10 µL solution incubate at 25°C for 15mins.
7. Store at 4°C.
8. (Use agarose gel electrophoresis to check sometimes)
9. Transform into cell.

PCR Overlap Extension – Tianjin – 2012

http://2012.igem.org/Team:Tianjin/Protocol

Procedure

1. Design Primers:
    1. These primers are like bridges between the two parts you want to assemble together.
    2. You will order two primers which are complements of one another.
    3. These primers will each have a 60°C Tm with one part and a 60°C Tm with the other part.

    4. The "end primers" will not have any complements and will likely only have restriction sites.
2. **"Extension PCR"** PCR amplify the necessary fragments separately
    1. Use a proofreading polymerase enzyme.
    2. Use an annealing temp of 60°C.
3. Clean up the product using a DNA column.
4. **"Overlap PCR"** Use cleaned up fragments as template in a PCR reaction:

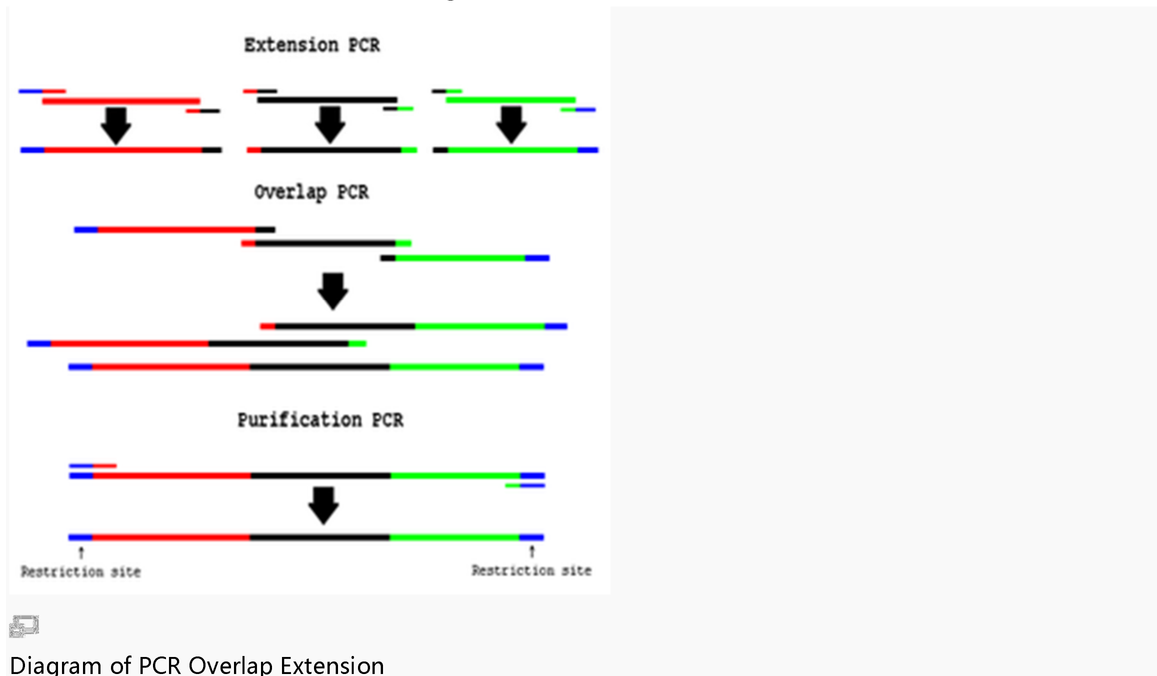

Diagram of PCR Overlap Extension

    1. About 1/2 to 3/4 volume of the Overlap PCR reaction should be equimolar amounts of purified fragments.
    2. Do not use Phusion polymerase. Try Pfu Turbo.
    3. Do not add any primers; the templates will prime each-other.
    4. Run 15 PCR cycles without primers.
    5. Use an annealing temp of 60°C.
5. "Purification PCR" Add end primers to the Overlap PCR reaction:
    1. Continue cycling for another 15-20 rounds.
    2. Use an annealing temp of 72°C
6. Gel extract the correct size fragment.
7. Clone into the desired vector.
    1. Digest
    2. Ligate
    3. Transform

4. Select
5. Sequence

DNA Assembler in Yeast – Tianjin – 2012

http://2012.igem.org/Team:Tianjin/Protocol

SC Minimal Medium and Plates

SC is synthetic minimal defined medium for yeast. 0.67% yeast nitrogen base (without amino acids) 2% carbon source (i.e. glucose or raffinose) 0.01% (adenine, arginine, cysteine, leuine, lysine, threonine, tryptophan, uracil) 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine) 2% agar (for plates)

1. Dissolve the following reagents in 900 ml deionized water (800 ml if preparing medium containing raffinose). Note: We make medium and plates as we need them and weigh out each amino acid. Many researchers prepare 100X solutions of each amino acid that they need.
   Reminder: Omit uracil to make selective plates for growing pYES2 transformants.</br>
2. If you are making plates, add the agar after dissolving the reagents above.
3. Autoclave at 15 psi, 121°C for 20 minutes.
4. Cool to 50°C and add 100 ml of filter-sterilized 20% glucose or 200 ml of filter-sterilized 10% raffinose.
5. Pour plates and allow to harden. Invert the plates and store at 4°C. Plates are stable for 6 months.

Induction Medium

If you are making induction medium, follow Steps 1–3 above except dissolve the reagents in 800 ml of deionized water. Cool the medium to 50°C and add 100 ml of filter-sterilized 20% galactose and 100 ml of filter-sterilized 10% raffinose to the medium.

When making stock solutions of raffinose, do not autoclave the stock solution. Autoclaving the solution will convert the raffinose to glucose. Filter-sterilize the stock solution.

Yeast Extract Peptone Dextrose Medium (YPD) 1 liter

1% yeast extract 2% peptone 2% dextrose (D-glucose)

1. Dissolve the following in 1000 ml of water:
   - 10 g yeast extract
   - 20 g peptone
   - 20 g dextrose (see note below if making plates)
2. Optional: Add 20 g agar, if making plates.
3. Autoclave for 20 minutes on liquid cycle.
4. Store medium at room temperature or cool the medium and pour plates. The

shelf life is approximately one to two months.

0.1 M Sodium Phosphate, pH 7.4

Materials needed:

Sodium phosphate, monobasic ($NaH_2PO_4{\cdot}H_2O$; Sigma-Aldrich S9638)

Sodium phosphate, dibasic ($Na_2HPO_4{\cdot}7H_2O$; Sigma-Aldrich S9390)

Protocol:

1. Prepare 100 ml of 1 M NaH2PO4·H2O by dissolving 13.8 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
2. Prepare 100 ml of 1 M Na2HPO4·7H2O by dissolving 26.81 g in 90 ml of deionized water. Bring volume up to 100 ml. Filter-sterilize.
3. For 1 liter of 0.1 M sodium phosphate, pH 7.4, mix together 22.6 ml of 1 M NaH_2PO_4 and 77.4 ml of 1 M Na_2HPO_4. Bring the volume up to 1 liter with deionized water.
4. Filter-sterilize and store at room temperature.

10X TE

100 mM Tris, pH 7.5 10 mM EDTA

1. For 100 ml, dissolve 1.21 g of Tris base and 0.37 g of EDTA in 90 ml of deionized water.
2. Adjust the pH to 7.5 with concentrated HCl and bring the volume up to 100 ml.
3. Filter-sterilize and store at room temperature.

Alternatively, you can make the solution using 1 M Tris-HCl, pH 7.5 and 0.5 M EDTA, pH 8.0.

1X TE

10 mM Tris, pH 7.5 1 mM EDTA Dilute 10X TE 10-fold with sterile water.

10X LiAc

1 M Lithium Acetate, pH 7.5

1. For 100 ml, dissolve 10.2 g of lithium acetate in 90 ml of deionized water.
2. Adjust pH to 7.5 with dilute glacial acetic acid and bring up the volume to 100 ml.
3. Filter-sterilize and store at room temperature.

1X LiAc

100 mM Lithium Acetate, pH 7.5 Dilute 10X LiAc solution 10-fold with sterile, deionized water.

1X LiAc/0.5X TE

100 mM Lithium Acetate, pH 7.5 5 mM Tris-HCl, pH 7.5 0.5 mM EDTA

1. For 100 ml, mix together 10 ml of 10X LiAc and 5 ml of 10X TE.
2. Add deionized water to 100 ml.
3. Filter-sterilize and store at room temperature.

1X LiAc/40% PEG-3350/1X TE

100 mM Lithium Acetate, pH 7.5 40% PEG-3350 10 mM Tris-HCl, pH 7.5 1 mM EDTA

1. Prepare solution immediately prior to use. For 100 ml, mix together 10 ml of 10X LiAc, 10 ml of 10X TE, and 80 ml of 50% PEG-3350.
2. Filter-sterilize and store at room temperature.

Small-Scale Yeast Transformation – Tianjin – 2012

http://2012.igem.org/Team:Tianjin/Protocol

Materials Needed

- YPD liquid medium
- 1X TE (see Recipe, page 12)
- 1X LiAc/0.5X TE (see Recipe, page 12)
- Denatured salmon sperm DNA (see recipe on the next page)
- pYES2 vector construct (or other plasmid DNA to be transformed)
- 1X LiAc/40% PEG-3350/1X TE (See Recipe, page 12)

- DMSO
- Selective plates

Protocol

1. Inoculate 10 ml of YPD medium with a colony of INVSc1 and shake overnight at 30°C.
2. Determine the OD600 of your overnight culture. Dilute culture to an OD600 of 0.4 in 50 ml of YPD medium and grow an additional 2–4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 ml 1X TE.
4. Pellet the cells at 2500 rpm and resuspend the pellet in 2 ml of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes.
6. For each transformation, mix together 1 µg plasmid DNA and 100 µg denatured sheared salmon sperm DNA with 100 µl of the yeast suspension from Step 5.
7. Add 700 µl of 1X LiAc/40% PEG-3350/1X TE and mix well.
8. Incubate solution at 30°C for 30 minutes.
9. Add 88 µl DMSO, mix well, and heat shock at 42°C for 7 minutes.
10. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
11. Resuspend the cell pellet in 1 ml 1X TE and re-pellet.
12. Resuspend the cell pellet in 50–100 µl 1X TE and plate on a selective plate.

Preparing Denatured Salmon Sperm DNA – Tianjin – 2012 http://2012.igem.org/Team:Tianjin/Protocol

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Materials Needed

- Salmon Sperm DNA (Sigma-Aldrich, Cat. no. D1626)
- 1X TE
- Sonicator
- 50 ml conical centrifuge tubes
- TE-saturated phenol
- 250 ml centrifuge bottle
- Chloroform
- Low-speed centrifuge
- 3 M sodium acetate, pH 6.0
- 95% ethanol (–20°C)
- 250 ml centrifuge bottle
- TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)

Protocol

1. In a 250 ml flask, dissolve 1 g salmon sperm DNA into 100 ml of TE

(10 mg/ml). Pipette up and down with a 10 ml pipette to dissolve completely.

1. Incubate overnight at 4°C.
2. Using a sonicator with a large probe, sonicate the DNA twice for 30 seconds at 3/4 power. The resulting DNA will have an average size of 7 kb. You may verify the size of the DNA on a gel.
3. Aliquot the sonicated DNA into four 50 ml conical centrifuge tubes (25 ml per tube).
4. Extract with 25 ml of TE-saturated phenol. Centrifuge at 10,000 × g for 5 minutes at 4°C. Transfer the DNA (upper layer) to a fresh 50 ml conical centrifuge tube.
5. Extract with 25 ml of TE-saturated pheno:chloroform:isoamyl alcohol (25:24:1). Centrifuge at 10,000 × g for 5 minutes at 4°C. Transfer the DNA (upper layer) to a fresh 50 ml conical centrifuge tube.
6. Extract with 25 ml of chloroform. Centrifuge at 10,000 × g for 5 minutes at 4°C. Transfer the DNA (upper layer) to a 250 ml centrifuge bottle.
7. Add 5 ml of 3 M sodium acetate, pH 6.0 (1/10 volume) and 125 ml ice-cold (–20°C) 95% ethanol (2.5 volume) to precipitate DNA.
8. Pellet the DNA at 12,000 x g for 15 minutes at 4°C.
9. Wash the DNA once with 200 ml 70% ethanol and centrifuge as described in step 9.
10. Partially dry DNA by air or in a Speed-Vac (cover tubes with parafilm and

poke holes in top) for 20 minutes.

1. Transfer DNA to a 250 ml sterile flask and dissolve DNA in 100 ml sterile TE (10 mg/ml).
2. Boil for 20 minutes to denature DNA. Immediately place on ice, aliquot in 1 ml samples, and freeze at –20°C.

According to INVITROGEN pYES2 Manual

Digestion – SJTU-BioX-Shanghai – 2012 http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Material

- EcoRI, BglII, PstI, XhoI, SpeI,XhoI,FD buffer,FD Green buffer, DNA,sterile water

Digestion temperature

- 37℃

Time period

1. Plasmid: 10-30min(the higher the concentration, the longer time it takes.
2. DNA fragment:1-2h(the higher the concentration, the longer time it takes.Since it is more difficult to digest DNA than plasmid, digesting DNA requires more time.

System amount

1. 50ul system is preferred if there is subsequent operations. If digestion is only for identification, 20ul system is used.
2. During the double digestion, the optimum buffer system which is suitable for both enzymes should be utilized. Without such shared system, digestion of two different enzymes is usually separated.

System

Enzyme	1μl
10×FD Buffer or FD Green buffer	2μl
DNA	≤1μg
Sterile water	up to 20μl

Gel extraction – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Material

- Agarose Gel DNA Extraction Kit (centrifugation)

Experimental Procedure

1. Add 500uL balance liquid(BL) to the spin column(CA2). Centrifuge at 12,000 x g for 1 min at room temperature. Discard the flow-through and put the column back to the collection tube.(optional)
2. Excise the DNA fragment from the agarose gel and weigh it in a clean centrifuge tube (A gel slice of 100 mg approximately equals to 100 μL).
3. Add 3 volume of sol solution(PN) to the centrifuge tube and incubate the mixture at 50℃ for 10 min with mixing the tube by tapping the bottom of the tube occationally till the gel has melted completely. Cool the tube to RT.
4. Transfer the mixture to an spin column (CA2) within a collection tube. Centrifuge at 12,000 x g for 1 min at room temperature. Discard the flow-through and put the column back to the collection tube.

5. Add 600 μL DNA Wash Buffer (PW) to the column and centrifuge at 12,000 x g for 1 min at room temperature. Discard the flow through and put the column back to the collection tube.
6. Repeat step 5.
7. Put the column back to the collection tube. Centrifuge at 12,000 x g for 2 min at room temperature to discard the PW totally. Keep the lid open for several minutes.
8. Put the column back to the clean collection tube. Add ddH2O to the column vacantly and keep it at room temperature for 2 minutes. Centrifuge at 12,000 x g for 2 min at room temperature to collect the DNA solution.
9. Transfer the eluted solution to CA2 and keep it at room temperature for 2minutes. Centrifuge at 12,000 x g for 2 min at room temperature.
10. Gel electrophoresis to verify the correctness and concentration of bands.

Important Notes

1. If the balance liquid turns turbid, incubate it at 37℃ for several minutes.
2. When cutting the gel, keep the UV irradiation time period as short as possible, otherwise DNA would be damaged.
3. For those DNA fragment shorter than 100bp or larger than 10kb, the sol solution volume should be increased as well as the time period for adsorption and elution.
4. Recovery depends on the initial DNA amount and elution volume. The less the DNA, the less the elution volume, the smaller of recovery.

PCR – SJTU-BioX-Shanghai – 2012 http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Reagents

- KOD-Plus-Neo
- Tap enzymes

Experiment system

- Experiment system with KOD enzymes (Mostly 50uL)
- Experiment system with taq enzymes:
- Reaction temperature:

```
Two-step method:
Predenature
  94℃, 2min
```

```
Denature and extension
  {
  98℃ 10sec
  68℃ 30sec/kb
  }
                × 25-45 cycles
  4℃
```

```
Three-step method:
Predenature
  94℃, 2min
Denature, annealing and extension
  98℃ 10sec
 {
  (Tm-5) ℃  30sec
  68℃  30sec/kb
  }
               × 25-45 cycles
  4℃
```

Important Notes

1. KOD enzymes should be operated in ice.
2. Two-step method is preferred if primer Tm is relatively high.
3. 1.2% agarose gel is used to separate 1kb-5kb DNA; 0.8% agarose gel is used to separate 3k-10k DNA; 2.5-3% agarose gel is used to separate DNA longer than 100bp.
4. PCR reaction system varies from 20uL to 100uL. Under most cases, 20uL system is preferred as long as PCR is for identification only. However, when subsequent experiments such as tailing, digestion which require certain amount of DNA are necessary, 50uL system is usually preferred.
5. KOD enzymes are preferred if the purpose of PCR is for gene amplification. At the same time, PCR for identification or tailing usually requires taq enzymes.

PCR Purification – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Reagents

- LifeFeng DNA PCR purification kit

Experimental Procedure

1. Transfer PCR system (< 250uL) to 1.5mL microcentrifuge tube. Add 5 volume Buffer PD and mix. Have a quick centrifugation to remove the liquid on lid.
2. Transfer the mixture to DNA spin column (A4) and put it in a collection tube.
3. Centrifuge at 7000g for 1 minute and replace the collection tube with a new collection tube.
4. To wash, add 500uL Buffer WB to the spin column (A4) and centrifuge at 7000g for 1 minute. Discard the flow-through and place the column back in the same tube.
5. Repeat step 4.
6. Centrifuge at 12,000g for 2 minutes.
7. Place spin column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add ddwater (>30uL) to the center of the membrane. Let the column stand for 1-2 min and centrifuge the column for 1 min at 12,000g.
9. Add elution liquid back to spin column and centrifuge at 12,000g for 1 min. (Inoculating ddwater at 65℃ prior would improve the elution efficiency)
10. Gel electrophoresis to verify the concentration of bands.

Important Notes

- Buffer PD contains irritant compounds, thus be careful not to avoid contamination of skin.

DNA Ligation – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Experimental Procedures

1. Set up the following reaction in a microcentrifuge tube on ice.
2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours
5. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Ligation System

COMPONENT	20 µl REACTION
10X T4 DNA Ligase Buffer	2 µl
Vector DNA	0.025 pmol
Insert DNA	0.076 pmol
T4 DNA Ligase	1µl

Nuclease-free water	to 20 µl

Bacteria Culture – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Strain

1. DH5a (F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–) use for cloning
2. BL21(DE3) (fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS,λ DE3 = λ sBamHIo Δ EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5) use for expressing

Medium – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

LB Medium

NaCl	10g
Tryptone	10g
Yeast Extract	5 g
Water	up to 1000mL

SOB Medium

Tryptone	20g
Yeast extract	5g
NaCl	0.5g
1M KCl	2.5ml
ddH2O	up to 1000 ml

Adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, and add 10 ml of 1 M MgCl2 before use

Antibiotics – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

	Storage	Use
Kana	50 mg/ml(Water)	50 µg/ml
Amp	50 mg/ml(Water)	50 µg/ml
Tet	10 mg/ml (Alcohol)	10 µg/ml
Cm	34 mg/ml (Alcohol)	68 µg/ml

Transformation – SJTU-BioX-Shanghai – 2012
http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

1. Remove competent cells from freezer and allow to thaw
2. Add 3-5 μl of DNA to the cells
3. Incubate on ice for 30 min
4. Heat shock the cells at 37°C for 1.5 mins
5. Add 500 μl of SOB medium, and incubate in a shaker at 37°C for 1 hour
6. Centrifuge for 3 min at 3,000 g, remove and keep 100 μl of the supernatant, and pour away the rest
7. Re-suspend the pellet in the 100 μl of supernatant that was removed earlier
8. Spread All onto one plate with corresponding antibotics, and then pour the rest onto another. Incubate the plates overnight at 37°C.

Violacein Protocols – SJTU-BioX-Shanghai – 2012 http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

Induction and Extraction

1. Incubate the monoclonal from medium in a shaker at 37°C overnight
2. Add 0.1% Arabinose, 1mM Ferrous ammonium sulfate and 40 μM to start induction for another 6 hours
3. Centrifuge for 1 min at 10000rpm, remove the LB Medium
4. Add 800 μL 10%SDS and break cells using ultrasound
5. Add 800 μL ethyl acetate to extract violacein/deoxyviolacein
6. Use rotovap to concentrate violacein and resuspend with methanol

HPLC

- Column: C18, 15cm

- Mobile phase: 75% methanol, 25% ddH2O
- Injection volume: 20 μL
- Speed: 0.5 mL/min
- Detection: 230 nm

Fatty Acid Protocols – SJTU-BioX-Shanghai – 2012

http://2012.igem.org/Team:SJTU-BioX-Shanghai/Notebook/protocol

1. Transfer a fresh transformed colony to 5ml LB liquid medium and shake at 37℃ overnight(12h).
2. Add seed broth to 50-100ml LB medium(volume ratio is 3% and loaded liquid is 20%) and shake until the OD600 reaches 0.6 or so. Add inducer of appropriate concentration to induce gene expression.
3. Continue shake culture for another 20h. Contrifuge at 8,000 for 5min. Collect supernatant and bacteria sediment respectively (prepare 3 parallel samples, each 10ml).
4. Extract the supernatant by chloroform-methanol(2:1) solution of the same volume (shake for 5min) place until stratification. Dry the lower organic layer at 50℃ for about 24h. Suspend the bacteria by 1ml ddH2O. Add 20ml chloroform-methanol solution and extract for another 2 times.(Attention: weigh the empty flask before drying).
5. Weigh the crude extract and record the results. (For reference only)
6. Dissolve the crude extract with 1ml methanol and add 2ml tetrafluoroboron. Methyl esterificate at 60℃ for 30min. After the reagent cools down, add 2ml N-hexane and extract for 2 times. Collect the upper layer and dry overnight at 50℃ (weigh the empty flask before drying as well).

7. Add the crude bacteria extract to 1ml chloroform-methanol(2:1) solution and shake by vortex to extract the total lipid of the cells. Add 2ml methanol-water(4:1) as saponification reagent. Saponificate in 60℃ water bath for 1h. Then esterificate as the 6th step.
8. Dissolve the obtained fatty acidmethyl ester(FAME)mixture with N-hexane(2mg/ml). Take 1ml sample for test.
9. GC-MS condition:

Sample injector temperature: 250℃;

Detector temperature: 280℃;

Temperature programming: begin at 100℃ and last 2min. Warm to 250℃ at the rate of 10 ℃/min and last 5min.

Use C15 FAME as the interior label for quantification. (Quantity of C15 FAME depends on the sample concentration)

Cell survival assay – Osaka – 2012 http://2012.igem.org/Team:Osaka/Protocols

1. 10 ml of LB broth was inoculated with 100 µl of an overnight culture and grown until the OD_{600} reaches 0.4-0.6.
2. Induce parts with IPTG addition to final concentration of 50 µM and incubate for 1h.
3. Cells were split into 3 ml aliquots in test tubes, and various concentrations of DNA damaging agents (we used Mitomycin C and Hydrogen peroxide) were added.
4. After incubation for 2 h with shaking, centrifuge and discard antibacterial agents spiked medium and resuspend with fresh LB medium.
5. [RECOMMENDED] Dilute pre-culture samples according to expected survival rate, as determined from a preliminary experiment (lower dilution rate is needed if fewer cells are expected to survive).
6. Pipette 100 µl to LB agar plates and spread evenly. Air-dry the plates to remove excess wetness.
7. Wrap plates in aluminium foil and incubate at 37℃.
8. After 16h, count number of colonies formed on control and mitomycin C-treated inoculum plates.

Promoter assay : Dual-GloR Luciferase Assay System (Promega) – Osaka – 2012 http://2012.igem.org/Team:Osaka/Protocols

1. 10 ml of LB broth was inoculated with 100 µl of an overnight culture and grown until the OD_{600} reaches 0.4-0.6.
2. Cells were split into 500 µl aliquots in 1.5ml tubes, and various concentrations of DNA damaging agents (we used Mitomycin C and Hydrogen peroxide) were added.
3. After incubation for 2 h with shaking, centrifuge the incubative tube at 3,000g for 15 min with soft brake.
4. Decant supernatant, wash with 1mL PBS
5. Centrifuge at 3,000g for 10 min with soft brake.
6. Decant supernatant, add 100 µl cell lysis buffer.
7. Remove lysate 10-50 µl to the vial.
8. Add a volume of Dual-GloR Reagent equal to the volume of cell lysate.
9. Wait at least 10 minutes to allow for chemiluminescence to become stable, then measure the firefly luminescence in a uminometer.
10. Add a volume of Dual-GloR Stop & GloR Reagent equal to the original culture medium volume.
11. Wait at least 5 minutes, then measure Renilla luminescence
12. Calculate the ratio of luminescence from the experimental reporter to luminescence from the control reporter.

1. Design of amiRNA Sequences – Nanjing-China – 2012 http://2012.igem.org/Team:Nanjing-China/Notebook

The target gene we want to silence is PGC-1a which is related to metabolism in several tissues. After inputting the sequence of PGC-1a to the Web MicroRNA Designer, the software will give us several potential amiRNA sequences which have high possibilities to silence the target genes. Although the sequences are picked out based on several principles, we still need to select the sequences by ourselves in the rules:

- It is preferable for all intended target genes to not have mismatches to the amiRNA at positions 2 to 12.
- AmiRNA candidates with one or two mismatches at the 3¢ end of the amiRNA (positions 18 to 21) should be preferred, since it has been suggested that perfectly matching amiRNAs might trigger so-called transitive siRNA formation, where amplification of sequences adjacent to the binding site is primed by the miRNA. These sequences could in turn themselves serve as silencing triggers and affect other, unintended genes.

- The absolute hybridization energy of the binding between amiRNA and the target sequence should be less than −30 kcal/ mole, and preferable be in the range between −35 and −40 kcal/ mole.
- The amiRNA binding site should be located within the coding region of the target gene, since UTRs are more likely to be misannotated. At least two amiRNAs per target gene or group of genes should be selected for experimental work. If several are selected, the amiRNAs should bind the target mRNA at different locations, since secondary structure is suspected to influence miRNA efficacy.

Of course, there is still an important thing to do before we use the sequences to do experiments. That is we should to do BLAST research to check whether the sequences have binding sites in other genes which will greatly affect the experiment.

After all these work we can get the amiRNA sequences which may work well during the experiments.

2. Construction of aMIRNA precursors by Site-Directed Mutagenesis – Nanjing-China – 2012

http://2012.igem.org/Team:Nanjing-China/Notebook

What we want to do is to make a miRNA precursor and make use of the Dicer in the plants to produce amiRNA which we design in the former step. MIRNA precursors fold back on themselves to form a hairpin structure, and it is important to preserve this structure for successful processing. Therefore, engineering of amiRNAs into MIRNA precursor templates not only requires the exchange of the miRNA by the amiRNA sequence, but also of the pairing region in the hairpin, called the (a)miRNA*, such that pairing positions as well as G:U pairs are retained. The WMD software (WMD-Oligo window) thus generates four oligonucleotides per amiRNA sequence input: I and II to engineer the actual amiRNA, and III and IV for the amiRNA* (with wobbles).

Endogenous MIRNA precursors that have been cloned into plasmids serve as templates for PCR reactions to exchange miRNA and miRNA*. These precursors include the hairpin and short pieces of flanking sequence on either side, which are known to be part of the longer endogenous MIRNA transcript.

Six PCR oligonucleotide primers are needed to produce an aMIRNA transgene. Four primer sequences are generated by WMD and are given in 5¢→3¢ orientation. They are 40 nucleotides long and specific for the intended amiRNA. The 5¢ most two and 3¢ most 17 nucleotides match the template MIRNA precursor, while the 21 nucleotides in between do not match and will generate the amiRNA and amiRNA* in the amplicon . An additional two general oligonucleotides (A and B) that match the harboring plasmid outside of the MIRNA precursor are also required. They have been placed such that the sizes of the resulting PCR products enable convenient purification and handling.

Using the six primers, the aMIRNA precursor is amplified in three pieces (a–c) as shown in the picture. The three pieces are subsequently fused to one amplicon (d) in a single PCR reaction which is called overlapping PCR. Then we can get the aMIRNA precursors.

3. Cloning – Nanjing-China – 2012

http://2012.igem.org/Team:Nanjing-China/Notebook

After getting the purified PCR fragment (d), we do blunt-end cloning by using linearized plasmids. The PCR products (d) are put into vectors, after that we do sequencing to ensure that the new plasmid is indeed transformed.

Once the result of sequencing is right, we do sub-cloning into binary plasmids. In this step we cut off the sequences from the sequencing plasmids and make it into binary plasmids. We transfect the new binary plasmids into the Agrobacterium.

Finally we pick out the Agrobacterium which has taken up the binary plasmids by using the Gentamycin and Specmycin to prepare for the infection in the next step.

4. Plant Transformation – Nanjing-China – 2012

http://2012.igem.org/Team:Nanjing-China/Notebook

We use the Agrobacterium strain delivering the above-described binary plasmid to generate transgenic plants. After that we pick out the transgenic plants by using Gentamycin. With several generations we can get some transgenic lettuce which can be put into soil.

5. Culture of lettuce – Nanjing-China – 2012

http://2012.igem.org/Team:Nanjing-China/Notebook

- Clean the seeds with 10% NaClO for 15min and then clean the seeds with water until the water is limpid. Put the seeds in the 1/2 MS medium uniformly. Put the seeds in the culture box for two days without light and then for one day in light.
- Trim the cotyledon through the vein and cut it down with scissors. Put them in a new 1/2 medium which has a filter paper on it for one day in light.
- Use the 5% sucrose wash and re-suspend the Agrobacterium. Put the cotyledon in the sucrose with 0.1%AS for 15min. Put them back onto the filter paper. Put in the culture box for two days without light.
- Wash the cotyledon with water which is mixed with Carb for 3 times. Put the cotyledon into MS medium which contain Gentamycin.
- After several generations put the plantlet which is still alive to a medium which is used for inducing roots.
- Put the plantlet which has roots into soil that experiences sterilization.

LB medium – KIT-Kyoto – 2012

http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

***per kilogram**

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
dH_2O	1000mL

1. Mix the reagents according to the previous components.
2. Adjust the pH (pH7.0) with NaOH.
3. Autoclave at 120℃ for 20min

LB plate – KIT-Kyoto – 2012 **http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol**

*per liter

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
dH_2O	1000mL

1. Mix the reagent according to the previous components.
2. Add NaOH and carry the enzymes pH7.0.
3. Add 15g Bacto agar and dissolve.
4. Autoclave at 120℃ 20min.
5. After cooling down approx. 60℃, add the suitable amounts of appropriate antibiotics.
6. Pour 20~30mL into laboratory dishes.

*Solution Ⅰ

Glucose	50mM
EDTA	10mM
Tris-HCl	25mM

*Solution Ⅱ

NaOH	0.2N
SDS	1%

*Solution Ⅲ

K-acetate	5M

Methods

Transformation into plasmid of E.coli.

1. Dissolve competent cell on ice.
2. Pour 100uL competent cell into 1.5mL tube, and mix with 1~5uL DNA.
3. Incubate for 30 minutes on ice.
4. Treat with heat shock the cells at 42℃ for 45 seconds.
5. Cool down on ice for 2 minutes.
6. Add 250uL S.O.C medium at room temperature.
7. Incubate at 37℃ with shaking for 1 hour.
8. Spread a suitable amount of the cells on LB plate (containing appropriate antibiotics).
9. Incubate overnight at 37℃.

Picking up a single colony

Pick up the colony grown on the LB plate by a platinum stick and put in 2.5mL of LB medium (containing appropriate antibiotics), and cultivate cells at 37˚C.

Miniprep by Alkaline-SDS method. – KIT-Kyoto – 2012
http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. Spin 1.5 mL of culture in microcentrifuge tube at 12,000 rpm for 5 min. at 4 ˚C. Discard the supernatant
and spin again for 2 min. Remove remaining liquid carefully.
2. Resuspend the bacterial cell pellet in 100 μL of solution I previously cooled on ice.
3. After 4 min. at room temperature, add 200 μL of solution II and mix by inverting several times.
4. Keep on ice for 4 min.
6. Add 160 μL of solution III and mix gently by inverting several times. Keep on ice for 5 min.
7. Centrifuge at 12,000 rpm for 10 min. at 4˚C
8. Transfer supernatant to new tube.
9. Add the same volume of phenol/chloroform and vortex well. Centrifuge at 12,000 rpm for 2 min at room
temperature.
10. Transfer top clear layer to new tube. Add the same volume of diethyl ether and vortex. Centrifuge at
12,000 rpm for 2 min. at room temperature.
11. Transfer the top clear layer to new tube. Add 2X volume of ethanol and vortex. Centrifuge at 12,000 rpm
for 10 min. at room temperature.
12. Remove supernatant and add 1 mL of 70% ethanol and mix gently. Centrifuge at 12,000 rpm for 5 min at
room temperature.
13. Remove supernatant and dry in vacuo. Dissolve pellet in 20 μL TE.

Miniprep by QIA prep Spin Miniprep Kit – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. Spin 1.5 mL of culture in microcentrifuge tube at 10,000 rpm for 5 min. at 4˚C. Discard the supernatant
and spin again for 2 min. Remove remaining liquid carefully.
2. Resuspend the bacterial cell pellet in 250 μL of buffr P1.
3. Keep at room temperature for 4 min.
4. Add 250 μL of buffer P2 and mix by inverting quickly.
5. Keep on ice for 4 min.
6. Centrifuge at 13,000 rpm for 10 min. at 4˚C.
7. Apply supernatant to QIAprepspin column.
8. Centrifuge at 10,000 rpm for 40 sec. at room temperature. Discard the flow-through.
9. Add 500 μL of buffer PB. Centrifuge at 10,000 rpm for 40 sec. at room temperature. Discard the flow-
through.
10. Add 750 μL of buffer PE. Centrifuge at 10,000 rpm for 40 sec. at room temperature. Discard the flow-
through.
11. Centrifuge again at 10,000 rpm for 1 min. at room temperature. Set a column on new 1.5 mL tube.
12. Add 30 μL of buffer EB and keep at room temperature for 1 min. Centrifuge at 10,000 rpm for 1 min at
room temperature.

13. Recover purified DNA in 1.5 mL tube.

Midiprep by Pure Link^{TM} HiPure Midiprep Kit – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. Centrifuge (4000xg,4℃,10min) E. coli culture in LB medium (50 mL) and remove the supernatant
2. Mix with suspension buffer (4mL) including RNase (20 micro g/mL) and the pellet
3. Add Lysis buffer (4mL) and turn upside and down several times in order to mix well
4. Incubate at room temperature for 5min
5. Add precipitation buffer (4mL) and shake the tube quickly
6. Centrifuge and remove the supernatant (15000 x g, room temperature, 10 min)
7. Transfer the supernatant to balanced column and elute out the solution by gravity
8. Wash the column twice with Wash buffer (10 mL) and elute out the solution as gravitation every after
   washing.
9. Put sterilized 15 mL centrifuge tube under the column
10. Add Elution buffer (5 mL) to the column and elute out the solution by gravity
11. Add isopropanol (3.5 mL) to the centrifuge tube and mix well
12. Centrifuge and remove the supernatant (15000 x g, 4℃, 30min)
13. Add 70% ethanol and mix well
14. Centrifuge and remove the supernatant (15000 x g, 4℃, 5min)
15. Dehydrated ,then suspend purified DNA into TE buffer (200 uL)

Purification of DNA from gel by QIA quick Gel Extraction Kit – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. Cut out the DNA fragment from agarose gel and put it in a 1.5mL tube and weigh the gel
2 .Add Buffer QG (x 3 volume of the gel weight)
3 .Vortex every 2 - 3 minutes to dissolve the gel completely
4 . Add isopropanol (equal weight to the gel, 100uL per 100mg)
5 . Trabsfer to spin column
6 . Centrifuge (10000rpm, room temperature,1min)
7 . Remove the solution
8 . Add Buffer PE (750 uL) and centrifuge (10000rpm,room temperature,1min)
9 . Remove the solution and centrifuge (17900 x g, room temperature, 1min)
10. Set a new 1.5 tube under the column, add TE (30uL) and centrifuge (10000 rpm, room temperature,1min)
11. Collect the purified DNA

BP reaction by Invitrogen gateway system – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. PCR using primers containing the attB sequence.
2. Purify PCR product.
3. Add the following components to a 1.5 mL microcentrifuge tube at room temperature and mix:

attB PCR product	75 ng/reaction (1-7 μL)
pDONR vector	150ng/reaction (1 μL)
TE Buffer	to 8 μL

4. Vortex BP ClonaseII enzyme mix briefly. Add 1 - 2 μL to the components above and mix well by vortexing
   and spin them down.
5. Incubate reaction at 25˚C for more than 1 hour.
6. Add 1 μL of 2 μg/μL Proteinase K solution and vortex briefly.
7. Incubate at 37˚C for 10 minutes.

LR reaction by Invitrogen gateway system – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. Add the following components to a 1.5 mL microcentrifuge tube at room temperature and mix:

Entry clones	50-150 ng/reaction (1-7 μL)
Destination vector	150ng/reaction (1 μL)
TE Buffer	to 8 – 9 μL

2. Vortex LR ClonaseII enzyme mix briefly. Add 1 – 2 μL, to the components above
   and mix well by vortexing and spin down.
3. Incubate reaction at 25˚C for 16 hours.
4. Add 1 μL of 2 μg/μL Proteinase K solution and vortex briefly.
5. Incubate at 37˚C for 10 minutes.

Purification of plasmid DNA by High Pure PCR Product Kit – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. Add 500 uL of Binding buffer to DNA sample.
2. Apply the sample to the Spin column. Centrifuge the column at 20,000 x g for 1 min.

3. Discard the flow through fraction. Add 500 uL of Binding buffer to the column and centrifuge at 13,000 x g for 1 min.
4. Discard the flow through fraction. Add 200 uL of Binding buffer to the column and centrifuge at 13,000 x g for 1 min.
5. Discard the flow through fraction. Add 50 uL of Elution buffer to the column and centrifuge at 13,000 x g for 1 min.
Eluted sample can be collected in 1.5 mL centrifuge tube. The purified plasmid DNA is recovered in this tube.

Protocol for Transfection of Adherent Cells (24 Well Plates) – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. The day before transfection, inoculate 24-well plates with an appropriate number of cells in serum-containing medium such that they will be 50 to 70% confluent the following day. For most cell lines, we recommend plating
2.5×10^5 cells in 0.5 ml of medium. Incubate the cells at 37℃ in 0% incubator overnight.
2. Fifteen to sixty minutes prior to transfection, carefully aspirate the medium from the wells and add 250 µl of fresh growth medium to each well.
3. For each well to be transfected, prepare 25 µl of serum-free medium containing 1 pj of siLentFect as a starting point.
4. For each well to be transfected, prepare 25 µl of serum-free medium containing DNA .Use a final concentration of 10 nM as a starting point. For example, for a 24-well plate with 250 µl of growth medium per well, prepare 25 µl of serum-free medium containing 120 nM of DNA. After mixing with the diluted siLentFect from step 3 and addition to cells, the final concentration will be 10 nM. The optimal concentration of siRNA may vary from 5 to 20 nM depending on the cell line used and the gene to be targeted.
5. Add the diluted DNA to the diluted siLentFect. Mix by tapping or pipetting.
Incubate 20 minutes at room temperature.
6. Add 50 µl of complexes directly to cells in serum-containing medium. Rock the plate back and forth to mix.
Incubate the cells at 37℃ in incubator.
7. Gene silencing can be monitored at the mRNA or protein levels from 4 to 72 hours after the transfection. If toxicity is a problem, change the medium 4 hours post transfection.

Embryo microinjection protochol – KIT-Kyoto – 2012 http://2012.igem.org/Team:KIT-Kyoto/Notebook-Protocol

1. w; pΔ2,3 (female-virgin) yw (male)
- (prepare the flies from 3-4 days before)
2. mating
w; pΔ2,3 (female-virgin) X yw (male)
- (keep at 25℃ for 3-4 hrs ; usually from 9:00 AM)
3. prepare
1) NaCl/Triton X-100, 10% Na-hypochloride, D.W. keep on ice.

2) 50ml tube for embryo collection
3) nylon mesh
4) cover glass (3M tape, Cot No. W-18 pastes on the center)
5) slide glass
6) paraffin oil
7) 1mg/ml DNA for micro injection
8) glass needle
9) Microinjection buffer
5 mM KCl, 0.1 mM Sodium Phosphate, pH6.8
DNA EtOH ppt, wash, dissolve to 1 mg/ml in the Microinjection buffe

4. collect the embryos
- wash 3-4 times with NaCl/Triton X-100
- dechorinate with 5 % Na-hypochloride
- wash 7-8 times with D.W.
5. put the dechorinated embryos on the prepared cover glass then drop parafin oil onto the embryos.
6. injection
7. move the tape containing the injected embryos to new egg plate.
caution: the tapes keep upside down from avoiding dry for embryo.
8. after 1 day, move the hatched larva to new vials.
9. mating
the hatched adult flies X yw female or male
10. take the flies having red eye then mate with yw.
- screening

Colony PCR – KAIT Japan – 2012

http://2012.igem.org/Team:KAIT_Japan/Protocol

Reagent

- TaKaRa Ex Taq(5units/μL) 0.5μL
- 10×Ex Taq buffer 10μL
- dNTP Mixture(2.5Meach) 8μL
- Primer F(10μM) 4μL
- Primer R(10μM) 4μL
- Template(E.coli DH5α)
- sterilized water(73.5μL)

Conditions of the thermal cycler

1. 95℃(5min)
2. 94℃(30sec)
3. 61℃(30sec)
4. 71℃(40sec)

5. 72℃(1min)
6. 4℃(Save)
    - 2-4:30cycle
    - gradient:57-62℃(+0.1c)

Ligation – KAIT Japan – 2012 http://2012.igem.org/Team:KAIT_Japan/Protocol

Reagent

- sterilize water 2μL
- PCR product 2μL
- vector DNA 1μL
- Ligation Mighty Mix 5μL

Method

1. Incubation(1h,16℃)
2. Storage Overnight(-4℃)

DNA extraction and purification of *P.aeruginosa* – KAIT Japan – 2012 http://2012.igem.org/Team:KAIT_Japan/Protocol

1. Centrifuge culture medium(6,000rpm,5min,4℃)
2. Remove supernatant,Add saline[0.85%](1.5mL)
3. Centrifuge(6,000rpm,5min,4℃)
4. Add 5mMEDTA 1mL
5. Add 10%SDS 100μL
6. Add proteinase K 50methodL
7. Vortex
8. Incubation(30min,55℃)
9. Add phenol mixture(TE saturated phenol:chloroform:isoamyl alcohol=25:24:1)
10. Shake vigorously(1min)
    - At this time,It became muddy white in color.
11. Centrifuge(16,000rpm,10min,4℃)
12. Pick up supernatant,remove new microtube
13. Repeat step 7-11
14. Add 3M-sodium acetate 40μL,chilled isopropanol 400μL
15. Vortex

16. Wind the DNA by a thin glass rod.
17. Rinse chilled 70%-ethanol(500μL)
18. Pick up DNA,air dry
19. Add TE buffer 500μL
20. Add RNase A 50μL
21. Incubation(20min,37℃)
22. Add proteinase K 50μL
23. Incubation(1h,37℃)
24. Add phenol mixture
25. Vortex(1min)
26. Centrifuge(16,000rpm,10min,4℃)
27. Pick up supernatant,remove new microtube
28. Add 3M-sodium acetate 40μL,chilled isopropanol 400μL
29. Wind the DNA by a thin glass rod
30. Rinse chilled 70%-ethanol(500μL,about 30s)
31. Pick up DNA,air dry
32. Add TE buffer 200μL
    - Melt DNA in buffer

Transformation – KAIT Japan – 2012

http://2012.igem.org/Team:KAIT_Japan/Protocol

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1. Put competent cells on ice(10-15min)
2. Add Ligation reaction solution(10μL) and tapping
3. On the ice(30min)[Transformation]
4. Add LB medium(0.7mL)
5. Incubate(60min,37℃)
6. Add X-gal(40μL) and ampicillin(10μL)[200μg/mL] on LB agar medium(IPTG)
7. Add one incubated(100μL)
8. Cultivation(overnight)

Confirmed of electrophoresis by PCR product and Ligation of the TA vector – KAIT Japan – 2012

http://2012.igem.org/Team:KAIT_Japan/Protocol

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1. Electrophoresis
    - Gel concentration:1.2%,Migration time:30min
    - Marker:Flash Gel 5μL

- Sample:dye 1μL,sample 5μL

2. Check and Colony PCR
3. Add to TA vector
    - PCR product 2μL
    - pMD20-Tvector 1μL
    - D_2W 2μL
    - Ligation Mighty Mix 5μL
4. Heat insulation(16℃,30min)
5. Storage(-20℃)

The purified DNA – KAIT Japan – 2012

http://2012.igem.org/Team:KAIT_Japan/Protocol

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1. Electrophoresis
    - Marker:dye 1μL,DNA molecule 2μL,TE buffer 3μL
    - Sample:dye 1μL,sample 5μL
    - Gel concentration:1.2%,Migration time:30min
2. Storage

PCR Product – KAIT Japan – 2012

http://2012.igem.org/Team:KAIT_Japan/Protocol

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1. Electrophoresis
    - The gel check and cut
2. DNA purification
3. Confirmation of electrophoresis
4. PCR
    - 50cycle
5. Storage

Miniprep – KAIT Japan – 2012

http://2012.igem.org/Team:KAIT_Japan/Protocol

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1. Add culture medium 1mL in a microtube
2. Centrifuge(1min,4℃,10,000rpm)
3. Remove the supernatant to new microtube
4. Repeat 1-3

5. Add SolI 100μL and Vortex
6. Centrifuge(1min,4℃,10,000rpm)
7. Add SolII 200μL and invert
8. ice-cold 3min
9. Add SolIII 150μL and invert
10. ice-cold 5min
11. Centrifuge(5min,4℃,10,000rpm)
12. Add the supernatant to new microtube
13. Add RNase 2μL
14. Incubation(20min,37℃)
15. Add phenol:chloroform 200μL
16. Tapping
17. Centrifuge(5min,4℃,10,000rpm)
18. Add the supernatant to new microtube
19. Add chloroform 200μL
20. Tapping
21. Centrifuge(1min,4℃,10,000rpm)
22. Add the supernatant(200μL) to new microtube
23. Add 3M-acetic acid 20μL
24. Add 100%Et 400μL and invert
25. Centrifuge(20min,4℃,10,000rpm)
26. Remove the supernatant to new microtube
27. Add 70%Et 400 μL
28. Tapping
29. Centrifuge(20min,4℃,10,000rpm)
30. Remove the supernatant to new microtube
31. Dry
32. Add TE buffer 50μL
33. Storage

1.1 Gene Amplification with PCR – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Put the reaction tubes on ice.

2. Add the following components into each reaction tube:

- 5x Phusion HF Buffer* (1X final concentration is recommended)
- 10 mM dNTPs (200 μM final concentration is recommended)
- Primer solution (0.5 μM final concentration of each is recommended)
- DNA Template

- Phusion DNA Polymerase (0.02 U/µl final concentration is recommended)

3. Mix well and run the following program:

2-step PCR Cycling Program

Initial denaturation:

98℃ 30 seconds

25–35 cycles

98℃ 5–10 seconds

72℃ 15–30 seconds/kb

Final extension:

72℃ 5–10 minutes

Hold:

4℃

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1.2 PCR Purification* – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix.
2. If pH indicator I has been added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate (pH 5.0), and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60s.
5. Discard flow-through. Place the QIAquick column back into the same tube.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

*Protocol adopted from QIAquick PCR purification kit protocol

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1.3 Overlapping PCR – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Put the reaction tubes on ice.
2. Add the following components into each reaction tube:

- 5x Phusion HF Buffer* (1X final concentration is recommended)
- 10 mM dNTPs (200 µM final concentration is recommended)
- Primer solution (0.5 µM final concentration of each is recommended)
- DNA Template
- Phusion DNA Polymerase (0.02 U/µl final concentration is recommended)

3. Mix well and run the following program:

2-step PCR Cycling Program

Initial denaturation:

98℃ 30 seconds

25–35 cycles:

98℃ 10 seconds (denaturation)

60℃ 10 seconds (annealing)

72℃ 15–30 seconds/kb (extension)

Hold:

4℃

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1.4 Agarose Gel Electrophoresis – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

A. Cast gel

1. Dissolve 0.55 g agarose into 55 ml 0.5X TBE buffer.
2. Microwave (high power, 800W) for 1 min.
3. Cool it down using running water for 1 min.
4. Add 1 μl GelRed
5. Pour the solution to tightened tank with gates and gel comb and allow it to solidify.
6. Transfer the gel to gel tank once it becomes solid.

B. Run gel

1. Orient the gel with wells facing the black negative electrode. Check if the gel is covered by TBE buffer in the tank. If not, add TBE buffer to cover it to about 1mm.
2. Mix loading dye and the insert/plasmid before adding to the wells. For example, if the DNA we have got is 45μl, and the loading dye we have got is 10X, then add 5μl of loading dye to the samples. Mixture should be in blue.
3. To run the gel, add all samples to the wells of gel. Then add 1kb DNA ladder to a separate well. 1μl should be enough for detection under UV.
4. Connect the electrodes to the power supply with correct colour. Set the power supply to 120V. Check if there are bubbles on the negative electrodes.
5. Allow it to run for about 40 - 60 min. To avoid running the band off the gel, the yellow band (position of the smallest fragments) should stay on the gel.

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1.5 Preparation of Competent Cells – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

A. Preparation of detergent-free glassware and media

1. Autoclave glassware filled with 3/4 dd-H2O to remove most residual detergent.
2. Autoclave media and buffers in detergent-free glassware

B. Preparation of the competent cells

Reagents:

- Glycerol stock
- LB plate
- MgCl2-CaCl2 solution
  - MgCl2･6H2O 3.25g
  - CaCl2･2H2O 0.6g
  - Add H2O to 200 ml
- 100mM CaCl2

    - $CaCl_2 \cdot 2H_2O$ 2.95g
    - Add H_2O to 200 ml
- 80% glycerol
- Liquid nitrogen

Procedure:

Day 1

1. Flame the metal inoculating loop until it is red got and then cools it down.
2. Scrape off a portion from the top of the frozen glycerol stock [DO NOT THAW].
3. Streak it onto the LB plate.
4. Put the stock back to -80 oC immediately.
5. Leave the plates for 5 min and place them upside down in the 37oC incubator for 16-20 h.

Day 2

6. Pick a single colony into 5 ml of LB medium.
7. Inoculate the culture overnight at 37oC with shaking at 250 rpm.

Day 3

8. Inoculate 100 ml LB medium with 1 ml of saturated overnight culture.
9. Shake at 37oC until OD600 = 0.4 (usually 2-3 h).
10. Place in an ice bath for 10 min.

[After this point, the cells must be placed on ice!]

11. Pre-cool solution, centrifuge, pipette tips, falcon, and eppendorf.
12. Transfer the culture into two pre-chilled 50ml falcon.
13. Centrifuge at 2700 x g for 10 min at 4oC
14. Remove the medium, and resuspend the cell pellet with 1.6 ml ice-cold 100 mM $CaCl_2$ by swirling on ice gently.
15. Incubate on ice for 30 min.
16. Centrifuge at 2700 x g for 10 minutes at 4oC.
17. Remove the medium, and resuspend the cell pellet with 1.6 ml ice-cold 100 mM $CaCl_2$ by swirling on ice gently.
18. Incubate on ice for 20 min.
19. Pool all cells into one tube and add 0.5 ml ice-cold 80% glycerol and swirl to mix.
20. Freeze 100 μl aliquots in liquid nitrogen.
21. Store in -80oC.

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1.6 Bacterial Transformation – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Thaw competent cell on ice.
2. Add 50 - 100 ng DNA to competent cell culture.
3. Put in ice for 30 min.
4. Heat shock at 42oC for 1 min.
5. Put in ice for 2 min.
6. Add 1 ml SOC medium.
7. Incubate at 37oC for 90 min with shaking (~ 250 rpm).
8. Spread plate (with suitable antibiotics)
9. Spin down the remaining cells and discard large amount supernatant (1 ml).
10. Resuspend the cell pellet and spread plate.
11. Incubate at 37oC overnight (preferably ~16 – 20 h).

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1.7 Inoculation – Hong Kong-CUHK – 2012 http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Pick colonies and culture in 2 - 3 ml LB broth with antibiotics.
2. Incubate at 37oC for 12 - 16 h with shaking at 250 rpm.

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1.8 Plasmid DNA Preparation (Miniprep) – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4 – 6 times and incubate 2 min at room temperature. Do not vortex!
3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4 – 6 times.
4. Centrifuge for 10 min at 13,000 rpm in a table-top microcentrifuge. A compact white pellet will form.
5. Apply the supernatants from step 4 to the QIAprep spin column
6. Centrifuge for 30–60 s. Discard the flow-through.
7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.
8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30 – 60 s.
9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube.

11. Add 50 μl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min to elute the DNA.

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1.9 Double Digestion – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Mix the components as follows to prepare a 50 μl reaction mixture:

- 37.6 μl Insert/Vector# + ddH2O*
- 5 μl 10X NEB Buffer3
- 5 μl 10X BSA
- 1.2 μl Enzyme 1
- 1.2 μl Enzyme 2

At least 200ng DNA should be added

* Water is added first and the template the last

2. Incubate the reaction mixture at 37oC for 2 h.

Buffer Chart

http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/buffer_activity_restriction_enzymes.asp

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1.10 Gel Extraction* – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a 2 ml centrifuge tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 μl).
3. Incubate at 50℃ until the gel slice has completely dissolved (around 10 min). Mix by vortexing the tube every 2 min during the incubation.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 5-10 μl of 3 M sodium acetate (pH 5.0), and mix until it turns to yellow.
5. Add 1 gel volume of isopropanol to the sample and mix (for DNA fragments < 500 bp and > 4 kb).

6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 30 s. (The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again).
8. Discard flow-through and place QIAquick column in the same collection tube. Collection tubes are re-used to reduce plastic waste.
9. Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 30 s to remove any traces of agarose.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column, stand for 2 min and centrifuge for 30 s.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 30 s and air-dry for 2 min (This step can ensure all ethanol is removed and the column is NOT over-dry)!
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 35 μl of 50oC ddH2O to the center of the QIAquick membrane, wait for 2 min and centrifuge for 2 min at maximum speed.

*Adopted from CUHK iGEM 2010 protocol.

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1.11 Ligation – Hong Kong-CUHK – 2012 http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

3. Mix the components as follows to prepare a 20 μl reaction mixture:

- 2 μl 10X ligation buffer
- 1 μl T4 DNA ligase
- 14 μl Vector
- 3 μl Insert

4. Incubate the reactions at 16oC overnight, or 22oC for 1 h.

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2.1 Cell movement test – Hong Kong-CUHK – 2012

http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Pre-culture *E. coli* for 1 h in LB medium containing 2 μM all-trans retinal.
2. Transfer 6 μl of pre-cultured solution onto plate with 0.5% agar and 2 μM all-trans retinal

3. Incubate the plate for 24 h under specific wavelength of unidirectional light.
4. Measure the cell movement.

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2.2 Sensory Rhodopsin-induced Gene Expression Test – Hong Kong-CUHK – 2012 http://2012.igem.org/Team:Hong_Kong-CUHK/DOC_PROTOCOLS

1. Culture *E. coli* in LB medium with and without light for 24 hrs.
2. Measure the OD measurement of the corresponding reporter gene.

Transforming Competent E.coli with the Plasmid: - HKU HK - 2012

http://2012.igem.org/Team:HKU_HongKong#

We chose the biobrick K137076 to ligate to the pvdQ gene for the following reasons:

- Take out the competent E-coli cells from the -80 freezer. (Keep all tubes on ice).
- Add 1uL of the plasmid DNA in 100uL competent cells (if from Kit). For transformation of ligated product, add 10uL of the ligated plasmid DNA in 100uL competent cells.
- Incubate on ice for 10 min.
- Place in water bath at 42°C for 90s.
- Place immediately back on ice for at least 2 min.
- Add 800uL LB broth. Incubate for 1hr at 37°C shaker.
- Centrifuge at 130rpm for 5min to remove supernatant. Re-suspend the pellet in about 10uL of supernatant.
- Spread the entire re-suspended pellet on ampicillin agar dishes.
- Incubate for 12-16 hours at 37 °C.

Notes:

- Colonies grown on plate were selected for colony PCR screening.
- Correct colonies were then cultured in 5 mL LB Broth with 0.5uL ampicillin for subsequent screening or mass culturing.
- Mass culturing involved adding 200uL of the culture into 35mL LB Broth with 35uL ampicillin.

Miniprep to Extract the Plasmid from E.coli (QIAprep Spin Miniprep Kit): - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#

- Transfer some of the 5mL bacterial culture into a microcentrifuge tube. Pellet by centrifugation at 13,500 rpm for 1 min. Repeat till all the culture has been pelleted.
- Resuspend the pellet in 250uL Buffer P1.
- Add 250uL Buffer P2 (Lysis Buffer). Mix thoroughly by inverting the tube 4-6 times. Do not allow prolonged lysis.
- Immediately add 350uL Buffer N3 (Neutralization Buffer). Mix immediately by inverting the tube.
- Centrifuge for 10 minutes at 13,500 rpm.
- Apply the resulting supernatant to the QIAprep spin column by pipetting. Centrifuge for 1 minute at 13,500 rpm.
- Wash the QIAprep spin column by applying 0.75mL Buffer PE. Centrifuge for 1 minute at 13,500 rpm.
- Centrifuge for an additional 1 minute to remove residual ethanol.
- Place the QIAprep spin column in microcentrifuge tube. Elute DNA by adding 50uL warm H_2O.
- Centrifuge for 1 minute at 13,500 rpm.

Midiprep for Large Volumes of Culture (QIAGEN Plasmid Midi Kit): - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#

Refer to the QIAGEN website for the protocol.

PCR Amplification of Gene from Bacterial Genome/Standard Biobrick - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#*Parts*

37.5µL ddH2O

5.0µL 10x PCR Buffer

2.5µL dNTPs

1.0µL Forward Primer (Prefix)

1.0µL Reverse Primer (Suffix)

1.0µL Template DNA

1.0 µL RTaq DNA Polymerase

50.0µL Total

Note:

- For sequential PCR reactions: After the first PCR is complete, perform PCR clean-up. Then use the product to conduct the second PCR reaction, after which gel purification needs to be carried out.

- The volume of DNA used in the second PCR reaction depends on the concentration of the DNA after the PCR Clean-up.

PCR Clean-Up – Qiagen QIAquick PCR Purification: – HKU HK – 2012

http://2012.igem.org/Team:HKU_HongKong#

- Add 5 volumes of Buffer PB to 1 volume of PCR mix.
- Place this mix within the QIAquick column. Centrifuge at 13,500 rpm for 1 minute.
- Discard flow through and centrifuge again to allow all the sample to pass through.
- Wash with 750uL Buffer PE. Centrifuge at 13,500 rpm for 1 minute.
- Discard flow through and centrifuge again to remove all residual buffer.
- Place the column in a micro centrifuge tube.
- Apply 50uL of pre-warmed distilled H2O to the column. Stand for at least 2 minutes.
- Centrifuge at 13,500 rpm for 1 minute.

Colony PCR – HKU HK – 2012 http://2012.igem.org/Team:HKU_HongKong#

14.52µL ddH2O

2.0µL 10x PCR Buffer

1.6µL dNTPs

0.4µL Forward Primer (Prefix)

0.4µL Reverse Primer (Suffix)

1.0µL Template DNA

0.08µL RTaq DNA Polymerase

20.0µL Total

Notes:

- The mixture was pipetted into PCR tubes
- All materials were kept on ice
- Colonies will inoculated into 5uL broth prior to PCR
- Prefix and Suffix were used as Forward and Reverse Primers respectively while amplifying standard biobrick parts
- PCR Reaction: 95°C - 10 min

95°C - 30 sec

57°C - 30 sec (appropriate annealing temperature for prefix and suffix)

72°C - 30 sec

(*28 cycles all together)*

72°C - 5 min

Agarose Gel Electrophoresis – HKU HK – 2012

http://2012.igem.org/Team:HKU_HongKong#

- Prepare a 2% or 1% Agarose Gel (amount in grams depending on volume of TAE buffer used). Add 0.1% Ethidium Bromide of the total volume.
- Place the gel in the Electrophoresis Apparatus with the wells facing the Negative Electrode.
- Fill the apparatus with 1% TAE Buffer to fully submerge the wells.
- Load 5μL of 1kb Ladder for each Run
- Add 0.1% of 10% Loading Dye to the respective volume of sample. Mix well and spin down.
- Pipette the samples into the wells and run at 106 Volts.

Gel Purification of DNA (Qiagen QIAquick Gel Extraction Kit) - HKU HK - 2012

http://2012.igem.org/Team:HKU_HongKong#

- Exercise the DNA fragment from the Agarose Gel using a scalpel. Minimize the extra peripheral gel slice.
- Weigh the gel slice (0.1g = 100uL) and add 3 Volumes of Buffer QG to every 1 Volume of Gel.
- Incubate in 50°C water bath for 10 minutes to completely dissolve the gel slice.
- Add 1 Volume of Isopropanol to the sample. Mix well
- Apply the sample to the QIAquick column. Centrifuge at 13,500 rpm for 1 minute. [Repeat till the total volume of the sample has sieved through the column]. Discard the flow through
- Apply 0.5mL Buffer QG to the QIAquick column. Centrifuge at 13,500 rpm for 1 minute.
- Discard the flow through
- Wash the column with 0.75mL Buffer PE. Centrifuge at 13,500 rpm for 1 minute.
- Discard the flow through and Centrifuge at 13,500 rpm for an additional 1 minute to eliminate any residual ethanol.
- Place the QIAquick column into a 1.5mL Eppendorf tube.
- Apply the 30uL warm H_2O to the column. Let the column stand for at least 1 minute.
- Centrifuge at 13,500 rpm for 1 minute.

Determining DNA Concentration Using NanoDrop Spectrophotometry - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#

- Choose the Nucleic Acid Measurement option in the programme.
- Initialize the NanoDrop by adding 1uL clean H_2O. Clean the sensor gently with tissue.
- Set Blank by adding an additional 1uL of clean H_2O. Wipe off.

- Add 1uL of the DNA sample to be measured. Wipe off after each run.

DNA Digestion - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#

__µL ddH2O (to a total of 40uL)

4µL 10X NEBuffer

0.4µL 100X BSA

1µg DNA Sample

__µL 1st Restriction Enzyme

__µL 2nd Restriction Enzyme (optional)

40µL Total

Notes:

- The NEB official website should be checked for buffers suitable for each restriction enzyme. Results can vary depending on double or single digestion.

- Incubate the digestion sample at 37°C for 3 hours (digestion time can also vary depending on enzyme). Prolonged digestion may lead to Star Activity otherwise.

- The volume of DNA must be calculated from its concentration. In restriction digestion test, the minimum volume that is equals 1ug DNA can be utilized. However, for purification, a much greater volume of DNA should be used.

- Appropriate amount of enzyme is derived from its concentration and the fact that 5 units of enzyme digest 1ug DNA.

Dephosphorylation of 5' Ends of Vector Backbone - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#

- Add 0.5µL (0.5 units per 1 ug DNA) of Calf Intestinal Alkaline Phosphatase (CIAP) to the digested sample immediately after digestion.

- Incubate at 37°C for 30 minutes

Vector-Insert Ligation - HKU HK - 2012 http://2012.igem.org/Team:HKU_HongKong#

__µL Autoclaved ddH2O (to a total of 20uL)

2µL T4 Ligase Buffer

1µL T4 DNA Ligase

__µL Vector DNA

__µL Insert DNA

20µL Total

Notes:

- Insert and Vector must be in a 3:1 ratio. The amount of each depends on their concentration (ng/uL) and legnth (bp).

- Incubate at room temperature for 1 hour.

PCR Deletion (Site-Directed Mutagenesis) Reaction – HKU HK – 2012

http://2012.igem.org/Team:HKU_HongKong#

Note: Keep everything on ice and add all volumes in a PCR tube.

? µL ddH2O (? = whatever volume needed to bring the total volume up to 50µL)

5.0µL 10x PfuUltra buffer

1.0µL dNTPs

? uL forward primer = 125ng fwd primer ÷ fwd primer concentration (ng/µL)

? uL reverse primer = 125ng rvs primer ÷ rvs primer concentration (ng/µL)

? µL dsDNA= 20ng insert ÷ insert concentration (ng/µL)

1.0µL PfuUltra high-fidelity DNA polymerase

50.0µL Total

- Volumes of diluted primer based on calculations for our ng/µL concentrations

95°C for 2min

95°C for 30sec (18 times)

55°C for 30sec

72°C for 1 min/kb

1min/kb corresponds to: 3.20min (RFP), 3.50min (VioA), 5.40min (VioB), 3.00min (VioE)

The Composition of LB liquid Medium – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

Yeast extact 5g

Peptone 10g

NaCl 10g

Distilled water 1000ml pH 7.0

Range of application: Escherichia.coli

The Composition of LB Solid Medium – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

Yeast extact 5g

Peptone 10g

NaCl 10g

Agar 1-2%

Distilled water 1000ml pH 7.0

Boil the mixture in autoclave at 121℃ for 30min

Range of application: Escherichia.coli

The Preparation of Competent Cell of Escherichia.coli – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

1. Place one colony in 3~5mL LB media, grow overnight at 37℃;

2. Transfer overnight DH5α culture(1:100~1:50)into 100mL LB liquid media;

3. Allow cell to grow at 37℃ (250 rpm), until OD600= 0.4 (2-3 hours);

4. Place cells on ice for 10 min;

5. Centrifuge cells at 4℃ for 10 min at 3,000g;

Subsequent resuspensions is done in the same bottle. Cells remain cold for the rest of the procedure: transport tubes on ice and resuspend on ice in the cold room.

6. Pour off media and resuspend cells in 10mL cold 0.1 M CaCl2, and incubate on ice for 30 min;

7. Centrifuge at 4℃ for 10 min at 3,000g;

8. Pour supernatant and resuspend cells (by pipetting) in 4mL cold 0.1M CaCl2 containing 15% glycerol. Transfer 100 μL into 1.5mL centrifuge tubes placed on ice. Cells is stored at -80℃ and can be used for transformation for up to 6 months.

Double Digestion(Ecor Ⅰ、Xba Ⅰ)Reaction – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Add

10× Tango^{TM}	Ecor Ⅰ	Xba Ⅰ	DNA	nuclease-free water	Total volume
2μL	1μL	1μL	0.5-1μg	to 20μL	20μL

2. Mix gently and spin down for a few seconds.

3. Incubate at 37℃ for 1-16 hours

Double Digestion(Xba Ⅰ、Pst Ⅰ)Reaction – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Add

10× Tango^{TM}	Xba Ⅰ	Pst Ⅰ	DNA	nuclease-free water	Total volume
2μL	1μL	1μL	0.5-1μg	to 20μL	20μL

2. Mix gently and spin down for a few seconds.

3. Incubate at 37℃ for 1-16 hours

Double Digestion(Spe I 、Pst I)Reaction – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Add

10× Tango™	Spe I	Pst I	DNA	nuclease-free water	Total volume
2μL	1μL	1μL	0.5-1μg	to 20μL	20μL

2. Mix gently and spin down for a few seconds.

3. Incubate at 37℃ for 1-16 hours

Double Digestion(Ecor I 、Spe I)Reaction – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Add

10× Tango™	Ecor I	Spe I	DNA	nuclease-free water	Total volume
2μL	1μL	1μL	0.5-1μg	to 20μL	20μL

2. Mix gently and spin down for a few seconds.

3. Incubate at 37℃ for 1-16 hours

Sticky-end Ligation – HIT-Harbin – 2012

http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Prepare the following reaction mixture:

Linear vector DNA	20-100ng
Insert DNA	1:1 to 5:1 molar ratio over vector
10× T4 DNA Ligase Buffer	2μL
T4 DNA Ligase	1μL
Water, nuclease-free(#R0581)	to 20μL
Total volume	20μL

2. Incubate 10 min at 22℃

3. Use up to 5μL of the mixture for transformation of 50μL of chemically competent cells.

PCR Reaction – HIT-Harbin – 2012

http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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10μL 5x buffer(Mg^{2+} plus)

4μL dNTPs

1.0μL forward primer

1.0μL reverse primer

1.0μL template (10pg-1ng)

1.0μL DNA polymerase

ddH2O up to 50.0μL

Note: Based on primers, set an appropriate annealing tem.

Keep everything on ice and add all volumes in a PCR tube.

Agarose Gel Electrophoresis – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Prepare a 1% weight-to-volume agarose gel and add SYBR dye or ethidium bromide to stain DNA;

2. Place the gel in the apparatus rig with the wells facing the negative end (black-colored);

3. Fill the rig with 1x TBE buffer;

4. Load 2μL of 1kb ladder;

5. Add 2μL of 6x loading dye to each PCR reaction tube, adn load 20μL in each well;

6. Run at 120V.

Gel Purification of DNA – HIT-Harbin – 2012 http://2012.igem.org/Team:HIT-Harbin/notebook/protocol

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1. Cut out the DNA fragment from the agarose gel with a razor blade, while minimizing the size of the gel slice;

2. Weigh the gel slice and add 3 volumes of Buffer QG to every 1 volume of gel(100mg = 100μL);

3. Dissolve the gel slice using a 60μC heat block;

4. Apply the dissolved gel to the QIAquick column and centrifuge at 13,000rpm for 1 minute;

5. Discard the flow-through and repeat Step 4 until all sample has passed through the column;

6. Add 750μL of rinse buffer to the QIAquick column and centrifuge at 13,000rpm for 1 minute;

7. Add 500L of rinse buffer to the QIAquick column and centrifuge at 13,000rpm for 1 minute;

8. Wash the column with 750µL of Buffer PE and centrifuge at 13,000rpm for 1 minute;

9. Discard the flow-through and centrifuge at 13,000rpm for 2 minute to remove residual EtOH;

10. Transfer the QIAquick column to a new Eppendorf;

11. Add 50µL elution buffer to the center of the column and wait at least 2 minutes;

12. Centrifuge at 13,000rpm for 1 minute.

Dope Making – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

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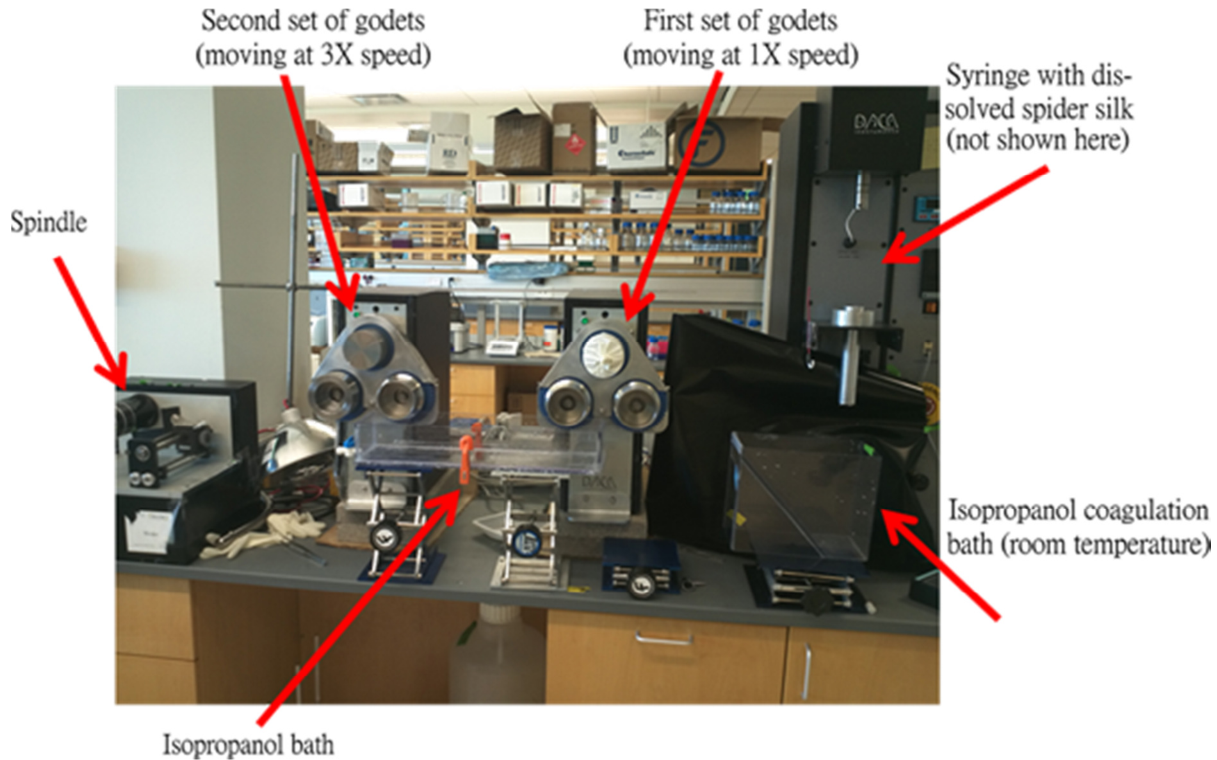
1. Decide the % w/v of your dope. For example, if you want 10 % w/v, that will be 0.125 g of spider silk protein and 1.25 ml of HexaFluoroIsoPropanol (HFIP). As you can notice 0.125g/1.25ml = 0.1 g/ml = 10% water density.
2. Weight the appropriate amount of solute in a balance and add it to your vial.
3. With gloves on, pipette the amount of HFIP in a fume hood and add it to your solute since the alcohol is highly toxic.
4. Close your vial and secure it with parafilm around the cap and around the y axis in order to prevent any spill.
5. Put your vial in a rotation device and let it mix approximately 24 hours.

DACA Silk Spinning – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook



1. After mixing or (doping) spider silk in HFIP it is pushed through a needle into an isopropanol bath.
2. Next the silk is taken out of the bath and passed over a hook.
3. The silk is then passed through the first set of godets, or winders.
4. The silk is then passed through an isopropanol bath (this helps with stretching the silk).
5. The second set of godets are spinning faster than the first set to help with this streteching.
6. The silk is then collected on a spool.

Carding of the Threads – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

1. Cut an X-ray film, with a gap 20-mm long.
2. Attach it to a microscope glass slide with strips of tape.
3. Take your fiber from the spool and place it on the card with strips of tape.
4. Secure the fiber with super glue at the ends.
5. Let the glue cure-dry for 24 hours.

Measuring Threads' Diameters – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

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1. Place your carding slide under an optical microscope with an appropriate camera installed.
2. With your camera software, take 3 pictures of the sample: at left, center, and right.
3. Of each picture measure 3 diameters with your software's tool: at left, center, and right. That will make a total of 9 measurements for your 20-mm long sample.
4. Take the average and standard deviation of those 9 measurements.

Testing of the Threads – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

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1. Take your glass slide card and cut the tape strips that attach the film to the glass.
2. Take your film card and attach its ends to the grips of your stretching-testing machine.
3. Cut the thin side of the film with scissors in order to allow the stretching of the fiber.
4. Start your testing software.
5. Write down your sample average diameter and press play.
6. The software will stretch the fiber until it breaks, collecting the necessary data in order to know its tensile strength, elongation, stress, strain, Young's modulus, etc.

Spidey Spinning

Electroporation Transformation of E. coli – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

Transformations are any procedure used to insert DNA into a bacteria (if you use a virus, the term becomes transfection). Electroporation uses a pulse of electricity to disrupt the cell membrane and create holes that would allow the DNA to enter the cell. Cells need to be made competent before doing this procedure, in order for them to efficiently take up the DNA. Transformations generally utilize millions to billions of cells and DNA molecules and, for a transformation to be successful, only one molecule of DNA needs to enter into one cell, which then grows into a colony. One issue with transformations is selecting and verifying which colonies have the desired DNA. This is usually done using a marker, a characteristic possessed by cells that have the DNA (or lost by those cells) that distinguishes it from the rest of the colonies that grow up. Commonly, this is the expression of an antibiotic resistance gene included on the transformed DNA, which allows only the cells that have taken up the DNA to survive on a plate in the presence of that antibiotic. Sometimes pigment producing or fluorescent/luminescent proteins can also be used in place of antibiotic resistance to allow visual determination of transformed colonies. Other ways of selection exist, but will not be discussed here.

1. Turn on ice machine
2. Thaw DNA solutions
3. Clean and sterilize the electroporation cuvettes by washing with double distilled water (ddH2O) twice and then fill the cuvettes with ethanol.
4. Let cuvettes sit with ethanol for 5-10 minutes, then wash 4-8 times with ddH2O
5. Place cuvettes on ice
6. Take competent cells out of the -80 ℃ freezer, and thaw them on ice
7. Add 3 µL of DNA to the cell solution. (This should be around 100-250 ng of total DNA, too much DNA causes arcing, too little gives few transformed colonies).
8. Incubate on ice for 5 minutes.
9. Add 60 µL WB buffer (10% glycerol). This helps reduce arcing, although too much can lower numbers of transformed colonies.
10. Set the electroporation machine to 2500 V, 200 O, and 25 µF for E. coli.
11. Transfer the cell/DNA/WB solution into the cuvettes by pipetting up and down in the 1.5 mL tube first to mix. Make sure the pipette tip is between the metal plates on the cuvette before ejecting the solution. Keep the cuvettes on ice.
12. Before electroporating, dry the cuvettes of with a KimWipe, to ensure no liquid on the surface that could create other paths for the electric pulse (and could cause arcing).
13. Pulse the cells and return cuvette to the ice. Check the time constant on the machine, a constant of 4.5+ is a very good transformation, and will yield many colonies. A constant of 2.5-4.5 is okay, and will still work. Constants below 2.5 will yield very low colony numbers, and may need to be redone. NOTE: addition of extra WB or lower amounts of DNA will reduce the time constant as well, so it is only a rough measure.
14. To remove the cells from the cuvette add 1 mL LB media or SOC media (no antibiotic in this media) to the cuvette. Pipette up and down a few times to mix.

15. Remove the solution to just above the two plates in the first removal pipetting (~1/2 of the volume) and transfer it to the original cell tube (NOT THE DNA TUBE). Then, tip the cuvette on its side so that the space between the plates is vertical, place the 1000 µL pipette tip between the plates, and slowly draw up the solution, while tipping the cuvette further. This should draw up the rest of the liquid in the cuvette.
16. Incubate the cell solutions at 37 °C for 1-2 hours (can go up to three, but try to avoid doing it for that long).
17. Plate the cells on plates containing the correct antibiotic. Each transformation requires two plates. Add 500 µL of solution to one plate, spread with the spreading stick, and then spread the spreading stick on the second plate without adding any solution to it. This creates a dilution plate in case you have thousands of colonies on the first plate. It is roughly a 1:100 to 1:200 dilution.
18. Grow the plates up overnight at 37 °C. Do not leave for longer than 24 hours, as contaminants might have a chance to grow and the plates could dry out.

<u>If your cuvette arcs (bright flash and loud popping noise during electroporation):</u>

1. Clean the electroporator lid.
2. Wash out and sterilize the cuvette with ethanol – the cells have been pretty much killed and will not be usable in plating, so you need to restart.
3. Add less DNA to the cells (reduce by 25%-33%).
4. Add an additional 15 µL of WB buffer to the solution.

Glycerol Stock Preparation – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

Glycerol Stocks are one of the best ways to store cells and DNA for long periods of time. These stocks are prepared so that the cells in the tubes are still alive, and capable of creating new liquid cultures from small amounts of frozen stock. This allows for quick growth and extraction of important DNA without spending an extra day and extra supplies re-transforming a construct every time it is needed. In order to freeze cells and still keep them alive, a cryoprotectant is added to the cultures. Cyroprotectants function by reducing the freezing point of the solution and act to reduce the formation of large ice crystals inside cells that could rupture membranes. Cryoprotectants are non-toxic to the cells, and are generally able to pass through the membranes into the cells.

1. Take 1 mL of overnight E. coli culture and add it to a clean, labeled 1.5 mL tube.
2. Add 200 µL of 80% glycerol to the tube (this creates a roughly 15% glycerol solution).
3. Mix well by inverting the tubes (unmixed glycerol will tend to stay separate from the cell solution.

4. Immediately place the cells into a -80 ℃ freezer box. There are often special freezing boxes that let the freezing occur more slowly, but for E. coli these are generally not needed.
5. To use the glycerol stock to establish a new culture, either scrape a very small amount of the frozen culture off with a pipette tip and add it to a culture (if it is still frozen), or add 10-20 µL of the liquid glycerol stock to the culture (if it is somewhat thawed). AVOID THAWING GLYCEROL STOCKS COMPLETELY – take what you need and quickly return them to the -80 ℃ freezer. If they do thaw completely, they can be re-frozen, but repeated thawing may reduce the number of live cells in the stock tube.

DNA Extraction Using Qiagen DNA Miniprep Kits – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

DNA Extractions are used to generate large amounts of plasmid DNA from E. coli cell cultures. This DNA can then be used for restriction digesting, PCR reactions, gel electrophoresis, or further transformations. By using a kit (Qiagen Qiaprep Spin Miniprep Kit) we are able to effectively remove most of the protein and RNA from a solution, leaving us with very clean DNA solutions.

1. Grow up 5 mL culture overnight in LB (6 mL if you want glycerol stock – 1 mL will be used for the frozen stock, which should be removed before pelleting the cells in the next step)
2. Pellet 5-10 minutes in centrifuge at 3000-3500 rpm
3. Pour off supernatant

   At this step, you can freeze the cell pellet if you don’t have time to finish the rest of the procedure. The pellet is fine for a week or two in the -20 ℃ freezer

4. Check P1 solution for the checked RNase added box
5. Add 250 µL solution P1 to the 10 mL culture tube
6. Suspend the pellet in the P1 by pipetting up and down
7. Transfer suspended pellet to a 1.5 mL tube
8. Add 250 µL solution P2 to the tube
9. Mix by inverting the tube by hand 10-20 times. Solution should turn blue throughout, if not, continue inverting until blue throughout
10. Allow lysis to occur for 3-4 minutes (no more than 5 minutes)
11. Add 350 µL solution N3 to the tube
12. Mix by inverting tube by hand 10-20 times. Solution should lose all blue color, if not, continue inverting until all blue is gone.
13. Centrifuge at 13,000 rpm for 10 minutes (keep the hinge out to get the pellet to form correctly)
14. Using a pipette, remove the supernatant from the tubes, and apply to a labeled blue spin column from the kit
15. Centrifuge at 13,000 rpm for 1 minute

16. Pour flow-through BACK into the column and centrifuge at 13,000 rpm for 1 minute 17. Discard flow through
17. Add 750 µL PE solution to tube and centrifuge at 13,000 rpm for 1 minute
18. Discard flowthrough and centrifuge again at 13,000 rpm for 1 minute
19. Transfer blue column to a fresh 1.5 mL tube (labeled)
20. Add 30-50 µL ddH2O (depending on how concentrated you want your final DNA). Buffer EB (supplied in the kit) can also be used.
21. Incubate on benchtop for 10-15 minutes
22. Centrifuge at 13,000 rpm for 1 minute and discard column
23. Nanodrop to determine DNA concentration

DNA Extraction using CTAB – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

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1. Preparation step: Make sure Lysozyme (50 mg/mL) and RNase A (10 mg/mL) stock solutions have been prepared.
2. Preparation step: Make sure CTAB solution is dissolved. If crystals of CTAB are on the bottom of the bottle, place in 37oC incubator and shake occasionally to re-suspend. CTAB is just above its soluble conditions at normal room temperature, and if the atmospheric pressure is low the day you are extracting DNA, it might come out of solution.
3. Prepare two water baths, one boiling and the other 68C (this can also be accomplished with just the digital waterbath, by bringing the temp to 99/100C, then adding some water to the tank to help it cool down to 68C by the time that water temp is needed)
4. Centrifuge the 12 ml tubes containing the 5 ml cultures in the large centrifuge at 3K RPM for 10 min. Discard supernatant liquid.
5. Re-suspend cells in 200 ul of “STET for CTAB” buffer. Transfer to 1.5 ml tubes.
6. Add 5-10 ul (10 uL if older preparation) Lysozyme (50 mg/ml) and incubate at room temperature for 5 min.
7. Boil for 45 seconds and centrifuge for 20 min at 13K RPM (or until pellet gets tight).
8. Use a pipette tip to remove the pellet by dragging it (it should be somewhat slimy, but if pelleted well enough, it will hold together), if it doesn’t hold together, re-centrifuge and retry.
9. Add 5 ul RNase A (10 mg/ml) and incubate at 68C for 10 minutes.
10. Add 10 ul of 5% CTAB and incubate at room temperature for 3 min.
11. Centrifuge for 5 min at 13K RPM, discard supernatant, and re-suspend in 300 ul of 1.2 M NaCl by vortexing.
12. Add 750 ul of ethanol
13. Optional Step (but gives better yield): incubate for 30 mins in -20oC freezer to help DNA precipitate

14. Centrifuge for 5 min at 13K RPM to compact DNA pellet. Make sure the hinge is away from the center of the rotor, this will make the pellet form on the bottom of the tube on the side of the hinge (it might be hard to see or invisible, so this way you know where it should be)
15. Discard supernatant, rinse pellet in 80% ethanol, and let tubes dry upside down with caps open.
16. Re-suspend DNA in ddH2O (50 uL). Vortex or pipette up and down to ensure re-suspension of DNA.

Making Agarose Gels – Utah State – 2012
http://2012.igem.org/Team:Utah_State/Notebook

Agarose gels are useful for DNA purification and analysis of DNA sizes. The gels are made up of an agarose matrix composed of long strands of agarose, and gaps of various sizes between the strands. The larger the DNA molecule, the longer it takes to fit through the gaps, making its progress through a gel slower than a small DNA molecule. The DNA is drawn through the gel using an electric current; the negatively charged phosphates on the DNA backbone being attracted toward the cathode ("Run towards red" is a helpful mnemonic as the cathode is generally red colored). By varying the concentration of the agarose gel, it is also possible to increase the separation of bands of certain sizes on the gel. A 1% agarose gel is generally used as it provides separation of bands from 200 bp to 3000 bp. For separating larger bands, a 0.7% gel is typically used and the smaller DNA fragments are run completely off the bottom of the gel. For separating smaller bands, a 2% or 1.5% gel can be used, and run normally. Gels are useful for purifying DNA bands of a particular size from restriction digests (to prevent multiple products from forming during ligations) and also for removing proteins from a DNA sample (such as restriction enzymes that are not inactivated by heat). Gel purification has the downside of losing some DNA, and reducing overall DNA concentration (a 120 bp band of DNA in a 2000 bp plasmid will only give .06 µg of DNA if 1 µg of total DNA digest is added to the lane). For small band sizes (< 200 bp), it may be necessary to use CIP or TAP dephosphorylation and ligation using the digested DNA solution without gel purification.

1. Determine the number of lanes you wish to run. Always plan for 1-2 lanes of the DNA ladder (2 especially if this gel will be cut up and DNA removed from it), or another suitable control. Most lanes can hold 20-25 µL of sample, so larger samples may need to be run on two lanes, or use the larger lane combs (40-50 µL capacity). The small gel box can hold 6 lanes of large capacity or 10 lanes of smaller capacity. The larger gel box can hold 12 lanes of large capacity or 20 lanes of small capacity. The large gel box is also capable of having 2 combs at a time (the second placed ½ down the box), and so its capacity can double at the cost of distance over which it can separate bands.
2. Once your gel box is selected, determine the concentration of gel you wish to make (see description for details). The concentration is the mass of agarose/mL of gel x 100%. The small gel box supports gels of 50 mL (potentially up to 75 mL, but 50 is easier to use) and the large gel box supports gels of 200 mL.

3. Set up the gel box by removing the gel tray. Make sure the rubber seals are still in their grooves. Apply a small amount of water to the inside of the side walls of the gel box and to the ends of the gel tray that have the rubber seals. Slide the gel tray into the gel box so that the open ends of the tray are against the box walls, and so that the rubber seals have not rolled up out of their grooves (if the seals moved, return them to their grooves and try sliding the tray in again).
4. Add the correct gel combs to the gel. The main comb for both gels goes into the first notch on the gel tray (should be 1-2 cm from an end), the secondary comb for the large gel is placed in the notch in the middle of the tray. The small gel combs have two sides (one thinner than the other) the thinner side has about 2/3 of the capacity volume of the thick side (which has the capacities listed in #1), so choose what you need.
5. In a flask that can hold at least 4x your gel's volume, add the correct volume of 1x TAE buffer. DO NOT ADD WATER – the gel will not work correctly.
6. Weigh out the correct mass of agarose (NOT AGAR) and add it to the flask.
7. Microwave the solution until it boils. There are two stages of boiling – where small white bubbles form (frothy) and where large clear bubbles form. You want to let it boil a bit past the frothy stage and into the clear bubble stage. These bubbles will pop naturally, and will keep you from having a bubble filled gel. It is necessary when using higher gel concentrations (and recommended for all other concentrations) that the microwaving occur in 30 second increments, with the solution being stirred by GENTLE rotation (wear protective heat gloves) after each 30 second period, to ensure proper agarose distribution.

8. After microwaving add Ethidium Bromide to the gel solution. WARNING – carcinogen, glove use is advised (if you get it on yourself, wash your skin with water for 5 minutes – its very water soluble). Add 1 µL of Ethidium bromide for EACH 10 mL of gel volume (5 µL for small gel, 20 µL for large gel).
9. Mix Ethidium Bromide into the solution by GENTLE swirling (to avoid bubbles).
10. The gel solution can be allowed to cool slightly before pouring into the gel box (pouring boiling solution into gel box can cause it to warp and bend over time).
11. Gently pour the gel into the gel tray by leaning it on the gel box wall farthest from the top comb, and slowly tipping it into the box. This prevents bubbles from forming in the gel, and if they do form, forms them near the bottom of the gel.
12. If additional bubbles form in the gel box, while the gel is still liquid take a pipette tip and push the bubbles to the bottom edge of the gel, where they won't interfere with DNA movement.
13. Allow gel to cool for 40 minutes to 1 hour. Test solidification by gently pressing on the bottom corner of the gel with a finger, it should feel solid and gel-like (not liquid).

Restriction Digests – Utah State – 2012

http://2012.igem.org/Team:Utah_State/Notebook

All restriction digests were carried out using Fermentas reagents and restriction enzymes. The Fermentas FastDigest® (FD) reagents were used.

In a tube the following was added:

- 4 µg DNA
- 4µl 10x FD Green buffer
- 1 µl FD restriction enzyme 1
- 1 µl FD restriction enzyme 2
- Volume brought up to 40µl total with nuclease free water

Tube was incubated at 37°C for 1hr.
The FastDigest® Green Buffer was used in this digestion, allowing the DNA to be directly loaded onto an agarose gel. As it contains a loading dye solution.

Gel Purification with Qiagen Gel Extraction Kit – Utah State – 2012 **http://2012.igem.org/Team:Utah_State/Notebook**

This procedure was carried out using the Qiagen QIAquick Gel Extraction Kit.

Ligation – Utah State – 2012 **http://2012.igem.org/Team:Utah_State/Notebook**

Ligation reactions are used to combine two linear fragments of DNA into a circular plasmid. The ligation procedure can be modified based on how much backbone DNA you have and how much insert you have, as well as if a phosphatase such as CIP (calf intestinal phosphatase) or TAP (thermo-sensitive alkaline phosphatase) was used in preparing the linear DNA molecules. Example protocol for ligation (to be added to a PCR tube)

- 10µl Insert DNA
- 3µl Vector DNA
- 2µl 10X ligation buffer
- 34µl H2O
- 1µl T4 DNA ligase

Note: This protocol can be adjusted depending on the concentration of insert and vector.

PCR – Utah State – 2012
http://2012.igem.org/Team:Utah_State/Notebook

Add the following reagents to a tube (50 µl reaction) in the following volumes and order:

- 32µl sterile H_2O
- 5 µl 10X buffer
- 2µl dNTP Mix
- 3µl $MgCl_2$
- 6µl cells/DNA
- 0.25µl Taq Polymerase
- 1µl Primer 1
- 1µl Primer 2

The thermocyler is setup beforehand with the desired protocol. Typically: 94℃ for denaturing, 50-60℃ for primer annealing, and 72℃ for polymerase extending. Example setup:

1. 94℃ 2min 1x
2. 94℃ 45sec
3. 55℃ 45 sec
4. 72℃ 1min 15 sec
5. 72℃ 5min 1x
6. 4℃ indefinitely

Repeat Steps 2 and 4 35x

DNA Mutagenesis – Utah State – 2012
http://2012.igem.org/Team:Utah_State/Notebook

- Use the QuikChange II Primer Design Program available online to create mutagenesis primers.
- Use the procedures in the QuikChange II XL Site-Directed Mutagenesis Kit Manual

Bacterial Glycerol Stocks – Nevada – 2012
http://2012.igem.org/Team:Nevada/Protocols

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1. Put 0.5mL bacterial culture in a sterile eppendorf tube.

2. Add 0.5mL of sterile 80% (v/v) glycerol solution
3. Freeze in liquid nitrogen and add to -80oC freezer
4. To recover, scrape frozen surface of culture with sterile inoculating needle, and then streak onto LB agar plate containing appropriate antibiotics, or inoculate liquid culture containing appropriate antibiotics.

DNA Quantification using NanoDrop Spectrophotometry – Nevada – 2012

http://2012.igem.org/Team:Nevada/Protocols

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1. Select *Nucleic Acids* measurement
2. Initialize the NanoDrop spectrophotometer with 2µL of autoclaved H_2O and wipe off
3. Blank (calibrate) the NanoDrop spectrophotometer with 2µL of the same elution buffer used during DNA purification and wipe off
4. Measure 1.5µL of DNA sample and record the concentration in ng/µL

Gel Purification of DNA *(Qiagen QIAquick Gel Extraction Kit)* – Nevada – 2012

http://2012.igem.org/Team:Nevada/Protocols

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1. Cut out the DNA fragment from the agarose gel with a razor blade, while minimizing the size of the gel slice
2. Weigh the gel slice and add 3 volumes of Buffer QG to every 1 volume of gel (100mg = 100µL)
3. Dissolve the gel slice using a 60°C heat block
4. Apply the dissolved gel to the QIAquick column and centrifuge at 13,000rpm for 1 minute
5. Discard the flow-through and repeat Step 4 until all sample has passed through the column
6. Add 500µL of Buffer QG to the QIAquick column and centrifuge at 13,000rpm for 1 minute
7. Wash the column with 750µL of Buffer PE and centrifuge at 13,000rpm for 1 minute
8. Discard the flow-through and centrifuge at 13,000rpm for 1 minute to remove residual EtOH
9. Transfer the QIAquick column to a new Eppendorf

10. Add 35µL elution buffer to the center of the column and wait at least 2 minutes
11. Centrifuge at 13,000rpm for 1 minute

PCR Purification of DNA – Nevada – 2012

http://2012.igem.org/Team:Nevada/Protocols

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1. Add 5 volumes of Buffer DB to 1 volume of PCR sample
   - ex: Add 250µL Buffer DB to 50µL PCR sample
2. Apply this mixture to a QIAquick column and centrifuge at 13,000rpm for 1 minute
3. Discard flow-through and repeat Step 2 until all sample has passed through the column
4. Wash column with 750µL Buffer PE and centrifuge at 13,000rpm for 1 minute
5. Discard flow-through and centrifuge at 13,000rpm for 1 minute to remove residual EtOH
6. Transfer QIAquick column to new Eppendorf
7. Apply 50µL elution buffer to center of the column and wait at least 2 minutes
8. Centrifuge at 13,000rpm for 1 minute

Phusion PCR – Nevada – 2012

http://2012.igem.org/Team:Nevada/Protocols

- Thermocycling conditions:

1. Initial Denaturation: 98℃ for 30 seconds
2. 25-35 cycles:

- 98℃ for 10 seconds
- 55℃, 60℃, 65℃ for 15 seconds
- 72℃ for 15 seconds

1. Final Extension: 72℃ for 5 minutes

Qiagen Miniprep kit – Nevada – 2012

http://2012.igem.org/Team:Nevada/Protocols

www.qiagen.com/hb/qiaprepminiprep

Primers– Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
Primers were designed using sequences obtained from the National Center for Biotechnology Information. To design the primers, we used ClustalX to align sequences and observe conserved regions of DNA.
Organisms:

- • Pseudomonas aeruginosa
- • Sphingomonas yanoikuyae
- • Sphingomonas paucimobilis
- • Sphingomonas aromaticivorans
- • Novosphingobium pentaromativorans

Operons:

- • Rhamnolipid synthesis genes:
- o rmlBDAC
- o rhlABG
- o MFST-rhlC
- o algC

- • Biphenyl degradation:
- o bphA1a
- o bphA2
- o bphA3
- o bphA4

- o bphB
- o bphC
- o bphD

top

Competent Cells– Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
Electrocompetent cells were created with Escherichia coli JM109 following previously published protocol (Sambrook, 2001).

top

DNA Purification– Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
DNA was extracted and purified from P. aeruginosa, S. yanoikuyae, and S. paucimobilis using Promega's Wizard Genomic DNA Purification Kit.

top

Using Standard Parts– Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
The following standard parts were used:

- • BBa_I13521 – RFP
- • BBa_I13522 – GFP
- • BBa_E0435 – lacZ alpha

Standard kit instructions were followed to rehydrate the DNA. The rehydrated plasmid DNA was then transformed into electrocompetent E. coli JM109 and plated onto selective media. Pure cultures were obtained and stock cultures made.

top

Polymerase Chain Reaction (PCR) – Clemson – 2012
http://2012.igem.org/Team:Clemson/Notebook

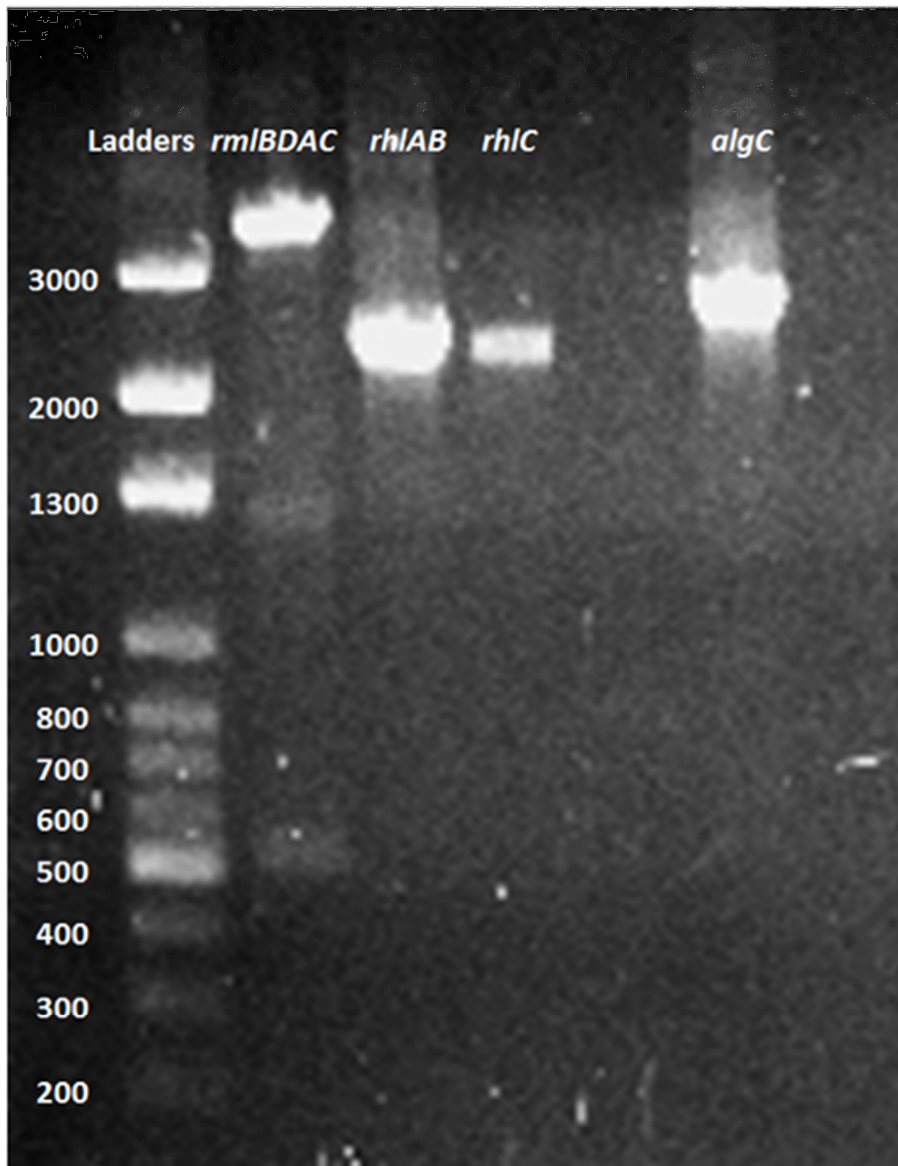
PCR was done using colony PCR or from template DNA extracted from genomic DNA isolation. Various PCR conditions were used to optimize the PCR products:

- • Varied annealing temperatures
- • Varied magnesium levels to optimize polymerase activity
- • Varied buffers

The following polymerases were used:

- • GoTaq polymerase from Promega
- • GoTag Long PCR Master Mix from Promega
- • Q5 High Fidelity from New England BioLabs
- • OneTaq Master Mix from New England BioLabs

PCR reactions were checked by DNA gel electrophoresis. An example of one such gel is below:



PCR of Rhamnolipid biosynthesis genes/operons from *Pseudomonas aeruginosa*. VWR 100bp DNA ladder. PCR products and their predicted sizes: *rmlBDAC* (3,396 bp), *rhlAB* (2,169bp), *rhlC* (2,232 bp), *algC* (2,607 bp).

top

PCR Purification– Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
PCR products were purified using two different protocols based on the quality of the electrophoresed DNA gels:

- • QIAGEN QIAquick PCR Purification Kit was used for DNA that gave one clear band on the gel.
- • QIAGEN QIAquick Gel Extract Kit was used to extract DNA that show multiple PCR products.

top

Joining DNA Fragments– Clemson – 2012
http://2012.igem.org/Team:Clemson/Notebook
PCR fragments were joined by two methods:

- • Chimeric PCR – Fragments of DNA are often joined together by restriction endonuclease digestion followed by ligation, but the fragments of DNA can also be joined together using just PCR. We used the following method to join multiple fragments of double-stranded DNA products together:

- • PCR products rhlA and rhlG (for Rhamnolipid biosynthesis) had already been amplified from P. aeruginosa, and we wanted to join them together so they could be part of a single operon on our recombinant plasmid. A new primer was designed that would serve as the forward primer for rhlG such that it contained a 5' extension with the ending sequence (approximately the

last 20 bp) of rhlAB. The original reverse primer for rhlG could be used to amplify, and the resulting PCR product contained a sequence identical to the end of rhlAB.

- • Next the two PCR fragments were combined along with only the forward primer for rhlAB and the reverse primer of rhlG. The denaturing step of PCR separates the double-stranded DNA, and during the annealing step, the overlapping sequences of rhlAB and rhlG anneal to each other along with the primers. Then during the elongation step, a single double-stranded DNA product is formed that contains both of the original DNA fragments.

- • Restriction digest and ligation – Restriction enzymes from New England BioLabs and Promega, and T4 ligase from Promega were used.

top

Transformation– Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
DNA fragments were ligated into plasmids pSB1C3 and transformed into E. coli JM109. Plasmids were purified and sequenced using Sanger's sequencing to verify successful cloning.

top

BioBricks – Clemson – 2012 http://2012.igem.org/Team:Clemson/Notebook
Three BioBricks were designed for rhamnolipid biosynthethis. Each BioBrick contains a promoter, three to four coding sequences for

rhamnolipid biosynthethis enzymes, a reporter gene, ribosome binding sites for all coding sequences, and a double terminator.

Materials – Carnegie Mellon – 2012

http://2012.igem.org/Team:Carnegie_Mellon/Met-Protocols

1. Cloning strains: DH5-alpha, Top10F', & BL21Pro.
2. Expression cell strains: BL21(DE3) & BL21(DE3)-pLys
3. Media: Luria Broth media, M9 media , Ampicillin, & Chloramphenicol.
4. Dyes: Malachite Green ester [from MBIC], 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI)

Kit Protocols – Carnegie Mellon – 2012

http://2012.igem.org/Team:Carnegie_Mellon/Met-Protocols

1. Z-Competent E. Coli Transformation Kit for making competent cells for easy transformation. Modified to incubate cells on ice for 1 hour during transformation
2. Phusion High Fidelity PCR Kit for amplifying our inserts and DNA cassettes.
3. TAQ DNA Polymerase for verifying our cloned constructs
4. Qiagen Mini-prep Kit for extracting our plasmids from the cells.
5. Zymo Clean and Concentrator kit for cleaning up DNA before digestion and ligation.

Cloning Protocol – Carnegie Mellon – 2012

http://2012.igem.org/Team:Carnegie_Mellon/Met-Protocols

1. If necessary, first prepare the insert using PCR amplification. This was done to add the necessary restriction sites for cloning. The PCR protocol follows the kit protocol outlined above, except with our specific range of melting and annealing temperatures calculated using NEB's Tm calculator

2. Start digestion of vector and insert DNA using desired restriction enzyme manufacturer protocol. [NEB]
3. After 2 hours, add Phosphatase [CIP] according to manufacturer protocol to insert to prevent self-ligation
4. Purify and clean DNA using kit protocol. [Zymo Research]
5. Measure vector/insert concentration. [Nanodrop]
6. Divide the concentration by the length of the sequence and calculate ligation ratios of 1 vector to 3 insert. Mix the ratios according to the calculations, including T4 buffer and ligase.
7. Leave ligation products at room temperature for 1 hour.
8. Transform ligation products into competent cells using appropriate protocol. Incubate on ice for 1 hour if using Zymo competent cells
9. Plate cells and incubate at 37 degrees overnight. Check for colonies the next morning. If there are colonies present, inoculate 1-3 colonies into (selective) L.B. in the evening and culture overnight.

Gel Protocol – Carnegie Mellon – 2012

http://2012.igem.org/Team:Carnegie_Mellon/Met-Protocols

1. For 7x7 cm, 0.5cm thick gel, with 1.75% agarose Concentration.
2. Add 0.35 grams of agarose to 20ml of 1x buffer (TBE) Note: Place mark on container to keep track of liquid level using marker, so water can be added to bring the liquid back to original level
3. Place gel solution in microwave. Use low/medium, set timer for 5 minutes. Stop the oven every 30 seconds and swirl gently to suspend undissolved agarose.
4. Once dissolved, set aside to cool (~60 degrees). Once solution is warm to touch, add 0.5ug/ml ethidium bromide. [1μl of 10mg/ml]
5. Pour into casting tray, remember to add in comb at the cathode side (black). Gel will solidify in ~10 mins
6. Divide the concentration by the length of the sequence and calculate ligation ratios of 1 vector to 3 insert. Mix the ratios according to the calculations, including T4 buffer and ligase.

7. Remove comb for solidified gel, remove casting gates and submerge gel beneath 2 to 6mm of 1x buffer.
8. Mix loading dye with PCR/digested products, load mixture into wells, together with 10μl of ladder
9. Run gel at 75V for ~1 hour. Adjust voltage accordingly if require faster or more distinct gels.

Dosage Curve – Carnegie Mellon – 2012

http://2012.igem.org/Team:Carnegie_Mellon/Met-Protocols

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1. The night before the experiment, start an overnight culture by seeding 1:100 of expression cell stock to fresh selection media [Typically 30μl stock to 3ml LB+Amp]. Place tube in a shaking water bath at 37 degrees celsius overnight.
2. Culture a fresh batch of cells using 1:100 of overnight culture prepared to selection media. Culture in a shaking 37 degrees celsius water bath for around 2 hours or until the solution is lightly turbid.
3. Induce cells by adding 1μl of 1M IPTG. Incubate cells in a shaking water bath at 37 degrees celsius for 2 hours.
4. Aliquot 1ml of induced cells into an 1.5ml Eppendorf tube and spin down the cells at 10,000rpm for 1min and discard the supernatant. Wash the cells with 1ml of PBS, repeat spinning down the cells at 10,000rpm and discard the supernatant. Resuspend the washed cells in 1mL M9 media.
5. To increase DFHBI fluorescence, add 1μL of 50nM Mg^{2+} to the tubes (from $MgCl_2$ from PCR kit)
6. Aliquot (100μl/200μl) of cells into desired wells of a 96-well plate.
7. To each of the 96-wells, add 1μl of 1mM IPTG to maintain the production of FAP and Spinach. Add various doses of DFHBI to the wells intended for testing DFHBI dosage. To a separate set of wells, add the desired doses of MG.

8. Incubate for 45mins in 37 degrees celsius, as recommended by Paige et al. Plate read with plate reader (Tecan Safire II). First find cell density using OD600. Then use Excitation/Emission of 469/501 for Spinach and 635/660 for Malachite-green.

Time Lapse Protocol – Carnegie Mellon – 2012

http://2012.igem.org/Team:Carnegie_Mellon/Met-Protocols

For the temporal analysis, we will be characterizing different promoters. Culture fresh batches of cells using 1:100 of overnight culture of the different promoter constructs. Do include the wild type promoter to act a a form of comparison. Culture for around 2 hours or until the solution is lightly turbid.

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1. Culture a fresh batch of cells using 1:100 of overnight culture prepared to selection media. Culture in a shaking 37 degrees celsius water bath for around 2 hours or until the solution is lightly turbid.
2. For each ml of cells required for the assay: Aliquot 1ml of cells into 1.5ml Eppendorf tubes and spin down the cells at 10,000rpm for 1min and discard the supernatant. Wash the cells with 1ml of PBS, repeat spinning down the cells at 10,000rpm and discard the supernatant. Resuspend the washed cells in 1mL M9+3µl 50nM Mg media.
3. Reserve part of the cells for testing the un-induced construct for leaky expression. Add 1µl of 1M IPTG per ml of remaining cells
4. Aliquot 100µl of induced and uninduced cells per well into a 96-well plate
5. Add the maximum dosage of DFHBI and MG as determined by the dosage curve into separate wells for each of the clones.
6. To each of the 96-wells, add 1µl of 1mM IPTG to maintain the production of FAP and Spinach. Add various doses of DFHBI to the wells intended for testing DFHBI dosage. To a separate set of wells, add the desired doses of MG.
7. As soon as the dyes are added, measure samples using the plate reader (Tecan Safire II). Measure cell density using absorbance

@600nm, followed by Excitation/Emission of 469nm/501nm for Spinach and 635nm/660nm for Malachite-green. Set the plate reader temperature to 37 degrees and to shake the plate for 10 seconds every 10mins. Repeat the OD and fluorescence measurements every 30minutes until the fluorescence output begins to plateau (~3 hours).

2011

PROCEDURE OF SPORICIDE – Faith Turkey – 2011
http://2011.igem.org/Team:Fatih_Turkey/ecolicompetent

Organism Preparation

1. A 2 x SG-Schaeffer sporulation medium (stock solution) : 16 g LB broth/liter, 0,5 g MgSO4 . 7H2O, 2 g KCl/lt.

2. Additional stock solutions include %10 glucose, 1M Ca(NO3)2, 0,1 M MnSO4, 0,001 M FeSO4.

3. Compenents of stock solutions are dissolved one at a time in 1 lt of d-distilled H2O.

4. 100 ml aliquots are poured into media bottles.

5. The caps are loosened and autoclaved in a pan of water for 20 min. on slow exhaust.

6. The bottles are allowed to cool comletely, caps are tightened, solutions are scored in the dark until use.

- As prepared, the Schaeffer nutrient base lasts approximately 1 week.

7. Stock solutions are filter sterilized yusing a 0,22 um filter.

Fermantation (Producing Spores)

1. 10 ml of Schaeffer medium is added to a 50 ml falcon, inoculated with a 10 ul culture (or one colony from plate) of B. Subtilis.

2. 5 days are allowed for growth and sporulation. (30 C, moderate aeration)

3. The culture is heat shocked at 65 C for 15 min to kill vegetative cells.

4. The spores are harvested by centrifiguation (16,000 x g for 25 min), then washed with d-distilled water 10 times.

5. The spores are resuspend in 1.7 ml of sterile d-distilled water.

6. A dilution series is performed on the suspension to numerate the number of viable spores.

7. Growth on nutrient agar medium deposited on petri dishes to determine the population of spores.

Preperation of the modified Fenton Reagents

1. Cupric chloride and ascorbic acid (AA) are dissolved in deionized water, tap water, or salt water (2 M NaCl). The most effectıve reagent solution includes 2M NaCl in water

2. A 1% surfactant (3 M FC-170 or FC-100 fluorinated surfactant) is also added in to reduce the surface tension of the aqueous solution in order to get the highest killing level.

- The most effetice reagent solution includes FC-100 type of sulfactant

3. The exposure time of spores to the reagent istypically 30 min, and exposure is performed at 25°C.

Sporicide Procedure (Aqueous)

1. Prepared aliquots of 1 ml of spore culture are placed in test tubes.

2. The tubes are spun for 10 min at 16,000 x*g* to pelletize the cells. The supernatant is discarded.

3. Three tubes are resuspended in the Fenton reagent to be tested (the precise volume and tube size varied).

- A fourth sample tube is resuspended in sterile d-distilled water as a control.

4. The tubes are exposed to the Fenton reagents for the specified exposure times (30 min unless noted otherwise).

5. Dilution with cold (0°C) liquid (sodium thiosulfate) quenches the reaction.

6. The sample tubes and the control are then spun at 16,000 x*g* for 10 min to pelletize the cells. The supernatant is removed.

7. Spores are rinsed two times in an appropriate diluent.

8. Spores are then resuspended in 1 ml of fresh sterile phosphate buffer.

9. Serial dilutions (generally over 5 to 7 logs of dilution) on both samples and controls are performed using nutrient agar growth medium plated on petri dishes. The dishes are incubated for 24 h minimum, 48 h maximum at 30°C.

NOTE: As described above, serial dilutions are then prepared for each tube, using sterile phosphate buffer as the diluent. In all cases, a standard serial dilution of the stock spore

culture is carried out to verify that the culture is still viable and that the number of CFU (CFU per milliliter) is constant over time. All spore-handling procedures use aseptic bacteriological techniques in laminar flow biohazard hoods.

Sporicide Procedure (Solid Surface)

1. Sterile 2-cm2 glass coupons are scored and cut from frosted glass microscope slides

2. The glass coupons are then inoculated with *B. subtilis*stock solution, approximately 5 x 10^7 spores/coupon. The pipette is fitted with a new tip for each coupon.

3. The inoculated coupon(s) then is dried at room temperature overnight in a desiccator containing Drierite.

4. Following exposure to Fenton reagents, coupons are placed in a cold (_0°C) neutralization agent (sodium thiosulfate) and then sonicated for 60 min to remove spores from the substrate. Separate tests verifythat the sonication procedure is capable of recovery from the coupons and that sonication do not induce spore deactivation.

5. The spore population is then determined by performing triplicate serial dilutions of the resulting suspension. Serial dilutions are prepared for each sample, using sterile phosphate buffer as the diluent.

6. Serial dilutions (generally over 5 to 7 logs of dilution) on both samples and controls are performed using nutrient agar growth medium plated on petri dishes. The dishes are incubated for 24 h minimum, 48 h maximum at 30°C.

NOTE: The magnitude of kill is then determined by comparing the treated colony count with that of untreated colonies. It is noted that the preparation techniques resulted in clumped spores on the impervious glass coupons.(J. B. Cross, 2003)[1]

BACILLUS SUBTILIS (competent ve transformation) – Faith Turkey – 2011

http://2011.igem.org/Team:Fatih_Turkey/ecolicompetent

Media Preparation

10X Medium A base:

● Yeast extract 10g

● Casamino acids(pepton from casein/ triptone) 2g

● Distilled water to 900mL

● Autoclave, then add :

● 50% glucose, filter sterilized 100mL (50 g/100 mL)(%50 glikozu distile suyle birlikte bir flaskın içinde bunsen burnerda ısıtarak çöz)

10X Bacillus salts:

● $(NH_4)_2SO_4$ 20g

● Anhydrous K_2HPO_4 139.7g

● KH_2PO_4 60g

● Tri-sodium citrate 10g

● $MgSO_4 \cdot 7H_2O$ 2g

● SDW(sterile distile water) to 1000mL

● Then, autoclav

Medium A

● Sterile water 81mL

● 10X Medium A base 10mL

● 10X Bacillus salts 9mL

● L-Tryptophan (11mg/mL) 0.1mL (filter sterilized)

● Then, filter sterilized

Medium B

● Medium A 10mL

● 50mM $CaCl_2 \cdot 2H_2O$ 0.1mL (filter sterilized) (147 g/mol)

● 250nM $MgCl_2 \cdot 6H_2O$ 0.1mL (filter sterilized) (203.3 g/mol)(hazırlanışı için notlar kısmızı oku)

Important:

● Autoclave Medium A base before adding glucose, and autoclave Bacillus salts

● Store aliquots of 10X Medium A base 10mL and 10X Bacillus salts 9mL and keep them in the fridge, never use them twice to avoid contamination

Protocols

Making Bacillus competent

1. Grow one blank plate of *Bacillus subtilis* (or several if you want to transform different strains) for 20 hours at 37ºC (plate been kept on the bench for several days would be better)
2. Inoculate about 12mL of medium with several colonies. Mix the contents of the tube. Check with OD650. Start OD should be between 0.1 and 0.2. Be careful to pipette 0.8mL of this mixture into the cuvette to measure and dispose of it after measurement to avoid contamination in the main mixture.
3. Incubate at 37ºC with vigorous shaking. Read the OD650 every 20min (never keep the solution you used for measuring!)
4. Plot log(OD650) in function of time. After a brief lag, you should observe a exponential increase. After awhile, it will leave the exponential growth; the moment at which it leaves the exponential path is denoted as t0 (3 on the graph). It should take about 100min and the OD should be between 0.35 and 0.55.

1. At t0, incubate for 90 minutes at 37ºC with vigorous shaking.
2. Transfer 0.05mL of this culture into 0.45mL **of pre-warmed Medium B in** an Eppendorf tube. You have to prepare one tube for each transformation, plus an extra tube for a DNA-less control.
3. Incubate the diluted cultures at 37ºC with shaking for 90min. At this moment, the cells are HIGHLY COMPETENT.
4. To check for competency, you can look at cells under the microscope; competent cells are very motile.

Transforming

1. Spin Eppendorf tubes containing cells. Remove 400µL of liquid to keep only 100µL of the culture (to concentrate cells). Re-suspend the cell pellet in the remaining culture.
2. To transform from competent glycerol stocks, firstly thaw on ice and then spin the tube at about 1600rpm for 20min, remove the supernatant (glycerol), and add 100µL of pre-warmed medium B.
3. Mix the cells thoroughly. (pipeting slowly)
4. Add 0.6µg of DNA to the competent cells. (yarın belli oacak)
5. Incubate for 30min at 37ºC with shaking.
6. Plate 100µL of transformed cells onto selective agar.

Glycerol Stocks

1. To freeze competent Bacillus cells, spin down(8ooo rpm, 5 min) the fresh competent cells to obtain a pellet.
2. Remove all supernatant. (remove 450µLsupernatant with pipette)
3. Re-suspend cells in 500µL 60% glycerol. (slowly)
4. Add into liquid nitrogen
5. Freeze tubes at -80ºC.

E.coli TOP10 Transformation – Faith Turkey – 2011
http://2011.igem.org/Team:Fatih_Turkey/ecolicompetent

MATERIALS

- **Heat block**
- **Incubator with shaker**
- **Competent cell**
- **Lb broth**
- **Lb agar with antibiotic**
- **Parafilm**
- **Sterile MiliQ dH2O**

- **Ice**
- **0,5 and 1,5 epp**
- **Spreader**

SOLUTIONS

LB Agar Preparation

- Add 200 mL of dH2O to a graduated cyclindar.

- Transfer dH2O into glass bottle.

- Add 7 gr of LB-agar powder

- Autoclave the bottle.

- After cooling, add 200 uL antibiotic (The LB agar solution should be cool enough not to damage to antibiotic)

- Pour the plates .

LB Broth Preparation

- Add 200 mL of dH2O to a graduated cyclindar.

- Transfer dH2O into glass bottle.

- Add 4 gr of LB powder

- Autoclave the bottle.

- After cooling, add 200 uL antibiotic (The LB agar solution should be cool enough not to damage to antibiotic)

Transformation

- Aseptic conditions prepared (70% EtOH, Bunsen burner etc.)

- Place 500 uL LB in epp into heat block(42˚C).

- Thaw 50 uL competent cells on ice.

- Add 1 uL plasmid into the competent cell epp and spin for few sec.

- Incubate for 45 min on ice.

- Incubate at 42˚C for 80 sec in heat block.

- Incubate for 5 min on ice.

- Complete to 500 uL with pre-heated LB (42˚C).

-.Epp s adhered with tape to horizontal on shaker.

- Incubate at 37 C for 1 h at 240 rpm.

- Spread 125 uL from each tube on agar plates with suitable antibiotic.

- Incubate plates at 37˚C not longer than 12-14 h.

E.coli TOP10 Competent Cell – Faith Turkey – 2011
http://2011.igem.org/Team:Fatih_Turkey/ecolicompetent

MATERIALS

- **Centrifuge**
- **Autoclave**
- **Incubator with shaker**
- **pH meter**
- **Stock competent cell**
- **LB broth**
- **Falcon**
- **Ice**
- **Liquid nitrogen**

- **Bacto yeast extract**
- **Bacto tryptone**
- **Magnesium sulfate**
- **Potassium hydroxide**
- **Potassium acetate**
- **Rubidium chloride**
- **Calcium chloride**
- **Manganese chloride**
- **Glycerol**
- **Dilute acetic acid**
- **Filter**
- **MOPS**
- **Dilute NAOH**

SOLUTIONS

Preparation of Psi broth (per liter)
- 5 g bacto yeast extract
- 20 g bacto tryptone
- 5 g magnesium sulfate
- PH 7.6 with potassium hydroxide
- Autoclave 40 min

Preparation of Tfbl (per 200 ml)
- 0.588 g potassium acetate (final molarity/conc= 30 mM)
- 2.42 g rubidium chloride (final molarity/conc= 100 mM)
- 0.294 g calcium chloride (final molarity/conc= 10 mM)
- 2.0 g manganese chloride (final molarity/conc= 50 mM)
- 30 mL glycerol (15% v/v)
- Adjust PH 5.8 with dilute acetic acid
- Sterilize with filter

Preparation of TfbII (per 100 ml)
- 0.21 g MOPS (final molarity/conc= 10 mM)
- 1.1 g calcium chloride (final molarity/conc= 75 mM)
- 0.121 g rubidium chloride (final molarity/conc= 10 mM)
- 15 mL glycerol (15% v/v)
- Adjust PH 6.5 with dilute NaOH
- Sterilize with filter

- Inoculate streak plates from liquid stock competent cells and incubate overnight at 37˚C

- Put 10 mL LB + colony into 50ml falcon. Incubate overnight at 37˚C with shaker

- Inoculate 200 ul to 1000 ul from overnight culture into 100-500 ml Psi broth (scale up or down as needed). Incubate at 37 C with aeration to A600=0.6-0.7

- Ice 15 min. From this step onward the cells must remain COLD. (4C or on ice)
- Pellet cells in appropriate centrifuge tube 3-5000 x g 5 min (~5000 rpm in a Sorvall SS-34 rotor)
- Discard supernatant and add 0.4 volume (ie of original volume, here it is 40-400 ml) TfbI, resuspend and ice 15 min.
- Pellet cells in appropriate centrifuge tube 3-5000 x g 5 min (~5000 rpm in a Sorvall SS-34 rotor)
- Discard supernatant and resuspend in 0.04 volume TfbII, ice 15 min and either use immediately or quick freeze at -70C for storage. I usually save these in 100ul to 200ul aliquots. Quick freeze in ethanol-dry ice or liquid nitrogen prior to storage in a -70 to -80 C freezer. Thaw on ice just before using in a transformation experiment.

β-gal assay – DTU-Denmark – 2011
http://2011.igem.org/Team:DTU-Denmark/Protocols

This assay provides a way of measuring activity of β-Galactosidase which hydrolyzes β-galactosides into monosaccharides. One example of such reaction is hydrolysis of disaccharide lactose into glucose and galactose. Measurement β-Galactosidase activity is made using o-nitrophenyl-β-D-galactopyraniside (ONPG). Cleavage of ONPG results

in release of yellow comound, o-nitrophenol, which absorbs 420 nm light. Activity of the enzyme is expressed in terms of increase of yellow color per minute.

Reagents:

- Suited media
- Z-buffer
- 2.5 mg/ml Fresh Lysozym
- 10% Triton X100
- 4mg/ml ONPG
- 0.5 M Na_2CO_3

Procedure: Day 1

1. Inoculate 10 ml suited media with 2-3 dilutions

Day 2

1. At OD450 =0.4-0.8 harvest 2 ml culture and resuspend in 2 ml Z-buffer.
2. Measure OD600.
   - In the following use 2 ml eppendorf tubes and make blind sample containing all the things except cells!
3. Pipet X µl cells and add Z-buffer to total volume of 0,832 ml.
4. Add 160 µl Fresh Lysozym solution and 8 µl 10% Triton X100.
5. Vortex for 5 sec.
6. Incubate for 5 min. at 30°C.
7. Add 100 µl 4 mg/ml ONPG.
8. Vortex for 5 sec. Note time.
9. Incubate on 30°C.
10. When the reaction turns yellow, add 1ml 0.5 M Na_2CO_3 to stop reaction. Note time.
11. Place the sample in the refrigerator while finishing all the samples.
12. Spin the samples for 2 min. at 15.000 rpm.
13. Measure OD420 and OD550, use the blind sample as reference. Measure both ODs on each sample. If OD550 is higher than 0.05 spin again and measure again.

Beta-gal acitivity = 1000 x (OD420 - 1.75 x OD550) / (T x V x OD600)

Gel preparation and electrophoresis – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

Gel preparation (1% gel) (100 ml of the buffer). Reagents:

- 100 ml of 1x TBE buffer .
- 10 µl of ethidium bomide (10 mg/ml).
- 1 g of agarose.
- Assembled gel container.

1. Mix buffer with agarose and heat in microwave until the solution is clear
2. Add 10µl of ethidium bromide (10mg/ml).
3. Pour solution to the gel container and leave it to solidify (30-45 min).
4. Place gel container in the electrophoresis machine, remove combs and cover with the TBE buffer.

Gel electrophoresis:

- 2µl of DNA sample.
- 3 µl of distilled water.
- 1µl of 6x loading dye.
- 4.2 µl of Gene Ruler DNA ladder mix from Fermentas.

It is recommended to use 7V for each cm of the gel length and to run the gel for 45 min.

Ligation – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

Ligation mix (20 µl total):

- 2 µl 10x Buffer
- vector+insert in molar ratio 1:5 (DNA concentration less than 50 ng)
- 1 µl ligase
- fill up with water

Positive control (20 µl total):

- 2 µl 10x Buffer
- vector (DNA concentration less than 50 ng)

- 1 µl ligase
- fill up with water

Negative control (20 µl total):

- 2 µl 10x Buffer
- vector (DNA concentration less than 50 ng)
- fill up with water

Procedure:

1. Estimate insert and vector concentrations after gel electrophoresis.
2. Calculate amount of inser and vector to take for ligation.
3. Ligate - 1 hour at room temperature.
4. Transform competent cells; incubate; analyze growth on plates taking to account positive and negative control.

Competent cells – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

Day 1:

1. Inoculate 5 ml of LB from a colony or from a -80oC stock.

Day 2:

1. Dilute exponentially growing cells to OD600=0.05 and grow cells in a shaker to OD600=0.5-0.6 in pre-warmed LB. Remove the cells from the shaker and place on ice. Transfer liquid cultures to centrifuge tubes. Note: It's very important to keep the cell on ice from now on.
2. Centrifuge at 6000 rpm for 10min; discard supernatant.
3. Gently resuspend in 5-7 ml of ice-cold 10% glycerol; consolidate to half the number of centrifuge tubes.
4. Fill up with 10% glycerol and centrifuge 10 min at 6 rpm.
5. Repeat step 3 and 4 (no consolidation) two to three times.
6. Resuspend in 5-7 ml of 10% glycerol and move to 15 ml or 50 ml tubes.
7. Centrifuge at 5000 rpm for 5 minutes, discard supernatant and resuspend gently in about 2 ml 10% glycerol pro L culture.

8. Flash freeze into Eppies placed in −80ºC pure ethanol bath and store at −80ºC. Make aliquots of 85µl, 135 µl and 175 µl for 2, 3 and 4 electroporations respectively.

Day 3:

1. Transform cells with 1 µl of 10 pg/µl of pUC19 (AmpR) to test efficiency of the competent cells.
2. Plate 10 µl and 100 µl on LB+Amp (100 µg/ml) plates.

Day 4:

1. Count colonies and calculate the transformation efficiency as colonies/µg of pUC DNA.

PCR protocol – DTU-Denmark – 2011

http://2011.igem.org/Team:DTU-Denmark/Protocols

Reagent	TAQ + PFU [µl]	Phusion [µl]
Enzyme	0.5	0.5
Forward primer	2.5	5
Reverse primer	2.5	5
dNTP	4	4
Template	1	1
Buffer	10	10

Water	79.5	74.5

PCR program design – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

1. Initial denaturation for 2 minutes at 95ºC.
2. Denature for 1 minute at 95ºC.
3. Anneal primers for 30 seconds at temperature ~5ºC below melting temperature of primers.
4. Extend DNA at 72ºC using each 1-2 minutes per kilobase of product, depending on polymerase used (see manufacturer's instructions).
5. Repeat steps 2-4 for 25-30 cycles.
6. Final extension for 10 min at 72ºC

PCR product purification with NucleoSpin – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

1. Mix 1 volume of sample with 2 volumes of NT buffer in an 1,5 ml Eppendorf tube.
2. Place a column into a 2 ml collection tube and load the sample.
3. Centrifuge at 11.000 g for 1 min.
4. Discard flow through and place the column back into the collection tube.
5. Add 600 μl NT3 buffer and centrifuge at 11.000 g for 1 min.
6. Discard flow through and place the column back into the collection tube.
7. Centrifuge at 11.000 g for 2 min to remove NT3 buffer. Discard flow through.
8. Place the column into a clean 1,5 ml Eppendorf tube.
9. Add 30 μl of water or NE buffer and incubate for 1 min to increase the yield of eluted DNA.
10. Centrifuge at 11.000 g for 1 min.

Plasmid puriification – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

This purification is based on the "Zyppy Plasmid Miniprep Kit"

Amounts of bacterial culture: According to Zyppy the purification can be done on 600 ul cell culture, but our experience suggests that it is not enough for further processing/use of the DNA. We use 2-4 ml of cell culture.

1. Initial steps:
   - Add 1.5 ml of cell culture in LB medium to 2 ml eppendorf tube.
   - Spin at 15.000 g for 2 min.
   - Discard supernatant
   - Add 1.5 ml of cell culture (for a total of 3 ml cell culture)
   - Spin at 15.000 g for 2 min.
   - Remove as much supernatant as possible - pipette carefully. This is (the only) point of no return! To stop, freeze the pellet.
   - Add 600 μL of TE-buffer. Ensure that the pellet is completely suspended.
2. Add 100 μL 7x lysis buffer. Remember not to process more than 10 minipreps at a time.
3. Add 350 μL cold neutralization buffer. Mix gently and thoroughly (= all the way through ≠ violently)!
4. Spin at 15.000 g for 5 min.
5. Transfer the supernatant to the columns; be careful not to get some of the pellet! It's better to leave some supernatant than to get some of the pellet. Several "lysis" can be poured together to up-concentrate.
6. Spin at 15.000 g for 30 sec.
7. Discard flow-through.
8. Add 200 μL endo-wash-buffer
   - Spin at 15.000 g for 30 sec.
9. Add 400 μL zyppy wash buffer
   - Spin at 15.000 g for 30 sec.
10. Transfer columns to clean 1.5 ml eppendorf tubes. Be careful when removing the tubes, the buffer may not touch the tip of the column! (if it happens, spin again).
11. Elute DNA in 30-100 ul of buffer of choice (TE/H2O/restriction buffer/Zyppy elution buffer). Add the buffer to the center of the column, but without touching the column material! If H2O, wait 5 min before proceeding to the final centrifugation step, as DNA is not easily suspended in water.
12. Spin at 15.000 g for 30 sec.

13. Check the purification by running a gel (at least until we get experienced with a high success rate).

Recombineering – DTU-Denmark – 2011 http://2011.igem.org/Team:DTU-Denmark/Protocols

The recombineering procedure is an efficient way of introducing multiple chromosomal gene deletions in E. coli in gradual manner. It exploits activity of two recombinases, Red and Cre. In the first step the Red recombinase inserts a linear DNA in place of a gene to be deleted based on sequence homology. Inserted DNA carries antibiotic resistance gene flanked with LoxP sites which can be selected for when growing bacteria on plates containing corresponding antibiotic. Then, Cre recombinase is activated and cuts out resistance gene from chromosome using the LoxP sites, which results in loss of resistance. Afterwards, the procedure can be repeated for subsequent gene deletions.

E. coli strain W3110, prior to be used with this protocol, was transformed with two helper plasmids: (1) pSLD18 containing the phage Lambda recombination system (also called Red) under temperature inducible promoter and erythromycin resistance gene, donated by our supervisor Sebastién Lemire; and (2) pHC3220 containing Cre recombinase under arabinose inducible promoter and ampicillin resistance gene, donated by Flemming G. Hansen. Both plasmids are temperature sensitive and temperature at which bacteria was grown is restricted to 30°C. Linear pieces of DNA where prepared (PCRed), that contained antibiotic resistance gene of choice, flanked by LoxP sites and homology regions to chromosomal regions flanking gene to be deleted.

Day 0 – Prepare overnight culture.

1. Pick wild-type E. coli colony from plate and whirl in 5 mL liquid LB + ery_{150} + amp_{100}, grow at 30°C with aeration/shaking.

Day 1 – Gene knock-out

1. Prepare competent cells, (small batch):

- Add 50 µL of ON culture to 5 mL LB, grow at 30°C for 2.5 hours (until exponential growth).
- Bottles with cells are transferred to 42°C for EXACTLY 15 minutes, NO MORE.
- The washing-buffer (10% w/w ultra pure glycerol in water) is put on ice

- The cells are harvested for 5 minutes at 6000 rpm, 4°C in 2ml tubes. Repeat once for each tube to increase cell concentration.
- The pellet is carefully re-suspended in 1 ml ice-cold glycerol 10%, and the cells are centrifuged for 5 minutes at 6000 rpm., 4°C. Discard the supernatant. This washing step is repeated three tims.
- Resuspend in 50 µl cold 10% glycerol.

2. Transform cells with linear piece of DNA (see protocol for transformation).
3. Leave in recovery medium at 30oC for 2 hours with aeration/shaking.
4. Plate on plates containing appropriate antibiotic and grow overnight at 30oC.

Day 2 – Test transformation with PCR and remove resistance gene.

1. Do colony PCR for 4-8 colonies from yesterday transformations:
   - Pick a colony using a toothpick.
   - Whirl it in an eppendorf tube containing 100 µL TE.
   - Use 1 µL as template in PCR.
2. Move cells to minimal medium arabinose + selection. Use the eppendorf tubes from the colony PCR as a source of cells. The minimal medium is ABT medium with arabinose (0.15%) and ery150, amp100. Grow at 30°C.
3. At the end of the day restreak onto ery150 and/or amp100 plates, and grow at 30°C.

Day 3 – Select colonies that lost resistance.

1. Restreak onto LB and LB+kan50.

Day 4 – Check colonies by PCR.

1. Do colony PCR for 4-8 colonies from yesterday transformations:
2. Pick a colony using a toothpick.
3. Whirl it in an eppendorf tube containing 100 µL TE.
4. Use 1 µL as template in PCR.
5. Prepare ON culture for the next round of red-swap. Pick wild-type E. coli colony from plate and whirl in 5 mL liquid LB + ery_{150} + amp_{100}, grow at 30°C with aeration/shaking.

Proceed with the subsequent gene deletion by starting the protocol from day 1, using a new PCR product.

After the last deletion the Red and Cre plasmids can be curated by growing cells at 37°C.

Restriction digestion – DTU-Denmark – 2011
http://2011.igem.org/Team:DTU-Denmark/Protocols

Reagents:

- 25 µL DNA
- 10 µL Buffer 10x
- 2.5µL of each restriction enzyme
- Add H2O to total volume of 100 µL

1. Mix everything. Leave for 2 hour at 37 C in thermo block/incubator - check the temperature with enzyme manufacturer.
2. Heat-deactivate enzyme - check the temperature with enzyme manufacturer.
3. Skip point 2 if proceeding immediately to DNA purification.

SAP treatment to prevent re-circularization of the plasmid:

1. Mix 50µL of the restricted DNA with 2.5µL SAP and 5µL SAP-buffer.
2. Put it in the incubator 37°C for 60 min.
3. 15 minutes on heat block 65°C to deactivate enzyme

Transformation and selection – DTU-Denmark – 2011
http://2011.igem.org/Team:DTU-Denmark/Protocols

Part 1: Transformation by electroporation

Material:

- ice
- test-tubes
- cuvettes
- LB medium at room temperature
- your strain
- DNA

Procedure:

1. Get competent cells and store them on ice (the number on the lid indicate the number of transformation the amount corresponds to).
2. Pool the competent cells into one tube. This is done to ensure a homogen batch.
3. Put cuvettes for electroporation on ice.
4. Label as many glass tubes and cuvettes as samples and in addition to that, prepare some extra tubes. Add 1 ml recovery media (LB) to each. Check the LB media by shaking to ensure that it is sterile and there's no bacteria growth.
5. Electorporation:

- Use program EC2, and set the form to 'time ms', press the right blue square bottom to get started.
- Add 2 µl of the ligation (DNA) to one side of the cuvette.
- Add 40 µl of the competent cells to the same side of cuvette.
- Mix by title gently, ensures that all liquid is at the bottom and that there are no bubbles. Dry the cuvette with paper towel.
- Pipette 1 ml recovery media quickly.
- Place the cuvette in the machine and press the pulse bottom, write down the time(usually is above 5 and below 5.8)
- Immediately add 1 ml recovery media to the cuvette, pipette gently up and down for 1-2 times and try to get as much of the liquid retransferred to the tube where you took recovery media from.
- Keep track of the time constant, it should preferentially be around 5.60. Mark the tube if the time constant is below 5.00 as the cells might be dead/weak. If the time-constant is 0.00 (ARG) the cells are dead! Try again, maybe with less ligation mix.
- ARG can be due to different factors:

Too much DNA

Too many ions

Bubbles

Liquid on the outside of the cuvette (i.e. the cuvette was not dried properly)

Part 2: Selection of transformants

Material:

- antibiotic plates
- LB plates

Procedure:

1. You will be plating 100 ul on each plate (with antibiotic corresponding to the selection marker on the plasmid).
2. First, decide if you need to make a dilution series which depends on how well transformation goes. If it is high, then without diluting there will be an over-grow of bacteria on the plate and you can't select a colony from there.
3. Choose your dilutions, e.g. from 10^{0} to10^{-7} in order to get an appropriate number of bacteria for plating out. High efficiency transformation: use dilution -5, -6 or -7.
4. Plate 100 µL from your 10^{-X}</sups> dilutions onto the antibiotic plates.
5. Negative control: Plate 100 µL from your undiluted bacteria culture (100 dilution) that was not transformed onto the selective plate.
6. Positive control: Plate 100 µL from your 10^{-x} dilution onto a non-selective plate and spread.
7. Incubate your plates overnight at 37°C (depends on the bacteria or experiment).
8. Place the rest of the LB-media with transformed cells on the bench overnight. If colonies are lacking the next day; plate again.

Making of LB medium (*Lysogeny Broth*, also referred as *Luria-Bertani* Medium) – Debrecen Hungary – 2011

http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

Giuseppe Bertani published the original formula of LB, which is used for the cultivation of E. coli. It provides the main essential nutrition, containing peptides. For transformation, we use LB agar but for small and big cultures we use the original liquid LB.

- **Overview**

The presparation is to dissolve LB powder in distilled (MilliQ) water, and autoclave it.

- **Materials**

For 1 L LB Broth

- 1 L dH20
- 20g LB powder

- a 1 l bottle
- measuring cylinder
- Laboratory scale

- **Procedure**

1) Take a jar of LB powder

2) Measure 20 g of LB powder in a scale (it depends on the amount of LB you need, take 10 g if you'd like to make 500ml of a LB medium)

3) Put the powder into a 1 liter flask or bottle, (again, depends on how much you need)

4) Add the distilled water into the bottle till it reaches 1 liter

5) Shake the bottle carefully

6) Close it and stick a tape from one side of the wall vertically over the cap to the other side of the wall (which means not sterile) which will be black striped after autoclave sterilization

7) Sterilize the bottle (autoclave)

- **References**

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed.,1.25-1.28. Cold Spring Harbor Laboratory Press, Cold Spring harbor, NY, USA.

Bacterial transformation by heat shock – Debrecen Hungary – 2011

http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

Transformation is the process of introducing foreign DNA (e.g plasmids, BAC) into a bacterium. Bacterial cells into which foreign DNA can be transformed are called competent. Some bacteria are naturally competent (e.g B. subtilis), whereas others such as E. coli are not naturally competent. Non-competent cells can be made competent and then transformed via one of two main approaches; chemical transformation and electroporation (our lab tends to use a protocol based on treatment with calcium-chloride-pipes-glicerol solution). We tried several protocols last year, but we found that this is the best yielding among them. This protocol may be particularly useful if you are finding that your transformations are not working or yiedling few colonies.

- **Overview**

Put the DNA to be introducend and your competent cells together, change incubation temperature dramatically some times then spread the cells on LB agar plates and incubate at 37 degrees overnight.

- **Materials**

- Competent cells (we always use DH5α strain - [2])

- DNA to be introduced
- Crushed ice, 1,5 ml Eppendorf tubes
- 42℃ water bath
- 37℃ incubator
- Petri dishes with LB agar and appropriate antibiotic

- **Procedure**

1. Start thawing the competent cells on crushed ice (we find this cells in the -70℃ fridge)
2. Put 50µl competent cells and 100-250ng DNA into a 1,5 ml tube kept on ice
3. Incubate the cells for 20 minutes on ice
4. Heat shock at 42℃ for 90 seconds in water bath (do not shake!)
5. Incubate for 5 minutes on ice again
6. Spread the transformed bacteria on the Petri dishes with LB agar and the appropriate antibiotic(s) with the part name, plasmid backbone and antibiotic resistance
7. Incubate the plate at 37℃ for 14 hours

- **References**

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed.,1.25-1.28. Cold Spring Harbor Laboratory Press, Cold Spring harbor, NY, USA.

MiniPrep – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of E. coli in LB (Lysogeny Broth, Luria-Bertani) medium.

- **Overview**

This year we used GeneJet Plasmid Miniprep Kit (Thermo Scientific), and followed the provided protocol with some minor changes.

- **Materials**

- Kit containts

- table-top centrifuge at >12000 x g(10 000-14 000 rpm)
- centrifuge for 50 ml tubes with relative high performance

- **Notes**

All purification steps should be carried out at room temperature. All centrifugations should be carried out in a table-top microcentrifuge at >12000 x g(10 000-14 000 rpm, depending on the rotor type). Use 1-5 ml of E. coli culture in LB media for purification of high-copy plasmids. For low-copy plasmids use up to 10 ml of culture.

- **Procedure**

0. [small culture] Next day after transformation of the bacteria pick up a colony with a pipette tip from the agar plate, wash into 1-5 ml LB medium (with the proper antibiotic) and put the tube into a shaker to 37℃ for 12 hours. After incubation, centrifuge the tube in a adequate centrifuge at 10000xg for 10 minutes. Pour off the used medium.

1. Resuspend the pelleted cells in 250 µl of the Resuspension Solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain. Note. Ensure RNase A has been added to the Resuspension Solution.

2. Add 250 µl of the Lysis Solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. Note. Do not vortex to avoid shearing of chromosomal DNA. Do not incubate for more than 5 min to avoid denaturation of supercoiled plasmid DNA.

3. Add 350 µl of the Neutralization Solution and mix immediately and thoroughly by inverting the tube 4-6 times. Note. It is important to mix thoroughly and gently after the addition of the Neutralization Solution to avoid localized precipitation of bacterial cell debris. The neutralized bacterial lysate should become cloudy.

4. Centrifuge for 5 min to pellet cell debris and chromosomal DNA. 5 Transfer the supernatant to the supplied GeneJET™ spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate. Step Procedure

6. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. Note. Do not add bleach to the flow-through, see p.7 for Safety Information.

7. Add 500 µl of the Wash Solution (diluted with ethanol prior to first use as described on p.3) to the GeneJET™ spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.

8. Repeat the wash procedure (step 7) using 500 µl of the Wash Solution.

9. Discard the flow-through and centrifuge for an additional 1 min to remove residual Wash Solution. This step is essential to avoid residual ethanol in plasmid preps.

10. Transfer the GeneJET™ spin column into a fresh 1.5 ml microcentrifuge tube (not included). Add 50 µl of the Elution Buffer to the center of GeneJET™ spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min. Note. An additional elution step (optional) with Elution Buffer or water will recover residual DNA from the membrane and increase the overall yield by 10-20%. For elution of plasmids or cosmids >20 kb, prewarm Elution Buffer to 70℃ before applying to silica membrane.

11. Discard the column and store the purified plasmid DNA at -20℃.

References

Birnboim, H.C., and Doly, J. (1979) A rapid alkaline lysis procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513–1522.

MidiPrep – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

This protocol is designed for the preparation of High Copy Number plasmids. We used Genopure Plasmid Midi Kit (Roche).

- **Materials and notes**

- Sample: 50 ml E. coli culture, transformed with a high copy number plasmid. Harvest cultures at a density between 2.0 and 6.0 A600 units per ml bacterial culture.
- Media: The isolation method is optimized for cultures grown in LB media; other rich media may require increased volumes of Suspension-, Lysis- and Neutralization Buffer, and an additional wash step
- Plasmid size: The isolation procedure is suitable for all plasmid sizes; lysates of larger constructs (up to 100 kb) should be cleared by filtration to avoid shearing
- Suspension Buffer/RNase A: To dissolve the lyophilized enzyme in Suspension Buffer, pipet 1 ml of Suspension Buffer (bottle 1, black cap) into the glass vial containing the lyophilized RNase (bottle 2, black cap). Reinsert the rubber stopper and invert the vial until all lyophilizate (including any that might stick to the rubber stopper) is dissolved. Transfer the dissolved enzyme back to the Suspension Buffer bottle (bottle 1). This is enough working solution for 60 Midi preps (isolation of up to 100 µg plasmid DNA/preparation)
- If preparing aliquots of the working solution, remember that the final concentration of RNase A in the working solution must be 100 g/ml. Reconstituted buffer is stable for 6 months is stored properly (+2 to +8℃)
- Neutralization Buffer: Before starting, cool it down to 4℃
- Elution Buffer: Before starting, warm up the buffer to 50℃
- Ethanol: Use 70% ethanol. Before starting, cool it down to 4℃

- **Procedure**

1. Centrifuge bacterial cells from 30 ml culture grown in LB medium by centrifuging for 10 min at 5000 rpm,4℃. Discard the supernatant. Carefully resuspend the pellet in 4 ml Suspension Buffer + RNase and mix well.

2. Add 4 ml Lysis Buffer to the suspension and mix gently by inverting the tube 6 times. Incubate 2-3 min at room temperature. To avoid shearing genomic DNA, do not vortex the suspension in Lysis Buffer. To prevent release of chromosomal DNA from the cell debris, do not incubate for more than 5 minutes.

3. Add 4 ml chilled Neutralization Buffer to the suspension. Immediately mix the suspension gently by inverting the tube 6 times until a homogenous suspension is formed. Incubate the tube 5 min on ice The solution becomes cloudy and a flocculent precipitate will form.

4. Clear the lysate by filtration. Put a folded filter into a funnel that has been inserted into a 50 ml plastic tube. Moisten the filter with a few drops of Equilibrium Buffer. Load the lysate onto the wet, folded filter and collect the flowthrough. The SDS precipitates with cellular debris when Neutralisation Buffer is added; this white precipitate should not be loaded onto the column. If the solution obtained after step 14 is not clear, remove the remaining precipitate by passing the solution over a folded filter.

5. Mount the sealing ring to the column to fix the column in the collection tube. Insert one column into one collection tube. Equilibrate the column with 2,5 ml Equilibration Buffer. Allow the column to empty by gravity flow. Discard the flowthrough.

6. Load the cleared lysate from step 4 onto the equilibrate column. Allow the column to empty by gravity flow. Discard the flowthrough.

7. Wash the column with 5 ml Wash Buffer. Allow the column to empty by gravity flow. Discard the flowthrough.

8. Repeat step 7 Discard flowthrough and collection tube.

9. Re-insert the column into a new collection tube Elute the plasmid with 5 ml prewarmed Elution Buffer (50℃) Allow the column to empty by gravity flow The collection flowthrough contains the plasmid Elute the plasmid again with the flowthrough Allow the column to empty by gravity flow Plasmid concentration is higher, than after only one elution.

10. Precipitate the eluted plasmid DNA with 3,6 ml isopropanol Total volume ~6,8 ml Divide the eluted plasmid into five 1,5 ml eppendorf tubes and one tube which contains 73 μl. Centrifuge immediately 30 min at 15000×g (rcf), +4℃. Carefully discard the supernatant.

11. Divide the 6 tubes into two groups. Wash the plasmid DNA with 1,5 ml chilled (+4℃) 70% ethanol in the first tube and afterwards wash the other two with the same ethanol. Wash the other group (3 tubes) the same way.Centrifuge 10 min at 15000×g (rcf), +4℃. Carefully remove ethanol from the tube with pipet tip.Air-dry the plasmid DNA pellet for 10 min.

12. Carefully redissolve the plasmid DNA pellet in 100-200 μl TE buffer.

13. Measure the concentration of DNA with NanoDrop.

Restriction digestion – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

BioBrick standard biological parts are flanked by well characterized upstream and downstream sequences which are technically not considered part of the BioBrick part (aka prefix and suffix). These up/down stream segments contain restriction sites for specific restriction enzymes, which allows for the simple creation of larger BioBrick parts by chaining together smaller ones in any desired order.

In the process of chaining biobrick parts together, the restriction sites between the two parts are removed, allowing the use of those restriction enzymes without breaking the new, larger BioBrick apart. To facilitate this assembly process, the BioBrick part itself should not contain any of these restriction sites.

One such type of assemblies is the “three antibiotic” assembly standard. This assembly begins with a restriction step.

- **Overview**

The following protocol contains detailed instructions on the restriction digestion step of the “three antibiotic” standard assembly. It starts with a medium amount of the two parts to be assembled and a medium quantity of the backbone that the parts will be assembled into. The result is a small amount of the insert part ready to be ligated into a linearized backbone.

Note: This protocol uses NEB High Fidelity restriction enzymes and buffers.

Note: One unit of restriction enzyme cuts 1ug of DNA in 1 hour between optimal conditions.

- **Materials**

- DNA sample
- appropriate restriction enzyme(s)/NEB
- Nuclease Free Water
- Buffers/NEB
- occasionally BSA
- PCR tubes
- crushed ice
- waterbath or thermocycler

- **Procedure**

1. Prepare the following mixes in 3 different PCR strips, work on ice.

Note: the restriction of Part A, Part B and the backbone at the same reaction is not mandatory and may be split over several reactions

basic components:

- DNA 3-5 ug
- Restriction enzyme(s): 0,5 - (0,5)ul
- NEBuffer (1-4): 1 ul
- (BSA: 0,1 ul, if one of the enzymes recquires it)
- Nuclease free water to 10 or 20 ul.

2. Resuspend with a pipette all the components to assure proper mixing

3. Incubate at 37℃ for 30 minutes

4. Run products on gel electrophoresis. (Nice bands on gel are made by 100-300ng of DNA).

5. Use PCR Purification Kit to get rid of salts and unnecessary reagents (this step is needed before every ligation)

- **Notes**

The volume of restriction enzymes used must not exceed 10% of the reaction volume (enzymes are dissolved in glycerol which damages the reaction).

The optimal total volume is 10 ul.

Before starting the reaction calculation it is vital to examine the enzymes information page in order to obtain:

Optimal working buffer -May cause a change in the procedure stated above

Optimal working temprature -May cause a change in the procedure stated above

- **References**

Sean C. Sleight, Bryan A. Bartley, Jane A. Lieviant, and Herbert M. Sauro "In-Fusion BioBrick assembly and re-engineering" Nucleic Acids Res. 2010 May; 38(8): 2624–2636.

DNA Ligation – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

The term recombinant DNA encapsulates the concept of recombining fragments of DNA from different sources into a new, and hopefully useful DNA molecule. Joining linear DNA fragments together with covalent bonds is called ligation. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another.

- **Owerview**

In a ligation process we put the DNA fragments to be ligated together, add T4 ligase enzyme with other supplements, and then incubate at 22,5℃.

-**Materials**

- PCR tubesized
- linearized DNA samples with appropriate ends (sticky or blunt but able to join)
- ligase buffer (with ATP)
- Nuclease Free Water
- waterbath
- crushed ice

-**Procedure**

1. Calculate the amuount of vector and insert by this equation:

vector mass is constant: 10 ng

insert mass(ng): 6x vector mass(ng)x insert bp/vector bp.

2. Put the appropriate amount of DNA to a tubeon ice, 1 ul Ligase Buffer (supplemented with ATP), 0,5 ul T4 ligase and fill the reaction volume to 10 ul.

3. Vortex, centrifuge shortly and incubate the mixure for 30 mins at 22,5℃ (room temp is also efficient).

4. Transform the mixture into competent cells to get the right clone with your DNA of interest.

NOTE: Heat inactivation will cause decrease in the number of colonies after transformation, because Ligase Buffer contains PEG which is damaged by heat and somehow don't let the DNA to get in the cell.

Gel electrophoresis – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

Gel electrophoresis is a technique used for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules using an electric field applied to a gel matrix. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. The measurement and analysis are mostly done with specialized software.

- **Overview**

After the preparation of the gel, we put DNA samples into the preformed wells with Loading Dye, than run by electric field.

-**Materials**

- agarose powder
- sterilized bottle
- 1x TAE solution
- microwave oven
- GelRed nucleic acid stain (non-teratogen, non-carcinogen, non-mutagen)
- laboratory scale
- 6y loading Dye (dextran/glycerol and bromothymol blue stain)
- 1 kb DNA ladder
- Gel electrophoresis apparatus

- **Procedure**

1. measure 1 g agarose (for 1% gel agarose).
2. put it into a sterilized bottle.
3. masure 100 ml 1x TAE.
4. put the bottle into the microwave (must not close the bottle totally), heat it still it will be fully clear.
5. Take the bottle out from the microwave with the plastic gripping.
6. Cold down the bottle in water bath until you can touch it.
7. Add 100 µl GelRed into the bottle (because 1000x must attenuate).
8. Shake gently the bottle still the red color disappears.
9. Put the liquid from the bottle into the gel tray (if there are any bubbles in it you can punch them with a tip)
10. After it became solid turn the gel rack to 90°.
11. Put 1x TAE into the gel tray still the level of line on the wall
12. Take out the comb
13. Put a ladder (3-6 µl) into the first hole
14. Into a new tube mix the loading dye(1 µl) and your sample(5 µl)

- **References**

Theriogenology. 2010 Sep 10 ,Huang HW, Su YF, Yao CT, Hung YC, Chen CC, Cheng CC, Li SS, Chang HW. "High-throughput gender identification of three Columbidae species using melting curve analysis"

PCR cleanup of the restricted DNA – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific background**

Some kits are available commercially to purify DNA or RNA from reactions like PCR, restriction digestion, dye labelling etc. Some are based on gel purification, to clean up DNA cut from agarose gel or on the well establisher spin column technique for liquid samples. Our team used this kit for the purification of mixes after restriction digestion to get rid of salts and enzymes of the solution (the rest went on a gel).

- **Overview**

The method used by out team were based on the well established spin column approach including binding, washing and elution steps for purification.

- **Materials**

- kit contents
- restriction mix
- table-top centrifuge

- **Procedure**

For detailed steps see: [[3]]

Protocols from tissue culture room

Preparation of DMEM medium and PEI solution – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

I. The Medium

Eukaryotic cells, derived from multicellular animal eukaryotes, can be maintained in culturing media. Aside from temperature and gas mixture, the most commonly varied factor in eucaryotic culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The type of the medium used depends on the type of the cell line.

II. PEI solution

PEI (10 mM) is a cationic polymer used for PEI-mediated transfection.

- **Overview**

I. The Medium

For culturing cells and for ligand treatment we use DMEM (Dulbecco's Modified Eagle's Medium) completed with 10% FBS, 1% Penicillin-streptomycin and 2% L-Glutamine.

For transfections we use DMEM medium without FBS, but other constitutives are included.

The preparation procedure takes 40-50 minutes including the FBS melting time (stored in 50 ml aliquots at -20℃).

II. Preparing PEI solution

The reconstitution consists of 3 steps including dissolving PEI, adjusting pH and sterilization by filter sterilizer.

- **Materials**

I. For the Medium:

500 ml Basic DMEM (Dulbecco's modified Eagle Medium) ordered from Sigma (D5671)

50 ml FBS (Foetal Bovine Serum)

10 ml 200 mM L-Glutamine ordered from Sigma(G7513)

5 ml 100x Penicillin-Streptomycin ordered from Sigma(P4333)

Serological pipettes, Pipettor

Sterile laminar flow box, kimwipes, 70% ethanol sprinkle bottle

II. Preparing PEI solution:

Pipette, pipet tip, glass, analitical scale

MQ water (double distilled, sterilized)

MW=25.000 Pei (ALDRICH 408727)

15, 50 ml tubes

pH measuring electrodes

HCl-solution

Sterile filter – with 0,2 um diameter pores

- **Procedure**

I. Preparing Medium:

1. Prepare the laminar flow box: turn on the ventillation, wait for 15 minutes, clean the bottom and the glass of the hood with 70% alcohol, and wipe it with kimwipes.

2. Put the Basic DMEM solution, the FBS, Penicillin Streptomycin and the L-Glutamine solutions into the 37℃ waterbath and wait for 30 minutes (The FBS is melting slowly)

3. Take out the melted solutions from the waterbath, wipe them, squeeze them down with 70% ethanol and load them into the hood as well as the serological pipettes and the Pipettor (squeezed down)

4. Put these amounts into the basic DMEM by using a pipettor and serological pipettes:

A. For 10% FBS medium:

Basic DMEM: 500 mL

FBS: 50 mL

L-Glutamine: 10 mL

Penicillin-Streptomycin: 5 mL

B. For Serum free medium:

Basic DMEM: 500 mL

FBS: -

L-Glutamine: 10 mL

Penicillin-Streptomycin: 5 mL

5. Invert the bottle, mark it with your name, actual date and with the constituents.

6. Put the bottle to 4℃, clean up after yourself.

II. Preparing PEI solution:

1. Dissolve 4,5 mg pure PEI in 8 ml MQ water, mix well (maybe it takes one day for proper dissolvation)

2. Neutralize the solution with HCl. The final pH should be between pH 6,5-7,5

3. Adjust the volume to 10 ml

4. Filter sterilize through 0,2 um pores

5. Store the solution at 4℃.

- **References**

Yves Durocher, Sylvie Perret, and Amine Kamen,"High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells". Nucleic Acids Res. 2002 January 15; 30(2)

Cell subculturing (also referred as "passaging") – Debrecen Hungary – 2011

http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

Cell passaging or splitting is a technique that enables an individual to keep cells alive and growing under cultured conditions for extended periods of time. Cells should be passed when they are 90%-100% confluent. You have to do the cell passage on every second to fourth day (i.e. on every Monday, Wednesday and Friday).

After reaching the confluency, the cells do not get enough nutrients and do not have enough place where they can extend. The colour of the medium switches from reddish-pink to orange or yellow which shows acidic metabolic products.

- **Notes**

While working in the Cell culture lab, always follow the rules of the laboratory. You should wear a lab coat, use gloves and keep the sterile box as clean as possible.

It is advisable not to take your mobile phone into the cell culture lab.

Never bring bacterial samples into the cell culture lab!

Carefully separate dangerous waste from communal waste.

- **Materials**

For COS1-cells, in 10cm Petri dishes:

I. Cell passaging

- Ethanol squirt bottle
- paper towels
- 5 mL and 10 mL sterile pipets
- confluent cells in Petri dishes
- Medium (DMEM) with 10% (50ml) serum and antibiotics
- Trypsin-EDTA
- 1% PBS (Phosphate Buffered Saline)

- Pasteur Pipettes+ vacuum for aspirating the used medium
- 15 mL centrifuge tube
- Petri dishes
- Automatic pipettors
- gloves
- 37℃ water-bath
- 37° thermostat
- laminar box
- tube holders

- **Procedure**

I. Cell Passaging for adherent culture (also called Splitting, subculturing):

1. Warm media, trypsin-EDTA and PBS in 37℃ waterbath

2. Check cells in 10 cm Petri dish under Phase-contrast microscope to confirm that the cells are 90%-100% confluent

3. Clean hood with 70% alcohol

4. Sterilize all materials, bottles, etc. which are loaded into the hood. Spray hands with ethanol. Sterile pipets may be placed in the hood directly

5. Spray hands with ethanol. Remove Petri dishes from the incubator and quickly place them in the hood. (Do not spray flasks with ethanol)

6. Attach a Pasteur pipette to vacuum. Turn on vacuum system by opening vacuum valve in hood

7. Using the empty liquid media covering cells. Be careful to not touch the pipet to anything outside of the Petri dis

8. Add 2-3 mL of PBS to Petri dish. Lightly swirl PBS on base of Petri dish. Aspirate PBS from dishes

9. Add 2 mL trypsin-EDTA to Petri dish. Lightly swish trypsin

10. Place flask in incubator until detached (2-3 mins, epends on the cell-type)

11. Remove cells from incubator. Tap side of Petri dish on hard surface or your hand. Repeat several times. Visually check to ensure lumps of cells are dispersed

12. Check cells under microscope to confirm that cells are detached from the surface

13. Add 5 mL of media to dilute trypsin. Media contains antitrypsin. (Note: The liquid suspension now contains the cells.) Carefully re-suspend cells by using pipettor and pipettes

14. Aliquot appropriate volume of cell suspension into freshly prepared Petri dishes with media (The total volume in a Petri dish is 10 mL; 2mL Trypsin-EDTA, 5 ml DMEM for trypsin dilution, 3 further mL of DMEM)

15. Replace media and cells to mix. Place Petri dishes in incubator

16. Turn off aspiration

17. Dispose of liquid and solid biohazards wastes properly

18.Clean hood with ethanol. Spray ethanol liberally over surfaces and wipe clean with kimwipe

Transfection with FuGENE6 – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

This protocol is written by our team members for nuclear receptor transfections, but can be used for any kind of transfections as well.

- **Scientific Background**

FuGENE 6 transfection reagent is a multi-component lipid reagent that forms a complex with the DNA, then transports it into animal cells. FuGENE 6 transfection is used as standard method in many different laboratories due to its simple methodology, low cytotoxicity, and ability to provide high transfection efficiency even in the presence of serum. Overview

The transfection is divided into two main steps: 1. plating COS1 cells into glass cell culture vessel 2. the transfection itself

The aim is to get cells of 50-80% confluency in the chosen vessel for the day of transfection.

- **Required materials**

- COS1 cells in a T75 flask (regularly passaged, proliferating well - best in the log-growth phase) - Trypsin/EDTA solution - 1 x PBS solution - 10% FBS containing DMEM medium - LAB-TEK glass chamber slide with 8 chambers - 15 ml centrifuge tube, tube holders - Bürker chamber, pipettor, serological pipettes, pipettes, pipet tips, Pasteur pipettes - sterile laminar air flow box, 37℃ incubator, 37℃ waterbath - 70% ethanol, kimwipes

- **Procedure**

I. *Plating*

1. Prepare the sterile box: Open the sterile laminar box (Hood), turn on the ventillator and wait for 15 minutes to reach the optimal level of sterility (0,45 um filter). PUT ON YOUR GLOVES, wipe the box with 70% alcohol.

2. Prewarm the DMEM medium, Trypsin/EDTA and the 1x PBS in the 37℃ waterbath (10-15 minutes)

3. Take the DMEM, Trypsin-EDTA and PBS and squirt the tubes and bottles with alcohol beforeyou put them in the sterile box. Put the pipettor and the tube holder into the box (after you sprayed them down with 70% ethanol), and load the serlogical pipettes directly,without spraying down into the box.

4. Spray hands with ethanol. Remove the flask from the incubator and quickly place in hood. Fire-sterilize the neck of the flask. (Do not spray flasks with ethanol).

5. Attach a Pasteur pipette to vacuum,turn on vacuum system by opening vacuum valve in hood. You should fire-sterilize the end of the pipette,after this step do not touch anything outside the flask. Aspirate the used medium from the cells by touching the bottom-side corner of the flask with the Pasteur-pipette.

6. Washing step:Add 2-3 mL of 1x PBS to flask by using pipettor and a serological pipette (Release the PBS onto the side of the flask, do not push the solution out directly onto the cells because they can come up easily). Lightly swirl PBS on base of the flask. Aspirate PBS from flasks by using a Pasteur pipette and vacuum.

7. Add 2 mL trypsin-EDTA to Flask. You can release the solution directly onto the cells, from now it does not matter if they come up. Lightly swish trypsin.

8. Place flask in 37℃ incubator until detached (3-5 minutes for COS1 cells, depending on the temperature of the Trypsin- opt. temp: 37℃)

9. Remove cells from incubator. Tap side of the flask on hard surface of your hand. Repeat several times. Visually check to ensure lumps of cells are dispersed.

10. Check cells under phase-contrast microscope to confirm that cells are detached from the surface.

11. Put the falsk back to the sterile box, add 4 ml of 10% FBS containing DMEM medium to dilute trypsin (you can change the dilution level depending on the cell number, in order to be able to count the cells easier). Medium contains antitrypsin.(Note: The liquid suspension now contains the cells.)

12. Carefully resuspend cells by using pipettor and serological pipettes. You can repeat this step until you get individual floating cells (microscope check needed).Put the cells into a 50 ml tube, for easier handling.

13. Prepare the Bürker chamber and do a cell counting:

- Cell counting in a Bürker chamber:

1) At first, clean the chamber with alcohol and water, put the thin glass slide onto the thicker slide, compress them together by using the metal screw.

2) Resuspend the cell suspension (for even distribution) in which you want to do the cell counting.

3) Take 10 ul from the cell suspension (using sterile tip),

inject into the chamber(marked with arrow) by touching the border of the two glasses with the pipet tip.

4) Place the chamber under the phase-contrast microscope and try to find the lines.

- count inside 3 big squares

- then we take the average of the 3 big squares, and it will give us the number of the cells in 0,1 ul we take it 10 in the factor of 4 times and it will give us the cell number in 1 ml cell suspension.

NOTE: Don't forget that the cell suspenson in the chamber is now not sterile, don't put it back into the cell solution. Clean the chamber with alcohol and water.

1. To reach the appropriate confluency the day after plating, we put 50.000 cells into each well. For an 8 well chamber, if we calculate with 12 wells (because the volume loss), we put into a 15 ml centrifuge tube: - 50.000 x 12 = 600.000 cells [in milliliter: counted cell number in 1 ml / 600.000 cells] - we fill the cell suspension up to 8 x 300 ul = 2, 5 ml with 10% FBS DMEM (total volume of the wells are 300 ul)

2. With a 1 ml pipette put 300 ul from this suspension into each well. Sometimes invert the cell suspension containing tube (the cells decent). After you finished the plate, swirl it circularly.

3. Incubate the cells for 1 day at 37℃, 5% CO2.

II. *FuGENE 6 transfection:*

The goal is to introduce foreign plasmid DNA into the plated COS1 cells 24 hours after the plating.

Materials required: - Plated cells in a LAB-TEK 8 well glass chamber slide (with 50-80% confluency) - FBS/antibiotics/other additives Free DMEM medium - Sterile FuGENE 6 transfection reagent in a tightly capped glass vial - Plasmids on ice, in known concentrations: Beta-Gal (normalizer plasmid), Luciferase (tracer), Nuclear receptor, VDR- as negative control, the plasmids are solved in sterile TE-buffer - 1,5 ml sterile Eppendorf tube, pipettes and tips - Sterile laminar air flow box, 37℃ incubator, 37℃ waterbath - 70% ethanol squirt bottle, kimwipes

Steps:

1. Prepare the sterile box: Open the sterile laminar box (Hood), turn on the ventillator and wait for 15 minutes to reach the optimal level of sterility (0,45 um filter). PUT ON YOUR GLOVES, spray down the base of the box with 70% ethanol, and wipe down with kimwipes.

2. Spray down your hands, the DMEM medium, the glass Reagent vial and the pipettes and tips with 70% ethanol. Put these and the plasmids and the eppendorf tube into the sterile box.

3. Transfection mix reconstitution:

3.1 Invert the room temp. FuGEGE 6 Transfection Reagent glass vial 2-3 times to distribute the components.
3.2 Dilute the FuGENE reagent with Serume/antibiotics/other additives free DMEM medium – the order and manner of addition is critical: Label a 1,5 ml eppendorf tube. Pipet 75 ul FBS/antibiotics/other additives Free DMEM into the eppendorf tube. Pipet 6 ul FuGENE 6 Transfection Reagent directly into the medium, without

allowing contact between the plastic wall and the undiluted reagent. 3.3 Vortex the mix for 1 second. 3.4 Incubate the mix for 5 minutes at room temperature. 3.5 Add 500 ng from each of the three plasmids (receptor, Luciferase, beta – Gal.) into the diluted FuGENE 6 transfection reagent. 3.6 Vortex the Transfection Reagent:Plasmid mixture for 1 second. 3.7 Incubate the mixture for 20 minutes at room temperature.

4. Remove glass chamber slide with plated cells from the incubator, place in the sterile box without spraying down with ethanol.

5. Note: We don't need to remove the culturing medium (10% FBS containing DMEM) from the cells, it does not have any effect on the transfection efficiency. Add 9 ul Transfection mix in a dropwise manner to each well. Swirl the chamber slide to ensure distribution over the entire surface.

6. Put on the cap of the slide chamber. Return the cells to the 37℃ incubator until the assay for gene expression is to be performed. Note: it is not necessary to remove and replace the transfection mixture-containing medium with fresh medium until the assay, only if you used FBS Free medium during the whole experiment (to avoid the cell starvation).

7. Clean up after yourself, place the FuGENE reagent to +2 - +8 ℃ and be sure if the cap is tightly turned on the Reagent.

NOTES:

- store the reagent at +2 - +8, with the lid very tightly closed, in the original glass vial. - Do not allow the reagent to contact plastic walls (pipet directly into serum free medium) to keep the maximal biological activity. - Do not use siliconized pipet tips and tubes. - To prepare transfection complexes for larger experiments or parallel experiments, proportionally increase the quantity according to the total surface area of the cell culture vessel being used. (ul FuGENE Reagent: ug DNA = 4:1, the used vessel in this case has a 79,21 cm2 surface area)

- **References**

1. Horbinski C, Stachowiak MK, Higgins D, Finnegan SG. Polyethyleneimine-mediated transfection of cultured postmitotic neurons from rat sympathetic ganglia and adult human retina. .BMC Neurosci. 2001;2:2.

2. Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D: Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells.J Biol Chem 1998, 273:7507-7511

Ligand treatment after transfection – Debrecen Hungary – 2011 http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

- **Scientific Background**

Ligand treatment is a procedure when we add the appropriate ligand (specific ligand, oil sample dairy product - in short the ligand of interest) to the nuclear receptor (NR, transcription factor). The NR got into the cells through a previous transfection step. After treatment the ligand-binded NRs will dimerize and bind to the DNA

at specific Nuclear receptor Response Elements and this will promote the gene expression of the downstream gene. In further examinations we detect the expression level of the target gene by Luciferase assay.

- **Overview**

The target cells in our case are COS1 cells. Performing the protocol from the beginning to the end takes no more than 1 hour, and the following steps are included:

1. Preparation of the ligand solutions

2. Removal of the transfection medium

The goal is to get rid of the transfection medium from the COS1 cells 5-7 hours after transfection, because:

- PEI can damage the cells

- FBS Free medium used for transfection can cause starvation

3. Cell refeeding with Ligand-containing medium

Our aim is to refeed the cells with 10% FBS containing DMEM and also give appropriate amounts of Ligand in one step. The 10% FBS in the medium will provide the necessary proteins and other molecules for proliferation and for life functions. The ligand will activate the nuclear receptor and will promote gene expression.

Preparation of the ligand solutions

- **Materials**

- Nuclear receptor-transfected cells in a 48-well plate
- 10% FBS containing DMEM medium
- Ligand solution in known concentration
- 50 ml, 15 ml tubes
- Pasteur pipettes, pipettor, serological pipettes, pipettes, pipet tips
- Sterile laminar air flow box with flame and vacuum system, 37℃ waterbath, 37℃ incubator, 70% ethanol squirt bottles

- **Procedure**

- in the case of 1-type receptor transfection (GAL4PPARg):

We use different concentrations of ligand, so as to make a dose-response curve in the subsequent measuring process:

10 µM: 200 * (x/10) µL Ligand up to 200µL with 10% DMEM /per well/

2 µM: 200 * (x/2) µL Ligand up to 200µL with 10%FBS DMEM /per well/

0,4 µM: 200 * (x/0,4) µL Ligand up to 200µL with 10%FBS DMEM /per well/

0,08 μM: 200 * (x/0,08) μL Ligand up to 200μL with 10%FBS DMEM /per well/

0,016 μM: 200 * (x/0,016) μL Ligand up to 200μL with 10%FBS DMEM /per well/

0 μM(negative control): 1 μL DMSO-ethanol(1:1) up to 200μL with 10%FBS DMEM /per well/

if x = original ligand concentartion[mM]

we use 48 well plates

DMSO-ethanol is the dissolvent of the ligand

Prepare the Ligand dilutions:

- We use 6 wells for 1 concentration / plate
- the total concentration of one well is 200 μL
- For this reason, from 1 concentration we need 6x200=1200 μL
- NOTE: these dilutions are examples, and can vary depending on the system itself.

We are doing serial dilutions (5-fold-dilutions) in 15 ml tubes:

1st dilution (10μM): I calculate with 5000 ul, put 5000*(x/10) μL Ligand solution and fill it up to 5000 μL with 10% FBS containing DMEM. Mix it with pipetting.

2nd dilution (2μM): 1 ml from the 1st dilution + 4 ml DMEM 10%

3rd dilution (0,4μM): 1 ml from the 2nd dilution + 4 ml DMEM 10%

4th dilution (0,08μM): 1 ml from the 3rd dilution + 4 ml DMEM 10%

5th dilution (0,016μM): 1 ml from the 4th dilution + 4 ml DMEM 10%

6th solution (0 μM): 6 μL-ethanol 1:1 solution + 4,994 ml DMEM 10%

(you can use serological pipettes and pipettor, or micropipettes with sterile pipette tips.

these solutions will be enough for three 48-well plates, if you have less than 3 plates you can store the tubes at +4℃ and use them next time.)

- in the case of a 2-type receptor transfection:

The receptor to be activated is Vitamin D Receptor (VDR) and the Ligand is Vitamin D. We use 10e-8 μM Vitamin D. We treat 24 wells only, the other 24 will be negative controls.

-Prepare the Vitamin D solution in a 15 ml tube:

Fill 2000 / (original cc. of the ligand solution [mM]/10e-8) μL ligand solution up to 2000 μL with 10% FBS containing DMEM. Use micropipettes and sterile pipet tips. Removal of the transfection medium

- **Materials**
- Nuclear receptor-transfected cells in a 48-well plate
- Pasteur pipettes

- Sterile box with flame and vacuum system Procedure

- **Steps:**

1. Prepare the hood : put on your gloves, turn on the ventillation (it needs 15 minutes to filter the air in the box), clean the inner space of the hood with 70% ethanol. Spray hands with ethanol.

2. Place the plate into the hood.Turn on the vacuum system, insert the Pasteur pipette into the vacuum tube. Fire-sterilize the Pasteur pipette.

3. Aspire the used medium from all of the wells, pay attention so as not to aspire the cells (touch only the bottom-side of the wells).

4. Put back the cap of the plate, we don’t want the cells to go dry. Cell refeeding with Ligand-containing medium

- **Materials**

- Ligand solutions prepared in point 1.

- 48 well plate without medium (point 2.)

- 10% FBS containing medium

- Repeating pipet and tips

- 37℃ incubator, 37℃ waterbath, Sterile laminar air flow box, 70% ethanol squirt bottles Procedure

- **Steps:**

1. Warm up the Ligand solutions for 1- receptor transfected cells and the Ligand solution + 10% FBS DMEM for 2-receptor transfected cells

2. You use the same hood which you prapared: put everything what is needed into the hood after spraying down with 70% alcohol.

3. Take down the cap of the plate. Vortex the Ligand solutions, after that put 200-200 ul from these solutions into the adequate wells quickly (in other case the cells will go dry). Release the solutions onto the wall of the wells

After 1-receptor transfection - by using a 200 μl or a 1 ml pipet with sterile tips

10 mM: A1-A3 and E1-E3

2 mM: A4-A6 and E4-E6

0,4 mM: B1-B3 and F1-F3

0,08 mM: B4-B6 and F4-F6

0,016 mM: C1-C3 and G1-G3

0 mM: C4-C6 and G4-G6

After 2-receptor transfection – by using a repeating pipet

Ligand treated /well A1-D6/ : 200 ul 10e-8 mM Ligand- DMEM solution

Non-treated /well E1-H6/ : 200 ul "0 μM" solution

4. Put the „treated" COS1 cells into the 37℃ incubator for 2 days, after 2 days comes the Luciferase assay to make sure that the transfection worked and to measure the Nuclear Receptor's response to the ligand.

5. Clean the surface

Measurement of Luciferase reporter – Debrecen Hungary – 2011

http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

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- **Scientific Background**

Luciferase assay / cotransfection assay is a laboratory technique used by our team to measure the expression of the reporter firefly luciferase enzyme in order to study the effect of nuclear receptors/zinc fingers in different conditions. Luciferase substrate is transformed and emits light that can be measured (directly proportional to the expression of the enzyme and hence to the upregulation by Nuclear receptors/zinc fingers. The luciferase assay system is an extremely sensitive and rapid method. Linear results are seen over at least eight orders of magnitude of enzyme concentration. Generally, 100-fold greater sensitivity can be achieved over the chloramphenicol acetyltransferase (CAT) assay.

- **Overview**

After transfection with nuclear receptors and/or zinc fingers and ligand treatment we measure the expression of the firefly luciferase reporter (MH-100) which has a response element to a specific receptor upstream the protein coding sequence.

- **Materials**

- Transfected and ligand treated cells is plates 2 days after ligand treatment
- Luciferase plates (96 wells, transparent and non transparent plates)
- Deep freezer of -70 degrees
- luciferase substrate (home made, see below)
- multi-channel pipettes
- X-gal
- Wallace VICTOR plate reader

- **Procedure**

I. *Sample preparation*

1. 2 days after ligand treatment the used medium on the cells has to be discarded (Pasteur pipettes and vacuum). 2. Wash the cells with PBS (150 ul/well), discard with pasteur pipette and vacuum. 3. Wash again with 150 ul 1xPBS. 4. Add 200 ul lysis buffer to the washed cells 5. Put the plate on a plate shaker for 2 hours (chemical lysis) 6. Place the plate to -70℃ for 30 minutes (physical lysis) 7. Divide the lysed cells into 2 plates, these will be the plates for beta-Gal measurement (for normalisation) and for luciferase activity measurement.

– Measurement of β-GAL:

1. Put 80 ul lysate from each well to the transparent β-GAL measurement plate. 2. Put 100 ul Luciferase substrate to the cells. 3. Wait for 3-5 minutes and the wells will become visibly yellow. 4. Measure the intensity with VICTOR plate reader

– Measurement of Luciferase:

1. Put 20ul lysate from each well into the luciferase measurement plate 2. Put the plate into the VICTOR plate reader 3. Place the luciferase reagent into the VICTOR plate reader (dark vial). The instrument will add the substrate automatically before plate reding. 4. Measure the intensity of light emission.

Analize the data.

Prepare substrate solutions:

• β-GAL substrate:

– 10ml β-GAL buffer – 20mg ONPG (light sensitive) – 35μl mercaptoethanol

NOTE: We always have to use a BLANK plate which contains lysis buffer triplicates.

• Normalisated luciferase assay measurement:

NLa=((Luc act-Luc. BASE)*10)/((β-GAL-β-GAL BASE)/TIME)

NLa= Normalised Luciferase Assay Luc act= luciferase activity Luc BASE= luciferase BASE

II. *Measurement vith VICTOR Plate Reader*

- **Scientific Background**

The Wallac 1420 VICTOR2 is a multilabel, multitask plate reader designed to support the future demands of industrial and academic laboratories for multiple assay technologies on a single platform. An extended version of the successful Wallac VICTOR multilabel reader, the VICTOR2 allows immediate access to more than 10 counting modes, covering all of the main nonradioactive counting technologies.

VICTOR2 accepts all types of microtitration plates with between 1 and 864 (1536 fluorescence and time-resolved fluorescence) wells, as well as Petri dishes, slides, filters and Terasaki plates. All models include scanning, shaking and kinetics modules. To support individual applications in cell or molecular biology, binding studies, environmental and food testing, toxicology and drug screening, VICTOR2 can also be supplied with dispenser module, temperature control, bottom reading, high density reading and various other options.

- **Procedure**

1. Pepare the following approximately half an hour before you start the B-gal and the Luciferase measurements

Switch on the machine VICTOR

Turn on the computer

Log in with your username

Open the program WALLAC 1420 WORKSTATION

2. Open main controll panel

Open the TOOLS tab and choose EXPLORER, after it you choose the BRAZDA* map

Open the corresponding file to measure the B-gal absorbance

choose the number of wells

save settings

3. go back to the main controll panel and choose the file programm within the PROTOCOL tab

4. Put the plate into the machine in right direction

5. Start the measurement

you may check live the measured absorbance values, if you click to the LIVE DISPLAY tab

6. To measure Luciferase activity, follow steps 1-4 with the corresponding file. Before step 4. add the Luciferase substrate

7. Use the icon which is a part of the main control panel for Dispenser maintenance

8. Appoint fill with the volume 500μL

9. Start measurement You may check live the measured Luciferase values, if you click to the LIVE DISPLAY tab

10. After the measurements empty the machine from the substrate

11. To wash the machine with water have to appoint flush (and the volume 500μL after it click ok) and then empty again

11. Turn off the VICTOR and then the computer.

Apoptosis – Debrecen Hungary – 2011

http://2011.igem.org/Team:Debrecen_Hungary/Protocols#Team.27s_notebook

DAPI staining

- **Scientific Background**

DAPI or 4',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to A-T rich regions in DNA. It is used extensively in fluorescence microscopy. DAPI can pass through an intact cell membrane therefore it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells and therefore the effectiveness of the stain is lower.

When bound to double-stranded DNA DAPI has an absorption maximum at a wavelength of 358 nm (ultraviolet) and its emission maximum is at 461 nm (blue). Therefore for fluorescence microscopy DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. The emission peak is fairly broad DAPI

will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA.

- **Overwiew**

In order to vizualize the nuclear morphology of living and dying cells the nucleus of the cells were stained with DAPI which intercalates to the double stranded DNA. Nuclear fragmentation can be seen in the apoptotic cells.

- **Procedure**

1. Washing cells which are attached to the surface of the plate with PBS for 5 minutes at room temperature.

2. Fixating cells with 4% paraformaldehyde for 10 minutes on ice.

3. Washing cells which are attached to the surface of the plate with PBS for 5 minutes at room temperature. Fixed cells can be stored at 4 C.

4. Stain cells with DAPI in a concentration of 1 ng/ml for 10 minutes at room temperature in the dark.

5. Washing cells which are attached to the surface of the plate with PBS for 5 minutes at room temperature.

6. Vizualizing the cells by fluorescent microscopy.

Amplification of DNA – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- Polymerase Chain Reaction : A method for amplifying a section of DNA.
- Colony PCR : PCR with cells as a template. Useful for checking the length of an insert in an introduced plasmid.

Analysis of DNA – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- Gel Electrophoresis : A method used to separate DNA fragments of different sizes.
- Gel Extraction of DNA : A technique used to isolate a desired fragment of intact DNA from an agarose gel following agarose gel electrophoresis.
- Rescue Precipitation of DNA : Creating a clean DNA solution after dissolving agarose gel.
- Restriction Enzyme Digestion : A method for creating a restriction map of a plasmid.

Preparation of DNA Constructs – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- Primer Design : Some general guidelines on how to design successful primers.
- Gibson Assembly : An extremely powerful technique for joining multiple, arbitrary DNA sequences in one step, compatible with standard assembly.

Transformation of Bacterial Cells – Cambridge – 2011
http://2011.igem.org/Team:Cambridge#/Protocols

- Making Electro-Competent Bacterial Cells : The methods required to make various cells competent.
- Transformation of E.coli : A simple method of transforming competent E.coli with your DNA of choice.
- Tranformation of B.subtilis : A technique used to introduce foreign DNA into Bacillus cells.
- Transformation of E.coli by Electroporation: A technique for rapidly inserting DNA into cells, typically plasmid DNA, providing the cells are made competent in the correct manner.

Bacterial Cultures – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- E.coli Cell Culture : A method for growing a cell culture in liquid medium.
- Glycerol Stocks: A method of storing E.coli cells preserving their viability.

Extraction of DNA – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- MiniPrep : Extracting DNA from bacterial cells.
- Extraction of Genomic DNA from Squid : Two methods to extract genomic DNA from squid tissue.
- Extraction of DNA from Filter Paper : Extraction of DNA from filter paper which is a safe way of shipping DNA.

Microscopy – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- Confocal Microscopy : A method to visualise reflectins from squid samples.
- Slide Preparation for Confocal Microscopy : A method of growing a monolayer of bacterial cells on a slide to aid their microscopic viewing.
- Trypsinisation : A method of dispersing cells from tissue, for microscopy.

Protein Purification – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- Buffer Preparation : Methods to prepare the various buffers used to purify his-tagged reflectin from inclusion bodies in E. coli.
- Inclusion Body Prep : Isolation of insoluble inclusion bodies of recombinant proteins.
- His-Trap Protein Purification : A method to purify reflectin from E. coli lysate using an affinity column.
- Acetone Precipitation of Proteins : A method to concentrate solutions of protein.
- Ethanol Precipitation of Proteins : A method to concentrate solutions of protein.
- Chloroform/Methanol Precipitation of Proteins : A method to concentrate solutions of protein whilst removing salts and detergents.
- Dialysis of Proteins : A method for removing salts, urea and contaminants by the use of a semi-permeable membrane and a concentration gradient.
- Norgen Proteospin Inclusion Body Prep : A proprietary kit.

Thin Film Preparation – Cambridge – 2011 http://2011.igem.org/Team:Cambridge#/Protocols

- Substrate Preparation:How to prepare a substrate for flow coating and spin coating.
- Spin Coating to Make a Thin Film: Our Spin Coating Protocol.
- Flow Coating: How to flow coat a thin film.

- Altering Substrate Surface Chemistry: Various methods to alter the surface chemistry of silicon and PDMS to achieve better wetting and thin film production

Gel Electrophoresis by SDS PAGE – Cambridge – 2011
http://2011.igem.org/Team:Cambridge#/Protocols

- Protein Identification by SDS PAGE: A method used to separate polypeptides of different lengths.

PCR (PrimeSTAR® HS DNA Polymerase) – Tokyo Metropolitan – 2011
http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook

- Ice
- Micro pipette
- Thermal cycler
- PCR Tube
- Micro Tube
- DW
- 5× PCR Buffer
- dNTP (2.5mM)
- Primer(Forward & Reverse) (20μM)
- Template DNA
- PrimeSTAR® HS DNA Polymerase (2.5U/μl)

1. Add following components on ice.
    - DW
    - 5× PCR Buffer
    - dNTP (2.5mM)
    - Primer(Forward & Reverse) (20μM)
    - Template DNA
    - PrimeSTAR® HS DNA Polymerase (2.5U/μl)
2. Dispense PCR solution to PCR tubes.
3. Set PCR tubes on thermal cycler
4. Run PCR

Electrophoresis – Tokyo Metropolitan – 2011
http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook

- Gel
- Electrophoresis bath
- Parafilm
- Micro pipette
- TAE Buffer
- 6× Loading Buffer
- DW

1. Set gel in a gel tank. Pour the TAE Buffer into the gel tank.
    - Loading Buffer
    - DNA solution
    - DW
2. Mix these components on a Parafilm by pipetting.
3. Pour the mixture sample into well.
4. Switch on the power-source and run the gel at 100V for 20min.
5. Observe the band by exposing ultraviolet radiation

Ethanol precipitation – Tokyo Metropolitan – 2011
http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook

- Centrifuge
- Micro Tube
- Micro pipette
- Aspirator
- 99.5%Ethanol
- 3.0M Sodium acetate (pH5.2)
- TE Buffer

1. Add 0.1 volume 3M Sodium acetate to the nucleic acid sample and vortex.
2. Add 4 μl of Dr. GenTLETM Precipitation Carrier and vortex.
3. Add 2.5 volumes of ethanol and vortex.
4. Centrifuge at 12,000 rpm at 4˚C for 15 min.
5. Discard the supernatant.
6. Rinse the pellet with 70% ethanol and centrifuge again at 12,000 rpm at 4˚C for 5 min.
7. Discard the supernatant and dry.
8. Dissolve the pellet in sterilized water or TE buffer.

Plasmid Extraction – Tokyo Metropolitan – 2011
http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook

- Ice
- Micro Tube
- Micro pipette
- Centrifuge
- 50mM glucose ; 10mM EDTA ; 25ml Tris-HCl (pH 8.0)
- 5N NaOH
- 10% SDS
- 3M Potassium Acetate (pH 4.8)

1. Centrifuge the E-coli pre-culture on 12,000 rpm for 5min, and then discard the supernatant.
2. Add 100µl of the solution (50㎜ glucose; 10mM EDTA; 25ml Tris-HCl(pH 8.0)), and mix it gently
3. Add 200µl of the 0.2N NaOH; 1% SDS, and mix it. Incubate on ice for 5min.
4. Add 150µl of 3M Potassium Acetate (pH 4.8, 5M Acetic acid; 3M Potassium), and mix it. Incubate #on ice for 5min.
5. Centrifuge on 12,000 rpm for 5min.
6. Take supernatant into Microtube.

Digestion – Tokyo Metropolitan – 2011
http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook

- Micro Tube
- Micro pipette
- Heat Block or Water bus
- Digest Enzyme
- 10×Buffer
- DW

1. Add below components into a tube and mix them.
    - DNA solution
    - DW
    - 10× Buffer
    - Digest enzyme

2. Incubate at 37°C, from 2h to 16h

Ligation – Tokyo Metropolitan – 2011 **http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook**

- Micro Tube
- Micro pipette
- Heat Block
- T4 Ligase
- 10× Buffer
- DW

1. Add below components into a tube and mix them.
    - DNA solution 1
    - DNA solution 2
    - DW
    - 10× Buffer
    - T4 Ligase
2. Incubate at 16°C, from 30min to 1h

Transformation – Tokyo Metropolitan – 2011 **http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook**

- Micro pipette
- Heat Block or Water bus
- ECOS™ Competent E. coli
- Plate (Antibiotic)
- Spreader

1. Keep competent cells on ice to thaw it.
2. Add plasmid DNA into E.coli cells.
3. Incubate on ice for 5 min.
4. Put tubes into water bath at 42℃ for 45 seconds.
5. Put tubes back on ice for 2 minutes.
6. Add four times volume of LB (with no antibiotic added). Incubate tubes for 30 min at 37℃.
7. Spread about 100 ul of the resulting culture on LB plates

8. Incubate overnight.

Colony PCR(Takara EX® Taq) – Tokyo Metropolitan – 2011 **http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook**

- Plate
- Ice
- Micro pipette
- Thermal cycler
- Burner
- 70%EtOH
- Toothpick
- PCRTube
- Micro Tube
- Spreader
- DW
- 10× PCR Buffer
- dNTP (2.5mM)
- primer(Forward & Reverse) (20μM)
- Taq Polymerase

1. Add the all following components on ice.
   - DW
   - 10× PCR Buffer
   - dNTP (2.5mM)
   - Primer(Forward & Reverse) (20μM)
   - Template DNA
   - Taq Polymerase
2. Dispense PCR solution to PCR tubes.
3. Pick up a colony and put it into PCR tube.then inoculate to master plate.
4. Set PCR tubes on thermal cycler
5. Run PCR

Making Medium for Culture – Tokyo Metropolitan – 2011 **http://2011.igem.org/Team:Tokyo_Metropolitan/Notebook**

- Erlenmeyer flask

- Graduated cylinder
- Test Tube or Plate
- Autoclave
- Stirrer
- Electronic balance
- Burner or Clean bench
- Yeast Extract
- Peptone
- NaCl
- Antibiotic
- Agar

1. Measure component of medium and mix them in Erlenmeyer flask.
2. Autoclave(121℃,20min).
3. (Add appropriate antibiotics)
4. Dispense medium to test tube or plate.
5. Store at 4℃

Protocols

LB medium and LB agar gel Medium for cultivation of E. coli **– NoKoGen – 2011** **http://2011.igem.org/Team:Tokyo-NoKoGen/protocols**

LB medium (1 L)

1; Add about 900 mL of distilled water to beaker.

2; Add 25 g of LB medium, Miller(MERCK) and stir.

3; Add distilled water up to 1 L and take LB medium to media bottle.

4; Autoclave for 20 min at 120°C.

LB agar gel (1 L)

1; Prepare LB medium without autoclave (Steps 1-3 of 1L scale of LB medium).

2; Add 15 g of agar and stirrer bar.

3; Autoclave for 20 minutes at 120°C.

4; Stir and cool LB medium with agar, add appropriate antibiotic (table).

5; Pour LB medium (Step 4) in plate and cool down in clean bench.

	f.c.
Ampicillin	100 µg/mL
Kanamycin	50 µg/mL
Chloramphenicol	30 µg/mL
Tetlacycline	11 µg/mL

Transformation **– NoKoGen – 2011**
http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Inserting plasmid into *E. coli*

1; Incubate frozen competent cell (DH5α) on the ice for a few minutes.

2; Add 1~5 µL of plasmid to competent cell (DH5α) on the ice.

3; Incubate for 20 – 30 minutes on the ice.

4; Incubate for 45 seconds at 42°C.

5; Add 1 mL LB medium and cultivate for 1 hour at 37°C.

6; Spread culture medium on LB agar plate with appropriate antibiotic.

Plasmid extraction **– NoKoGen – 2011**
http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Preparation of plasmid extracted from *E. coli*

1; Pick up single colony from agar plate and cultivate it in 1.5 mL LB medium containing appropriate antibiotic overnight at 37°C.

2; Move the culture medium to 1.5 mL tube.

3; Centrifuge for 5 seconds at 15,000×g and 4 °C and discard supernatant.

4; Add 100 µL Solution 1 to the pellet and resuspend and incubate for 3 minutes.

5; Add 100 µL Solution 2, invert tube gently 5 times and incubate for 3 minutes.

6; Add 100 µL Solution 3, invert tube 5 times and incubate for 3 minutes.

8; Add 200 µL Solution 4 and invert tube 5 times and centrifuge for 3 minutes at 15,000×g and 4 °C.

10; Take supernatant to new 1.5 mL tube and centrifuge for 3 minutes at 15,000×g and 4 °C.

11; Vortex Bind mix for 1 min and add 800 µL Bind mix to new 1.5 mL tube.

12; Add 400 µL supernatant after centrifugation (Step 10) to tube containing Bind mix (Step 11) and mix.

13; Incubate for 3 minutes, centrifuge for 3 seconds at 5,000×g and 4 °C, and discard supernatant.

14; Add 1 mL of 50% ethanol and resuspend.

15; Centrifuge for 3 seconds at 5,000×g and 4 °C and discard supernatant.

16; Repeat wash (Steps 14-15).

17; Dry pellet for a few minuet under a vacuum to remove residual ethanol.

18; Add 50 µL nuclease-free water or TE buffer and incubate for 3 minutes at 65°C.

19; Centrifuge for 3 minutes at 15,000×g and 4 °C.

19; 40 µL of supernatant into new 500 µL tube.

Restriction enzyme digestion of DNA **– NoKoGen – 2011**
http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Cleavage of insert DNA from plasmid

1; Mix DNA and restriction enzyme (Table).

2; Incubate for 2 hours at 37°C.

3; Incubate for 10 minutes at 65°C.

4; Confirm the band of DNA by agar gel electrophoresis.

reagent name	volume
DNA restriction enzyme A restriction enzyme B buffre MQ	5 µL 0.5 µL 0.5 µL 1.5 µL 7.5 µL
total	15 µL

Confirmation and separation of digested DNA

Preparation of agar gel

1; Add 1 g of agar to 100 mL of 1×TAE.

2; Boil and stir until solution is dissolved and clear.

3; Cool down, pour into container to set its shape.

4; Wait until gel dries.

5; Store gel in 1×TAE.

Agar gel electrophoresis **– NoKoGen – 2011**
http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

1; Place agar gel and pour 1×TAE in electrophoresis chamber.

2; Load DNA ladder and DNA sample mixed with loading dye on agar gel.

3; Electrophorese for 20 minutes at 100 V.

4; Stain gel by Sybr SafeTM Gel Stain (Invitrogen).

5; Visualize the band of DNA using UV light.

6; Confirm the length of digested DNA.

PCR

1; Add 25 µL of reagent solution (Table 1) to PCR tube.

2; Amplify target DNA with PCR program (Table 2).

3; Confirm the band of DNA by agar gel electrophoresis.

reagent name	volume
primeSTAR® GXL DNA polymerase 5X GXL buffer dNTP template DNA foward primer reverse primer MQ	0.5 µL 5 µL 2 µL 1 µL 1 µL 1 µL 14.5 µL
total	25 µL

Table 1

	Step 1	Step 2	Step 3
Cycle	1	30	1
	98℃ 1:00	98℃ 0:10 55℃ 0:10 68℃ 4:00	68℃ 2：00 4℃ ∞

Table2

Gel purification **– NoKoGen – 2011**

http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Purification of DNA from agar gel

GENECLEAN® II Kit(NaI、glass milk、NEW Wash)/Qbiogene

1; Cut the objective band in the agar gel after electrophoresis and stain with SYBR Safe.

2; Put the gel including objective DNA into 1.5 mL tube and measure the mass of that.

3; Add 2.5-3 fold volume NaI solution into the tube (Gel: NaI =1 mg : 1 µL).

4; Incubate the gel at 50°C for 5 minute.

5; Add 10 µL of glass milk and vortex.

6; Incubate for 5 minutes and vortex per a minute.

7; Centrifuge for 5 seconds at 15,000×g and 4°C and discard the supernatant.

8; Add the 500µL of New Wash and resuspend.

9; Centrifuge for 5 seconds at 15,000×g and 4°C.

10; Repeat wash (Steps 8-9).

11; Dry the pellet for 5-10 minutes under vacuum.

12. Add 20 µL of nuclease-free water and resuspend.

13. Centrifuge for 5 seconds at 15,000×g and 25°C.

14. Transfer supernatant including objective DNA into new tube.

Ligation – NoKoGen – 2011 http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Ligation inset DNA and vector
DNA Ligation kit Ver 2.1(Solution Ⅰ)/Takara

1; Mix the insert DNA, vector and solution I (Table).

2; Incubation at 16°C for 30 minute.

3; Transform E. coli with ligation sample.

reagent name	volume
insert DNA vector solution I	2 μL 2 μL 4 μL
Total	8 μL

Colony PCR – NoKoGen – 2011 http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Confirmation of insert DNA in plasmid, directly doing PCR on *E. coli*

1; Add 10 μL of reagent solution (Table 1) to PCR tube.

2; Pick up single colony from agar plate with tooth pick and sting replica plate (new LB agar plate).

3; Put and stir toothpick to reagent solution (Step 1).

4; Amplify insert DNA with PCR program (Table 2).

5; Electrophorese PCR sample with agar gel.

6; Check the band and length of insert DNA and decide the colony with insert DNA.

reagent name	volume
fowerd primer reverse primer Go taq® Green Master Mix(Promega) MQ	0.5 μL 0.5 μL 5 μL 4 μL
total	10 μL

Table 1

	Step 1	Step 2	Step 3
Cycle	1	30	1

	95℃ 1:00	95℃ 0:10 55℃ 0:10 72℃ 4:00	72℃ 2：00 4℃ ∞

Table2

Sequence analysis – NoKoGen – 2011 http://2011.igem.org/Team:Tokyo-NoKoGen/protocols

Identification of insert DNA

*Preparation of PCR product

Big Dye® Terminator Cycle Sequencing Kit Ver. 3.1 (Premix, Buffer) / Applied Biosystems

1; Add reagent solution (Table 1) to PCR tube and amplify insert DNA with PCR program (Table2). *Purification of PCR product and sequence analysis

Agencourt CleanSEQ® and 96 R ring Super Magnetic Plate® / Beckman Coulter

1; Add 10 µL of Agencourt CleanSEQ® 10 µL to PCR product.

2; Add 62 µL of 85% ethanol, mix and incubate for 3 minutes.

3; Incubate for 3 minutes on 96 R ring Super Magnetic Plate® and discard supernatant.

4; Add 100 µL of 85% ethanol and mix.

5; Incubate for 3 minutes on 96 R ring Super Magnetic Plate® and discard supernatant.

6; Repeat wash (Steps 4-5).

7; Dry for 10 minutes.

8; Add 40 µL nuclease-free water and mix.

9; Transfer 30µL of clear sample into a new plate for loading on the detector.

10; Load sample on sequencer and analyze.

reagent name	volume
plasmid primer premix buffer MQ	3 µL 0.5 µL 0.5 µL 4 µL 12 µL
total	20 µL

Table 1

	Step 1	Step 2	Step 3
Cycle	1	30	1

	95℃ 1:00	95℃ 0:10 60℃ 50℃ 4:00 0:10	60℃ 2 : 00 4℃ ∞

Table2

Point Mutation – NCTU Formosa – 2011

http://2011.igem.org/Team:NCTU_Formosa/protocol

The procedure is as follows :

1. Design primers
2. Find the best PCR condition by gradient PCR
3. KOD PCR condition

Plasmid 0.5 µl	94℃ 5 min
pF 0.5	94℃ 30 sec
pR 0.5	55℃ 30 sec
MgCl2 1	72℃ 5 min
dNTP 2.5	72℃ 7~10 min
buffer 2.5	Cycles : 25
KOD enzyme 0.5	
H2O 17	
Total 25	

4. Confirm the PCR product with electrophoresis
5. DPN1 37℃ for 3hr~overnight

DPN1 0.5
Buffer2 2
PCR product 17.5
Total 20

6. 80℃ 20mins to denature the DPN1
7. Confirm with electrophoresis
8. Self ligation room temperature for 2~3 hr

Enzyme 1
Buffer 2
ATP 2
H2O 5
PCR product 10
Total 20

9. Transform DH5alpha with the self-ligation product

Self-ligation product 20
DH5alfa 50

10. Incubate in Ap25 plate then transfer to Ap50 plate

PCR – KAIT Japan – 2011

http://2011.igem.org/Team:KAIT_Japan/Notebook

1. Add 100µL of reagent solution(Table) to microtube.
2. Dispense 20µL PCR solution to PCR tube.
3. Amplifty target DNA with PCR program.
4. Confirm the band of DNA by agar gel electrophoresis.

reagent name	Volume(µL)
TaKaRa Ex Taq	0.5
10×Ex Taw buffer	10
dNDP Mixture	8

template DNA	2
foward primer	4
reverse primer	4
sterile water	71.5
total	100

Transformation – KAIT Japan – 2011

http://2011.igem.org/Team:KAIT_Japan/Notebook

1.Incubate frozen competent cell on the ice.
2.Add 2µL of plasmid to competent cell.
3.Incubate for 15 minutes on the ice.
4.Incubate for 45 seconds at 42°C.
5.Incubate for 2 minutes on the ice.
6.Add 250µL LB medium and cultivate for 1 hour at 37°C.
7.Spread culture medium on LB agar plate with appropriate antibiotic.
8.Incubate over night at 37°C.

Restriction enzyme digestion of DNA – KAIT Japan – 2011

http://2011.igem.org/Team:KAIT_Japan/Notebook

1.Mix DNA and restriction enzyme(Table).
2.Incubate over night at 37°C.
3.Confirm the band of DNA by agar gel electrophoresis.

reagent name	Volume(µL)
DNA	15

EcoR I	1
Xba I	1
buffer M	2
steril water	1
total	20

Plasmid Prep – KAIT Japan – 2011

http://2011.igem.org/Team:KAIT_Japan/Notebook

1. Pick up single colony from agar plate and cultivate it in 20 mL LB medium containing
   appropriate antibiotic overnight at 37°C.
2. Move the culture medium to 50 mL falcon.
3. Centrifuge for 5 minutes at 6000rpm and 4 °C and discard solution.
4. Add 2 mL Solution1, the pellet.
5. Add 4 mL Solution 2, invert tube and stored 3 minutes on ice.
6. Add 3 mL Solution 3, invert tube and stored 5 minutes on ice.
7. Centrifuge for 10 minutes at 9,500rpm and 4 °C.

10.Take aqueous layer to new 50 mL falcon.
11.Add 40 µL RNase, invert tube and incubate for 30 minutes.
12.Add 2 mL phenol chloroform mixture.
13.Centrifuge for 5 minutes at 9,500rpm and 4 °C.
14.Take supernatant to new 50 mL falcon.
15.Add 3 mL chloroform solution and misce.
16.Centrifuge for 3 minutes at 9,500rpm and 4 °C.
17.Take 3 mL supernatant to new 50 mL falcon.
18.Add 300 µL sodium acetate.
19.Add 7.5 mL 100%ethanol.

20. Centrifuge for 20 minutes at 9,500rpm and 4 °C and discard solution.
21. Add 8 mL 70% ethanol.
22. Centrifuge for 20 minutes at 9,500rpm and 4 °C and discard solution.
23. Add 50 µL TE buffer and tapping.
24. Preserve at freezer.

Bacterial Culture

1. Single colony isolation on LB plate
2. Incubate the plate for 15-19 hrs at 37C
3. Lift a colony into 2 mL of LB
4. Culture cells at 37C for 12-16 hrs at 180-200 rpm
5. Transfer 30 uL, 100 uL, 300 uL of the culture into 100 mL SOB medium, respectively
6. Culture cells at 20C (for 24 hrs over) at 180-200 rpm (to ΔOD_{550nm} = 0.5~0.6)
7. Leave the 300 mL flask for 10 min on ice
8. Transfer the culture into two 50 mL Falcon tube
9. Centrifuge 7500 rpm at 4C for 20 min (TOMY TA-22 rotor), and discard sup
10. Suspend the pellet in ice-cold 15 mL of TB (Transformation Buffer)(7.5 mL/tube)
11. Centrifuge 7500 rpm at 4C for 2 min (TOMY TA-22 rotor), and discard sup
12. Suspend the pellet in ice-cold 3.2 mL of TB
13. Add 0.24 mL of DMSO (stirring, bit by bit)
14. Leave the 50 mL Falcon tube for 10 min on ice
15. Dispense 50 uL into 0.5 mL tube
16. Freeze the suspension in liquid nitrogen
17. Store at -80C

Bacterial Transformations – HokkaidoU Japan – 2011

http://2011.igem.org/Team:HokkaidoU_Japan/Protocols

1. Add DNA solution to thawed competent cells
2. Incubate the cells on ice for 30 min
3. Heat shock the cells by immersion in a pre-heated water bath at 42C for 60 sec
4. Incubate the cells on ice for 5 min
5. Add 200 uL of SOB broth
6. Incubate the cells at 37C for 2 hrs while the tubes are shaking
7. Plate 200 uL of the transformation onto the dish
8. Incubate the plate at 37C for 12-14 hrs

Mini-prep (Alkaline SDS Method) – HokkaidoU Japan – 2011

http://2011.igem.org/Team:HokkaidoU_Japan/Protocols

Reagents

Solution I

(at RT, filtration 0.2 um, 50 mL)

reagents	amount	Final concentration
Glucose (at RT)	0.45 g	50 mM
1 M Tris-HCl (pH8.0, at RT, autoclaved)	1.25 mL	25 mM
0.5 M EDTA (pH8.0, at RT, autoclaved)	1 mL	10 mM
Total	50 mL	

Solution II

(at RT, filtration 0.2 um, 20 mL)

reagents	amount	Final concentration
10 N NaOH (at RT)	0.4 mL	0.2 N
10% SDS (at RT, filtration)	2 mL	1%
Total	20 mL	

Solution III

(at RT, filtration 0.2 um, 50 mL)

reagents	amount	Final concentration
5 M CH_3COOK	30 mL	3 M
CH_3COOH	5.75 mL	
H_2O	14.25 mL	
Total	50 mL	

Procedure

1. Lift colony *E. coli* into 2 mL LB contained antibiotics
2. Culture cells at 37C for 16-20 hrs at 180-200 rpm
3. Transfer 1.2-1.5 mL of culture into 1.5 mL tube
4. Centrifuge the culture at 15,000 rpm for 1 min at 4C and discard sup
5. Suspend the pellet in ice-cold 100 uL of Solution I
6. Add 200 uL of Solution II to the suspension
7. Mix by inverting the tube 10-20 times
8. Add ice-cold 150 uL of Solution III to the suspension
9. Mix by inverting the tube 10-20 times
10. Leave the tube for 5 min on ice

11. Add 10 uL of Chloroform
12. Mix by inverting the tube 5-10 times
13. Centrifuge the suspension at 15,000 rpm for 5 min at 4C
14. Transfer the supernatant into new 1.5 mL tube↓
15. Add equal volume of isopropanol and mix by vortex
16. Leave the tube for 5 min at RT
17. Centrifuge the suspension at 15,000 rpm for 10 min at 4C and discard sup
18. Rinse the ppt by 70% EtOH and mix by vortex
19. Centrifuge the suspension at 15,000 rpm for 2 min at 4C and discard sup
20. Dry up the ppt
21. Dissolve the ppt in 50 uL of TE (pH 8.0)
22. Add 1 uL of 10 mg/mL RNase A (4C and stock at –20C)
23. Incubate for 30 min at 37C
24. PCIAA and CIAA extraction
25. Ethanol precipitation
26. Dry up the ppt
27. Dissolve the ppt in 50 uL of TE (pH 8.0)

PCR – HokkaidoU Japan – 2011

http://2011.igem.org/Team:HokkaidoU_Japan/Protocols

Vector

Standard reaction setup

Component	Volume
10x PCR Buffer	5 uL
2mM dNTPs	5 uL
25mM $MgSO_4$	3 uL

Suffix-F primer	1 uL
Prefix-R primer	1 uL
Template DNA	1 uL
KOD -Plus- Neo	1 uL
DW	X uL
Total	50 uL

Cycling conditions

(2-step cycle)

Stage	Temperature and Time
Predenature	94C 2 min
Denature	98C 10 sec
Extension	68C X sec (30 sec/kb)
Hold	4C

- 30-40 cycles

Insert

Standard reaction setup

Component	**Volume**
10x PCR Buffer	5 uL
2mM dNTPs	5 uL
25mM $MgSO_4$	3 uL
EX-F primer	1 uL

PS-R primer	1 uL
Template DNA	1 uL
KOD -Plus- Neo	1 uL
DW	X uL
Total	50 uL

Cycling conditions

(2-step cycle)

Stage	Temperature and Time
Predenature	94C 2 min
Denature	98C 10 sec
Extension	68C X sec (30 sec/kb)
Hold	4C

- 30-40 cycles

Colony PCR

- resuspend a colony into 10 uL of DW (template suspension)

Standard reaction setup

Component	**Volume**
template suspension	4.8 uL
Quick Taq	5 uL
Forward primer	0.1 uL
Reverse primer	0.1 uL

Total	10 uL

Cycling conditions

(2-step cycle)

Stage	Temperature and Time
Initial denature	94C 2 min
Denature	94C 10 sec
Extension	68C X sec (60 sec/kb)
Hold	4C

- 30-40 cycles

PCIAA and CIAA extraction – HokkaidoU Japan – 2011

http://2011.igem.org/Team:HokkaidoU_Japan/Protocols

Reagent

- PCIAA = Phenol : CIAA = 1 : 1
- CIAA = Chloroform : IsoAmyl Alcohol = 24 : 1

Procedure

1. Add equal volume of PCIAA and vortex vigorously
2. Centrifuge at 15,000 rpm for 2 min at RT
3. Transfer the aqueous phase to a new tube, being careful not to transfer the phase interface
4. Add equal volume of CIAA and vortex vigorously
5. Transfer the aqueous phase to a new tube
6. Ethanol precipitation

Ethanol precipitation – HokkaidoU Japan – 2011 **http://2011.igem.org/Team:HokkaidoU_Japan/Protocols**

1. Add 1/10 volume of 3M CH_3COONa
2. Add 2.5 volume of 100% ethanol (EtOH)
3. Incubate on ice for few min
4. Centrifuge at 15,000 rpm for 10 min at 4C and discard sup
5. Wash precipitation with 100 uL of 70% EtOH (EtOH has to be cold)
6. Centrifuge at 15,000 rpm for 5 min at 4C and discard sup
7. Dry up the ppt (no EtOH should be left)
8. Resuspend ppt in wanted volume of TE

Mini-prep (QIAprep Spin Miniprep Kit) – HokkaidoU Japan – 2011 **http://2011.igem.org/Team:HokkaidoU_Japan/Protocols**

1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a micro-centrifuge tube
2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times
3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times
4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge
5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting
6. Centrifuge for 30–60 s. Discard the flow-through
7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through
8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s
9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min

- see details (Official website)

Gel Extraction (Wizard® SV Gel and PCR Clean-Up System) – HokkaidoU Japan – 2011 **http://2011.igem.org/Team:HokkaidoU_Japan/Protocols**

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 mL microcentrifuge tube
2. Add 10 uL Membrane Binding Solution per 10 mg of gel slice
3. Vortex and incubate at 50–65C until gel slice is completely dissolved
4. Insert SV Minicolumn into Collection Tube
5. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly
6. Incubate at room temperature for 1 min
7. Centrifuge at 16,000 g for 1 min
8. Discard flowthrough and reinsert Minicolumn into Collection Tube
9. Add 700 uL Membrane Wash Solution (ethanol added)
10. Centrifuge at 16,000 g for 1 min
11. Discard flowthrough and reinsert Minicolumn into Collection Tube
12. Repeat Step 4 with 500 uL Membrane Wash Solution
13. Centrifuge at 16,000 g for 5 min
14. Empty the Collection Tube and recentrifuge the column assembly for 1 min with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol
15. Carefully transfer Minicolumn to a clean 1.5 mL microcentrifuge tube
16. Add 50 uL of Nuclease-Free Water to the Minicolumn
17. Incubate at room temperature for 1 min
18. Centrifuge at 16,000 g for 1 min
19. Discard Minicolumn and store DNA at 4C or –20C

Agarose Gel Electrophoresis – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Preparation of agarose gel

Pour 100 mL of 1X TAE buffer into a conical flask.

Add the agarose powder to the buffer in the amount with respect to the concentration of the agarose solution (e.g. add 1 g for preparing 1% agarose gel solution).

Use a plastic wrap to cover the opening of the conical flask and microwave for approximately 2 minutes or until the agarose dissolves completely.

Pour the agarose solution into another conical flask which specifies for holding ethidium bromide (EB) – containing solution.

Add 1 – 2 ul of EB into the agarose solution and mix well.

Pour the solution into a gel tray with a comb. Remove any bubbles formed.

Allow the gel to solidify which takes approximately 30 minutes.

Discard all the wastes into the EB waste box.

Electrophoresis – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Remove the comb and place the solidified gel into the electrophoresis tank.

Add TAE buffer to the tank when necessary.

Add 6X loading buffer to the DNA sample in the ratio of 1:6 and mix well.

Load the samples into the wells with care.

Load 2-3 ul of marker to a well for reference.

Run the electrophoresis at around 140V for about 30 minutes.

Take the gel photo in the UV-illuminating machine.

DNA Extraction from Agarose Gel

Gel extraction – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Wear UV protection glasses before the gel extraction.

Place the gel onto the transilluminator.

Turn on the transilluminator and quickly cut the desired gel band.

Place the cut band into an eppendorf tube for further processing.

Discard all the wastes into the EB waste box.

DNA extraction (Adopt from Qiagen)

Weight the Eppendorf tube and determine the weight of the cut band.

Add 3 volumes of extraction buffer to 1 volume of the cut get.

Place the Eppendorf tube (with the cut gel and the extraction buffer) into 55oC water bath to dissolve all the agarose gel.

After dissolving, add the mixture to the spin column with a collection tube.

Centrifuge at 11,000 rpm for 1 minute.

Discard flow through.

Add 750 ul of washing buffer and centrifuge at 11,000 rpm for 1 minute.

Discard the flow through and centrifuge again at 11,000 rpm for 1 minute.

Place the collection tube to a new Eppendorf tube.

Add 20 mL elution buffer directly to the centre of the membrane of the collection tube.

Let it stand for approximately 3 minutes.

Centrifuge at 11,000 rpm for 1 minutes and collect the flow through (i.e. product).

Take a small portion of the DNA product for confirmation by gel electrophoresis.

Protocol adopt from http://www.qiagen.com/literature/render.aspx?id=201083

DNA Digestion – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Add the following reagents, with the enzymes added at the last, into a tube

Table 1.jpg

Reagents for DNA digestion.

All steps should be carried out on ice.

Mix well after addition of all the reagent.

Incubate the mixture at 37oC for several hours.

Miniprep(Adopt from Qiagen)

Centrifuge the sample at 8,000 rpm for 1 minute.

Discard the supernatant.

Add 250 ul P1 buffer to resuspend the pellet (tap to suspend the pellet completely).

Add 250 ul P2 buffer and mix gently by inverting the tube for several times.

Add 350 ul N3 buffer and mix thoroughly. The solution should now turn cloudy.

Centrifuge the solution at 13,000 rpm for 10 minutes.

Transfer the supernatant to a spin column with a collection tube inside.

Centrifuge at 12,500 rpm for 1 minute. Discard the flow through.

Add 750 ul PE buffer to the collection tube and centrifuge at 12,500 rpm for 1 minute.

Discard the flow through and centrifuge again to remove all remaining washing buffer.

Place the collection tube into a new eppendorf tube.

Add 50 ul elution buffer directly at the centre of the membrane of the collection tube.

Let it stand for approximately 3 minutes.

Centrifuge at 12,500 rpm for 1 minutes and collect the flow through (i.e. the product).

Protocol adopt from: http://www.qiagen.com/literature/render.aspx?id=201081

Polymerase Chain Reaction

Colony PCR

Add the following reagents into a PCR tube (in order) and mix well.

Colony PCR.jpg

Reagents for colony PCR.

Set the following PCR program.

Colony pcr program.jpg

Reaction program for colony PCR.

Reverse PCR

Add the following reagents into a PCR tube (in order) and mix well.

Reverse pcr.jpg

Reagents for reverse PCR.

Set the following PCR program.

Reverse pcr program.jpg

Reaction program for reverse PCR.

Overlap PCR

First, two PCR reactions are set for amplifying the two genes, tetR and HNS, separately.

Add the following reagents into a PCR tube (in order) and mix well.

Overlap pcr.jpg

Reagents for amplifying genes.

Set the following PCR program.

Overlap pcr program.jpg

Reaction program for amplifying genes.

Set up another PCR reaction using a primer with a linker to link the two genes.

Add the following reagents into a PCR tube (in order) and mix well.

Overlap pcr program.jpg

Reagents for linking two genes.

Set the following PCR program.

Overlap pcr program 2.jpg

Reaction program for linking two genes.

Set up another PCR reaction to further amplify the fused product.

Add the following reagents into a PCR tube (in order) and mix well.

Overlap pcr 3.jpg

Reagents for amplifying the fused product.

Set the following PCR program.

Overlap pcr program 3.jpg

Reaction program for amplifying the fused product.

DNA ligation – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Add the following reagents, with the enzymes added at the last, into a tube.

DNA ligation.jpg

Composition for DNA ligation.

Incubate at 16oC overnight.

Sequencing

Send to BGI company for sequencing.

B. BACTERIAL WORK – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Overnight culture

Pipette 3 mL of LB broth into a culture tube.

Add 3 ul of Ampicillin or 3 ul of Chloramphenicol.

Pick a single colony by a sterile pipette tip.

Place the culture tube in the rotary shaker and incubate at 37oC overnight.

Preparation of competent cell – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Seed culture:

Pick a single colony from a plate with fresh grown cells (for 16 – 20 hours at 37oC) and transfer it into 3 mL of LB broth in a sterilized 15-mL polypropylene tube.

Incubate the culture overnight at 37oC in a rotatory shaker to provide vigorous shaking.

Main culture: – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Inoculate 1,000 ul of seed culture into 100 mL of LB broth in a sterile 250-mL flask.

Incubate the culture at 37oC with vigorous shaking (in a rotary shaker) for approximately 2 hours or until the OD600 value reaches 0.3 to 0.4.

Aseptically transfer the cells to a sterilized, chilled 50-mL polypropylene tube and cool the cultures to 0oC by placing the tube on ice for 10 minutes.

Centrifuge at 4,000 rpm for 5 – 15 minutes at 4oC.

Decant the media from the cell pellets.

Resuspend the cell pellets in 20 mL of filtered, sterilized, chilled 0.1M calcium chloride (CaCl2).

Vortex gently to mix it and place the tube on ice for 15 to 30 minutes.

Centrifuge at 4,000 rpm for 5 minutes at 4oC.

Add 1 mL of chilled glycerol to each tube of culture.

Pipette up and down to mix it gently.

Add 100 ul culture in each eppendorf tube.

Store the culture at -80oC for approximately 1 hour.

Spread plate – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Pipetting the liquid culture (about 200 ul) onto the surface of the LB agar plate.

Sterilize an L-shape glass rod.

Spread the cells evenly on the plate.

Incubate the plate at 37oC overnight with the bottom facing upward.

Streak plate

Sterilize the inoculating loop in flame.

Pick a portion of a single colony of the sample.

Make the first phase streak.

Flame sterilize the inoculation loop.

Cross the first phase of inoculum and make the second phase streak.

Repeat step d and e for making the third phase streak.

Flame sterilize the inoculating loop.

Incubate the plate at 37oC overnight with the bottom facing upward.

Transformation – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Mix 1 ul of DNA in 100 ul of competent cells.

Place the mixture on ice for 40 minutes.

Heat shock the cells in 42oC water bath for 90 – 100 sec.

Incubate on ice for 3 minutes.

Recovery:

Add 900 ul LB broth to the tube.

Incubate the mixture at 37oC for 1 hour in the rotary machine.

Spread 100 ul of each culture on a LB agar plate.

Incubate at 37oC overnight.

C. Preparation of materials – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Preparation of ampicillin – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Add 1 g of ampicillin powder to 10 mL of ddH2O. Mix well.

Filter the solution using 0.22 um filter.

Store the filtrate at -20 oC.

Preparation of LB agar plate – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Add 7 g of LB powder to 200 mL of ddH2O. Mix well.

Autoclave the solution.

Add 180 ul of ampicillin to 180 mL of molten agar, in a ratio of 1:1000 (if necessary).

Pour 10 mL agar solution per plate.

Let the agar solidify.

Preparation of LB broth – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Add 4 g of LB powder to 200 mL of ddH2O. Mix well.

Autoclave the solution.

Add 200 ul of ampicillin to 200 mL of LB broth (1:1000).

Preparation of 1X TAE buffer

Add 50 mL of 50X TAE buffer.

Add 2.5 L of ddH2O.

D. Protein Work – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Gel Shift Assays (Adopt from Promega)

Gel Preparation – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Clean all the glassware by distilled water (ions-free).

Prepare a non-denaturing 4% acrylamide gel according to the following formula:

Gel shift assay gel preparation.jpg

Formula for preparing acrylamide gel.

Allow the gel to stand until the gel is completely polymerized.

DNA Binding Reactions – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Set up four binding reactions (if necessary) with the following composition.

Gel shoft assay DNA binding reactions.jpg

The four binding reactions: Negative control, Positive control, Specific competitor and Non-specific competitor

Incubate the reactions at room temperature for 10 minutes.

Add 1 ul of labeled DNA sample to each reaction.

Incubate the reactions at room temperature for 20 minutes.

Add 1 ul of 10X loading buffer for each reaction.

Electrophoresis – HKU-Hong Kong – 2011 http://2011.igem.org/Team:HKU-Hong_Kong/Lab_Protocol

Pre-run the gel in 0.5X TBE buffer for 10 minutes at 350V.

Load the sample.

Run the gel at 350V until the loading dye reached three fourth of the gel.

Maintain the gel temperature under 30oC.

Protocol adopt from:

http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/0/Gel%20Shift%20Assay%20Systems.ashx

Ligation Protocol – UT Dallas – 2011 http://2011.igem.org/Team:UT_Dallas/protocols_new

Determine insert to vector ratios

Calculate the amount of insert needed if 50ng of vector is used (can use different amount of vector)

In a PCR tube add the following:

50ng of vector

Amount of insert based on ratios (calculated in second step)

2uL of buffer

2uL of DNA ligase

Amount of water to bring total volume to 20uL

Incubate overnight at 14 degrees Celsius

Note: We used T4 DNA ligase and buffer from NEB

Gel Purification Protocol (QIAquick Gel Extraction Kit) – UT Dallas – 2011
http://2011.igem.org/Team:UT_Dallas/protocols_new

Excise DNA fragment from the agarose gel with a clean, sharp scalpel

Weigh the gel slice in a microcentrifuge tube.

Add 3 volumes of Buffer QG to 1 volume of gel (100mg~100uL)

Incubate at 50 degrees Celsius for 10 min (until the gel slice has completely dissolved)

After the gel slice has dissolved completely, check that the color of the mixture is yellow

Apply the sample to a QIAquick column, and centrifuge for 1 min

Maximum volume of the column is 800uL. For samples larger than this, simply load and spin again.

Discard flow-through and place QIAquick column back in the same collection tube

To wash, add 750uL of Buffer PE to column and centrifuge for 1 min.

Discard the flow-through and centrifuge for additional 1 min. at 13,000rpm

Place QIAquick column into a clean 1.5 mL microcentrifuge tube

To elute DNA, add 50uL of Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min. and then centrifuge the column for 1 min.

Gel Electrophoresis Protocol – UT Dallas – 2011 http://2011.igem.org/Team:UT_Dallas/protocols_new

Making a 1% agarose gel

100mL 1X TBE buffer

1g agarose

microwave until agarose dissolves

let mixture cool

when cool add 8-10uL ethidium bromide

stir gently, let cool

pour into plate with comb already in place

let harden

Using the gel

Add loading buffer to DNA (for 100uL DNA, add 20uL loading buffer)

Load 2uL of DNA ladder into the gel

Load DNA into the gel

Run at 130V for 30min-1hr

Digestion Protocol – UT Dallas – 2011 http://2011.igem.org/Team:UT_Dallas/protocols_new

Using a microcentrifuge tube add the following:

~3000-5000 ng of DNA

10uL Buffer 4

10uL BSA

5uL of appropriate enzyme (if doing a double digest, use 5 uL of both enzymes)

Amount of H_2O needed to make final volume 100uL

Incubate at 37 degrees Celsius for 1hr and 30min

Note: We used the following enzymes from NEB: EcoRI-HF, PstI-HF, SpeI, and XbaI. All of which can be double digested with each other using Buffer 4.

Preparing LB+Appropriate Antibiotic Protocol – UT Dallas – 2011
http://2011.igem.org/Team:UT_Dallas/protocols_new

200 mL LB broth

Autoclave

Put control thermometer in H_2O (from the sink)

Select vented container mode (Do Not Change Program)

Let cool to 50 degrees Celsius

Add antibiotic (50-100 ug/mL) (10 mg total)

Weigh on paper

Add to 0.5 mL DI H_2O

Add to LB mixture when cool enough

Store at 4 degrees Celsius

Preparing Agar Plates Protocol (Makes 12 (15mm) Plates) – UT Dallas – 2011
http://2011.igem.org/Team:UT_Dallas/protocols_new

300 mL DI H_2O + 11 g LB agar

Autoclave

Put control thermometer in H_2O (from the sink)

Select vented container mode (Do Not Change Program)

Mix well after autoclaving; let cool to 50 degrees Celsius

Add antibiotic (50 to 100 µg/mL) (15 mg total)

Weigh on paper

Add to 0.5 mL DI H_2O

Add to LB mixture when cool enough

Plate

Under flame open lids of all plates

Slowly pour agar into plate, avoiding bubbles, when it touches all edges stop pouring

Let sit under flames until gel solidifies

Replace lids on plates

Store upside down at 4 degrees Celsius

Preparing Competent Cells Protocol – UT Dallas – 2011
http://2011.igem.org/Team:UT_Dallas/protocols_new

Place 1 colony in 5 mL of LB (with antibiotics if appropriate) Grow overnight at 37 degrees Celsius and 200-300 rpm

Inoculate 0.25 mL of the overnight strain into 25 mL of LB

Shake at 37oC until the OD650 is 0.6-0.7

Harvest cells and resuspend in 12.5 mL ice cold 0.1M $MgCl_2$

Harvest immediately and resuspend in 7.5 mL cold 0.1M $CaCl_2$

Leave on ice for 30 minutes. Harvest and resuspend in 2.5 mL cold 0.1M $CaCl_2$

Leave on ice for 30 minutes

For long term storage, use 0.1M $CaCl_2$ in 15% glycerol at step 6 and store cells at -800 degrees Celsius

Note: Harvest cells at 5000 rpm for 10 minutes at 4 degrees Celsius

Miniprep Protocol (from QIAprep Spin Miniprep Kit) – UT Dallas – 2011
http://2011.igem.org/Team:UT_Dallas/protocols_new

Harvest cells at 5400g 10 minutes 40 degrees Celsius (possibly program 1)

Resuspend pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube

Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times

Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times

Centrifuge for 10 minutes at 13000 rpm (~17900g) in a table-top microcentrifuge

Apply the supernatant (from step 4) to the QIA prep spin column by decanting or pipetting

Centrifuge for 30-60 seconds. Discard the flow-through

Wash QIA prep spin column by adding 0.75 mL Buffer PE and centrifuging for 30-60 seconds

Discard the flow-through, and centrifuge for 1 minute to remove residual wash buffer

To elute DNA, place the QIA prep column in a clean 1.5 mL microcentrifuge tube. Add 50 µL Buffer EB or water to the center of each QIA prep spin column, let stand for 1 minute and centrifuge for 1 minute.

Preparing Glycerol Stock Protocol – UT Dallas – 2011
http://2011.igem.org/Team:UT_Dallas/protocols_new

Add 150 µL of 50% glycerol to 350 µL of cells

Place in -80oC freezer

Transformation Protocol – UT Dallas – 2011 http://2011.igem.org/Team:UT_Dallas/protocols_new

With a pipette tip, punch a hole through the foil cover of the DNA plate

Add 10 µL of DI water

Thaw competent cells on ice

Add 1-2 µL of resuspend DNA and 50 µL of thawed competent cells to labeled tubes

Incubate the cells on ice for 30 minutes

Heat shock the cells at 42 degrees Celsius for 45 sec

Incubate the cells on ice for 2 minutes

Under flame, add 450 µL SOC broth

Incubate at 37 degrees Celsius for 1 hour while rotating or shaking at 300rpm

Spread cells on appropriate antibiotic LB plates (usually 100 µL)

Incubate at 37 degrees Celsius for 18-24 hours

Take a colony, put in 3 mL of LB + appropriate antibiotic

Use resulting culture to miniprep DNA and make your own glycerol stock

Point mutation Protocol – UT Dallas – 2011 http://2011.igem.org/Team:UT_Dallas/protocols_new

1) Create reaction mixture
5 ul 10x buffer
10-100 ng DNA
1 ul of foward primer
1 ul of reverse primer
1 ul of dNTP'S
1.5 ul of Quik Solution reagent
Bring to 50 ul with NF-H20
***Then add 1ul Quik Change Lightning Enzyme**
2)Run thermo-cycler (program--mutate)
1 cycle: @ 95C 2 minutes
18 cycles:
a) 95C x 20 seconds
b) 60C x 10 seconds
c) 68C x 30 seconds/kb per plasmid length
1 cycle: 68C 5 minutes
3) Then add 2 ul of DpnI enzyme directly to each amplification reaction
4) Pipette up & down several times
5) Incubate @ 37C x 5 minutes to digest the parent DNA (cuts methylated dna)
6)Then transform.

I. Electroporation Transformation of E. coli – Utah State – 2011 **http://2011.igem.org/Team:Utah_State/Notebook**

Transformations are any procedure used to insert DNA into a bacteria (if you use a virus, the term becomes transfection). Electroporation uses a pulse of electricity to disrupt the cell membrane and create holes that would allow the DNA to enter the cell. Cells need to be made competent before doing this procedure, in order for them to efficiently take up the DNA. Transformations generally utilize millions to billions of cells and DNA molecules and, for a transformation to be successful, only one molecule of DNA needs to enter into one cell, which then grows into a colony. One issue with transformations is selecting and verifying which colonies have the desired DNA. This is usually done using a marker, a characteristic possessed by cells that have the DNA (or lost by those cells) that distinguishes it from the rest of the colonies that grow up. Commonly, this is the expression of an antibiotic resistance gene included on the transformed DNA, which allows only the cells that have taken up the DNA to survive on a plate in the presence of that antibiotic. Sometimes pigment producing or fluorescent/luminescent proteins can also be used in place of antibiotic resistance to allow visual determination of transformed colonies. Other ways of selection exist, but will not be discussed here.

1. Turn on ice machine

2. Thaw DNA solutions

3. Clean and sterilize the electroporation cuvettes by washing with double distilled water (ddH2O) twice and then fill the cuvettes with ethanol.

4. Let cuvettes sit with ethanol for 5-10 minutes, then wash 4-8 times with ddH2O

5. Place cuvettes on ice

6. Take competent cells out of the -80 °C freezer, and thaw them on ice

7. Add 3 µL of DNA to the cell solution. (This should be around 100-250 ng of total DNA, too much DNA causes arcing, too little gives few transformed colonies).

8. Incubate on ice for 5 minutes.

9. Add 60 µL WB buffer (10% glycerol). This helps reduce arcing, although too much can lower numbers of transformed colonies.

10. Set the electroporation machine to 2500 V, 200 Ω, and 25 µF for E. coli.

11. Transfer the cell/DNA/WB solution into the cuvettes by pipetting up and down in the 1.5 mL tube first to mix. Make sure the pipette tip is between the metal plates on the cuvette before ejecting the solution. Keep the cuvettes on ice.

12. Before electroporating, dry the cuvettes of with a KimWipe, to ensure no liquid on the surface that could create other paths for the electric pulse (and could cause arcing).

13. Pulse the cells and return cuvette to the ice. Check the time constant on the machine, a constant of 4.5+ is a very good transformation, and will yield many colonies. A constant of 2.5-4.5 is okay, and will still work. Constants below 2.5 will yield very low colony numbers, and may need to be redone. NOTE: addition of extra WB or lower amounts of DNA will reduce the time constant as well, so it is only a rough measure.

14. To remove the cells from the cuvette add 1 mL LB media or SOC media (no antibiotic in this media) to the cuvette. Pipette up and down a few times to mix.

15. Remove the solution to just above the two plates in the first removal pipetting (~1/2 of the volume) and transfer it to the original cell tube (NOT THE DNA TUBE). Then, tip the cuvette on its side so that the space between the plates is vertical, place the 1000 µL pipette tip between the plates, and slowly draw up the solution, while tipping the cuvette further. This should draw up the rest of the liquid in the cuvette.

16. Incubate the cell solutions at 37 °C for 1-2 hours (can go up to three, but try to avoid doing it for that long).

17. Plate the cells on plates containing the correct antibiotic. Each transformation requires two plates. Add 500 µL of solution to one plate, spread with the spreading stick, and then spread the spreading stick on the second plate without adding any solution to it. This creates a dilution plate in case you have thousands of colonies on the first plate. It is roughly a 1:100 to 1:200 dilution.

18. Grow the plates up overnight at 37 °C. Do not leave for longer than 24 hours, as contaminants might have a chance to grow and the plates could dry out.

If your cuvette arcs (bright flash and loud popping noise during electroporation): 1. Clean the electroporator lid.

2. Wash out and sterile the cuvette with ethanol – the cells have been pretty much killed and will not be usable in plating, so you need to restart.

3. Add less DNA to the cells (reduce by 25%-33%).

4. Add an additional 15 µL of WB buffer to the solution.

II. Colony selection, Stock Streak Plating, and Culture Preparation – Utah State – 2011 **http://2011.igem.org/Team:Utah_State/Notebook**

After allowing transformed cells to grow up overnight, we can then select for the colonies that exhibit the characteristics indicating that they have taken up our DNA of interest. It is important at this point to know all the possible DNA combinations that could be in these cells, and which set of characteristics we want. For example, if we are converting a construct from one plasmid to another and replacing an RFP expression system on the final plasmid with our construct, we would want to select cells that did not express RFP, as these are clearly wrong. One important thing to note is that when using a RFP/GFP color or fluorescence to indicate the correct or incorrect colonies, sometimes these characteristics are not visible at the small colony stage, and a second round of selection on a streak plate is necessary. Luckily, streak plating is necessary for all types of selection, as it acts as an intermediate storage stock until glycerol stocks are created.

1. Carefully decide which cell characteristics you are looking for.

2. Identify colonies with the characteristic that are separated by a small distance from other colonies – this makes it easier to pick only the colony you want

3. With a sterile toothpick or sterile pipette tip, lightly touch down on the colony to pick up cells

4. Keeping the toothpick or tip oriented so that the cells are on the bottom, gently streak the cells on a new plate with the correct antibiotic. DON'T DISCARD THE TIP/TOOTHPICK. The streak should be around 2 cm or so long, and should be separated from other streaks on the new plate.

5. If you want to grow the cells up in a liquid culture (for glycerol stock and DNA prep), you can place the tip/toothpick into a tube with 5-6 mL LB. There should be enough cells still on the tip/toothpick to have a dense culture overnight. If there is a strong chance that not all colonies picked will be correct (see RFP example above), 2-3 of these cultures should be made for each construct; otherwise 1-2 will usually be enough.

6. If colonies are used to grow up liquid culture, label the streak and the culture tube with the same number, so if the colony is incorrect, you know which tube is incorrect.

7. Repeat for as many colonies as you think you will need (15-25 is generally good for streaks)

8. Put the plate into the 37 °C incubator overnight

9. Re-evaluate the streaks for indications that they are not the right colony. Toss any liquid cultures corresponding to incorrect colonies.

III. Glycerol Stock Preparation – Utah State – 2011 **http://2011.igem.org/Team:Utah_State/Notebook**

Glycerol Stocks are one of the best ways to store cells and DNA for long periods of time. These stocks are prepared so that the cells in the tubes are still alive, and capable of creating new liquid cultures from small amounts of frozen stock. This allows for quick growth and extraction of important DNA without spending an extra day and extra supplies re-transforming a construct every time it is needed. In order to freeze cells and still keep them alive, a cryoprotectant is added to the cultures. Cyroprotectants function by reducing the freezing point of the solution and act to reduce the formation of large ice crystals inside cells that could rupture membranes. Cryoprotectants are non-toxic to the cells, and are generally able to pass through the membranes into the cells.

1. Take 1 mL of overnight E. coli culture and add it to a clean, labeled 1.5 mL tube.

2. Add 200 µL of 80% glycerol to the tube (this creates a roughly 15% glycerol solution).

3. Mix well by inverting the tubes (unmixed glycerol will tend to stay separate from the cell solution.

4. Immediately place the cells into a -80 °C freezer box. There are often special freezing boxes that let the freezing occur more slowly, but for E. coli these are generally not needed.

5. To use the glycerol stock to establish a new culture, either scrape a very small amount of the frozen culture off with a pipette tip and add it to a culture (if it is still frozen), or add 10-20 µL of the liquid glycerol stock to the culture (if it is somewhat thawed). AVOID THAWING GLYCEROL STOCKS COMPLETELY – take what you need and quickly return them to the -80 °C freezer. If they do thaw completely, they can be re-frozen, but repeated thawing may reduce the number of live cells in the stock tube.

IV. DNA Extraction – Utah State – 2011 **http://2011.igem.org/Team:Utah_State/Notebook**

DNA Extractions are used to generate large amounts of plasmid DNA from E. coli cell cultures. This DNA can then be used for restriction digesting, PCR reactions, gel electrophoresis, or further transformations. By using a kit (Qiagen Qiaprep Spin Miniprep Kit) we are able to effectively remove most of the protein and RNA from a solution, leaving us with very clean DNA solutions.

1. Grow up 5 mL culture overnight in LB (6 mL if you want glycerol stock – 1 mL will be used for the frozen stock, which should be removed before pelleting the cells in the next step)

2. Pellet 5-10 minutes in centrifuge at 3000-3500 rpm

3. Pour off supernatant

a. At this step, you can freeze the cell pellet if you don't have time to finish the rest of the procedure. The pellet is fine for a week or two in the -20 °C freezer

4. Check P1 solution for the checked RNase added box

5. Add 250 µL solution P1 to the 10 mL culture tube

6. Suspend the pellet in the P1 by pipetting up and down

7. Transfer suspended pellet to a 1.5 mL tube

8. Add 250 µL solution P2 to the tube

9. Mix by inverting the tube by hand 10-20 times. Solution should turn blue throughout, if not, continue inverting until blue throughout

10. Allow lysis to occur for 3-4 minutes (no more than 5 minutes)

11. Add 350 µL solution N3 to the tube

12. Mix by inverting tube by hand 10-20 times. Solution should lose all blue color, if not, continue inverting until all blue is gone.

13. Centrifuge at 13,000 rpm for 10 minutes (keep the hinge out to get the pellet to form correctly)

14. Using a pipette, remove the supernatant from the tubes, and apply to a labeled blue spin column from the kit

15. Centrifuge at 13,000 rpm for 1 minute

16. Pour flow-through BACK into the column and centrifuge at 13,000 rpm for 1 minute 17. Discard flow through

18. Add 750 µL PE solution to tube and centrifuge at 13,000 rpm for 1 minute

19. Discard flowthrough and centrifuge again at 13,000 rpm for 1 minute

20. Transfer blue column to a fresh 1.5 mL tube (labeled)

21. Add 30-50 µL ddH2O (depending on how concentrated you want your final DNA). Buffer EB (supplied in the kit) can also be used.

22. Incubate on benchtop for 10-15 minutes

23. Centrifuge at 13,000 rpm for 1 minute and discard column

24. Nanodrop to determine DNA concentration

V. Restriction Digest – Utah State – 2011

http://2011.igem.org/Team:Utah_State/Notebook

All restriction digests were carried out using Fermentas reagents and restriction enzymes. The Fermentas FastDigest® (FD) reagents were used.

In a tube the following was added:

4 µg DNA

4µl 10x FD buffer

1 µl FD restriction enzyme 1

1 µl FD restriction enzyme 2

Volume brought up to 40µl total with nuclease free water

Tube was placed at 37°C for 1hr.

The FastDigest® Green Buffer was also used. When it was used the DNA could directly be loaded onto an agarose gel.

VI. Gel Making – Utah State – 2011

http://2011.igem.org/Team:Utah_State/Notebook

Agarose gels are useful for DNA purification and analysis of DNA sizes. The gels are made up of an agarose matrix composed of long strands of agarose, and gaps of various sizes between the strands. The larger the DNA molecule, the longer it takes to fit through the gaps, making its progress through a gel slower than a small DNA molecule. The DNA is drawn through the gel using an electric current; the negatively charged phosphates on the DNA backbone being attracted toward the cathode ("Run towards red" is a helpful mnemonic as the cathode is generally red colored). By varying the concentration of the agarose gel, it is also possible to increase the separation of bands of certain sizes on the gel. A 1% agarose gel is generally used as it provides separation of bands from 200 bp to 3000 bp. For separating larger bands, a 0.7% gel is typically used and the smaller DNA fragments are run completely off the bottom of the gel. For separating smaller bands, a 2% or 1.5% gel can be used, and run normally. Gels are useful for purifying DNA bands of a particular size from restriction digests (to prevent multiple products from forming during ligations) and also for removing proteins from a DNA sample (such as restriction enzymes that are not inactivated by heat). Gel purification has the downside of losing some DNA, and reducing overall DNA concentration (a 120 bp band of DNA in a 2000 bp plasmid will only give .06 µg of DNA if 1 µg of total DNA digest is added to the lane). For small band sizes (< 200 bp), it may be necessary to use CIP or TAP dephosphorylation and ligation using the digested DNA solution without gel purification.

1. Determine the number of lanes you wish to run. Always plan for 1-2 lanes of the DNA ladder (2 especially if this gel will be cut up and DNA removed from it), or another suitable control. Most lanes can hold 20-25 µL of sample, so larger samples may need to be run on two lanes, or use the larger lane combs (40-50 µL capacity). The small gel box can hold 6 lanes of large capacity or 10 lanes of smaller capacity. The larger gel box can hold 12 lanes of large capacity or 20 lanes of small capacity. The large gel box is also capable of having 2 combs at a time (the second placed ½ down the box), and so its capacity can double at the cost of distance over which it can separate bands.

2. Once your gel box is selected, determine the concentration of gel you wish to make (see description for details). The concentration is the mass of agarose/mL of gel x 100%. The small gel box supports gels of 50 mL (potentially up to 75 mL, but 50 is easier to use) and the large gel box supports gels of 200 mL.

3. Set up the gel box by removing the gel tray. Make sure the rubber seals are still in their grooves. Apply a small amount of water to the inside of the side walls of the gel box and to the ends of the gel tray that have the rubber seals. Slide the gel tray into the gel box so that the open ends of the tray are against the box walls, and so that the rubber seals have not rolled up out of their grooves (if the seals moved, return them to their grooves and try sliding the tray in again).

4. Add the correct gel combs to the gel. The main comb for both gels goes into the first notch on the gel tray (should be 1-2 cm from an end), the secondary comb for the large gel is placed in the notch in the middle of the tray. The small gel combs have two sides (one thinner than the other) the thinner side has about 2/3 of the capacity volume of the thick side (which has the capacities listed in #1), so choose what you need.

5. In a flask that can hold at least 4x your gel's volume, add the correct volume of 1x TAE buffer. DO NOT ADD WATER – the gel will not work correctly.

6. Weigh out the correct mass of agarose (NOT AGAR) and add it to the flask.

7. Microwave the solution until it boils. There are two stages of boiling – where small white bubbles form (frothy) and where large clear bubbles form. You want to let it boil a bit past the frothy stage and into the clear bubble stage. These bubbles will pop naturally, and will keep you from having a bubble filled gel. It is necessary when using higher gel concentrations (and recommended for all other concentrations) that the microwaving occur in 30 second increments, with the solution being stirred by GENTLE rotation (wear protective heat gloves) after each 30 second period, to ensure proper agarose distribution.

8. After microwaving add Ethidium Bromide to the gel solution. WARNING – carcinogen, glove use is advised (if you get it on yourself, wash your skin with water for 5 minutes – its very water soluble). Add 1 µL of Ethidium bromide for EACH 10 mL of gel volume (5 µL for small gel, 20 µL for large gel).

9. Mix Ethidium Bromide into the solution by GENTLE swirling (to avoid bubbles).

10. The gel solution can be allowed to cool slightly before pouring into the gel box (pouring boiling solution into gel box can cause it to warp and bend over time).

11. Gently pour the gel into the gel tray by leaning it on the gel box wall farthest from the top comb, and slowly tipping it into the box. This prevents bubbles from forming in the gel, and if they do form, forms them near the bottom of the gel.

12. If additional bubbles form in the gel box, while the gel is still liquid take a pipette tip and push the bubbles to the bottom edge of the gel, where they won't interfere with DNA movement.

13. Allow gel to cool for 40 minutes to 1 hour. Test solidification by gently pressing on the bottom corner of the gel with a finger, it should feel solid and gel-like (not liquid).

VII. Gel Purification – Utah State – 2011

http://2011.igem.org/Team:Utah_State/Notebook

This procedure was carried out using the Qiagen QIAquick Gel Extraction Kit.

VIII. Ligation – Utah State – 2011

http://2011.igem.org/Team:Utah_State/Notebook

Ligation reactions are used to combine two linear fragments of DNA into a circular plasmid. The ligation procedure can be modified based on how much backbone DNA you have and how much insert you have, as well as if a phosphatase such as CIP (calf intestinal phosphatase) or TAP (thermo-sensitive alkaline phosphatase) was used in preparing the linear DNA molecules.

Example protocol for ligation (to be added to a PCR tube)

10µl Insert DNA

3µl Vector DNA

2µl 10X ligation buffer

34µl H2O

1µl T4 DNA ligase

Note: This protocol can be adjusted depending on the concentration of insert and vector.

IV. PCR – Utah State – 2011

http://2011.igem.org/Team:Utah_State/Notebook

Add the following reagents to a tube (50 µl reaction) in the following volumes and order:

32µl sterile H2O

5 µl 10X buffer

2µl dNTP Mix

3μl $MgCl_2$

6μl cells/DNA

0.25μl Taq Polymerase

1μl Primer 1

1μl Primer 2

The thermocyler is setup beforehand with the desired protocol. Typically: 94˚C for denaturing, 50-60˚C for primer annealing, and 72˚C for polymerase extending.

Example setup:

Step 1. 94˚C 2min 1x

Step 2. 94˚C 45sec

Step 3. 55˚C 45 sec

Step 4. 72˚C 1min 15 sec

Step 5. 72˚C 5min 1x

Step 6. 4˚C indefinitely

Repeat Steps 2 and 4 35x

V. Luminescence Protocol – Utah State – 2011

http://2011.igem.org/Team:Utah_State/Notebook

Reagents:

http://www.promega.com/products/reporter-assays-and-transfection/reporter-assays/dual_luciferase-reporter-assay-system/

1X Phosphate Buffered Saline solution (PBS)

1X Passive Lysis Buffer (PLB)

1X LARII

1X Stop & Glo

Luminometer :

http://www.promega.com/products/instruments/luminometers/glomax-20_20-luminometer/

Luminometer software protocol should be set for 10 second integration.

1. Innoculate overnight cultures, incubate for 12 to 16 hours.

2. Add 0.5ml of culture to 1.5 ml centrifuge tube.

3. Pellet cells for 5 minutes at 10,000 RCF.

4. Discard supernatant.

5. Suspend cell pellet in 0.5 ml 1X PBS by vortex.

6. Pellet cells for 5 minutes at 10,000 RCF.

7. Discard supernatant.

8. Suspend cell pellet in 0.5 ml 1X PLB by pipette.

9. Agitate cells on shaker table at room temperature for 15 minutes.

10. Add 50 ul of LARII to a new 1.5 ml centrifuge tube.

11. Place 50 ul tube in luminometer, add 10 ul of lysed cells. Pipette up and down several times to ensure mixing.

12. Record first luminometer measurement.

13. Add 50 ul of Stop & Glo reagent to tube, vortex for 1 second.

14. Record second luminometer measurement.

Restriction Enzyme Double Digest – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials:

- **22 uL dH2O**
- **1 uL BSA**
- **5 uL Buffer**
- **20 uL Template**
- **1 uL Enzyme 1**
- **1 uL Enzyme 2**

Buffer Compatibility Chart:
Number indicates percent activity of enzyme in given solution.

	1	2	3	4
EcoRI	100	100	100	100
SpeI	75	100	25	100
PstI	75	75	100	50
NheI	100	100	10	100
XbaI	0	100	75	100

Procedure

- **Mix reactants thoroughly.**
- **Place at 37 C for 3 hours.**

- **Increase to 80 C for 20 minutes to kill enzymes (some enzymes need only a 65 C heatkill, check enzyme).**
- **Run on a gel and extract product.**

Print this protocol

Gel Extraction and Purification – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials

- **GeneJET Gel Extraction Kit**
- **Binding Buffer (1 uL for every mg of agarose gel)**
- **700 uL of Wash Buffer**
- **50 uL of Elution Buffer**

Procedure

- **Add the binding buffer to the gel slice in a microcentrifuge tube.**
- **Incubate the gel mixture at 50-60 °C for 10 minutes (until melted).**
- **Transfer the solution to a GeneJET purification column.**
- **Centrifuge for 30-60 seconds at 12000 x g and discard the flow through.**
- **Add Wash Buffer and centrifuge for 1 minute.**
- **Discard flow through, then centrifuge empty column for 1 minute.**
- **Transfer the column into a fresh 1.5 ml microfuge tube.**
- **Add Elution Buffer.**
- **Centrifuge for 1 minute and collect the flow-through.**

Print this protocol

Transformations – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials

- **Competent cells**
- **DNA template**
- **800 uL of LB**
- **LB+antibiotic plates**

Procedure

- **Thaw competent cells on ice.**
- **Transfer 50 uL of competent cells to chilled falcon tubes.**
- **Add 1 uL of template to cells (2.5 uL if dilute).**
- **Incubate on ice for 30 minutes.**
- **Heat schock in 42 °C water bath for 90 seconds.**
- **Immediately place back onto ice for 2 minutes.**
- **Add 800 uL of LB to each tube.**
- **Incubate at 37 °C for 1 hour.**
- **Place 200 uL of the transformed cells on plates containing LB and the appropriate antibiotic.**
- **Incubate overnight at 37 °C.**

Print this protocol

Liquid Cultures – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials

- **LB**
- **Plated colonies of cells**
- **Antibiotic stock**

Procedure

- **Working near a flame or in a laminar flow hood, add 5 mL of LB to each falcon tube.**
- **Add the appropriate amount of antibiotic.**
  - **For example, 5 uL of 35 mg/mL chloramphenicol stock or 10 uL of 100 mg/mL carbenicillin stock.**
- **With a tip, scoop a colony from your agar plate and place it in the falcon tube.**
- **Incubate overnight at 37 °C.**

Print this protocol

PCR – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Mix

- **10ul Q solution**
- **5ul 10x buffer**
- **1.25ul DNTPs**

- **1ul Forward primer**
- **1ul Reverse primer**
- **1ul Template**
- **.3ul Taq**
- **.15ul PFU**
- **30ul dH2O**

Print this protocol

Minipreps – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials

- **Liquid culture**
- **Miniprep kit (QIAprep Spin Miniprep Kit)**

Procedure

- **Centrifuge liquid culture of cells.**
- **Discard the supernatant.**
- **Resuspendd the pelleted cells in 250 uL of Buffer P1 and transfer to a microcentrifuge tube.**
- **Add 250 uL of Buffer P2.**
    - **Invert 4-6 times until the solution become clear.**
- **Add 350 uL Buffer N3.**
    - **Invert 4-6 times.**
- **Centrifuge for 10 minutes at 17900xg.**
- **Apply the supernatant to a spin column.**
    - **Centrifuge for 1 minute and discard the flow-through.**
- **Wash the spin column with 0.5 ml Buffer PB.**
    - **Centrifuge for 1 minute and discard the flow through.**
- **Wash the spin column with 0.75 ml Buffer PE.**
    - **Centrifuge for 1 minute and discard the flow through.**
- **Centrifuge an additional 1 minute to remove residual wash buffer.**
- **In a clean 1.5 ml microcentrifuge tube, elute DNA with 50 ul Buffer EB.**
- **Centrifuge for 1 minute and collect the flow-through.**

Print this protocol

Ligation – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials

- **Digested vector**
- **Digested insert**
- **Water**
- **T4 DNA ligase.**
- **T4 DNA ligase buffer.**

Procedure

- **Mix these materials in the amounts determined by the reaction volume calculator.**

media:UC_Davis_Reaction_Volume_Calculator.xls

Print this protocol

Error-Prone PCR – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

This PCR protocol generally introduces between one and seven mutations (i.e. single base substitutions) per 1000 bases of template DNA. This is due to an increase in the magnesium chloride concentration, the addition of manganese chloride, the low template concentration, an unbalanced solution of dNTPS (that is, A,G,C, and T are not all present at the same concentration) and the use of Taq polyermase without an error-checking enzyme.

Prepare Unbalanced DNTP Mix:

- **20uL dATP**
- **20uL dGTP**
- **100uL dCTP**
- **100uL dTTP**
- **260uL H2O**

(Store at -20 C)

Create a Mutant PCR Mix using:

- **5ul 10x buffer**
- **2.5ul Unbalanced DNTPs**
- **1ul Forward primer**

- **1ul Reverse primer**
- **10uL 25 mM MgCl2**
- **7.5uL 1 mM MnCl2**
- **22.4ul dH2O**

These values can be multiplied by 5x, 10x or however much you like, so long as the mixture is stored at -20C. Aliquot 49.4 uL of this solution into a PCR tube per each reaction and add:

- **0.1ul Template**
- **.5ul Taq**

Mix the reaction thoroughly and place it in a thermal cycler. You can use the same program that you would for a conventional PCR reaction, with run times appropriate for the length of your template. We used around twenty cycles and an elongation time of 30 seconds for our promoter mutants, which took only a few hours to complete per cycle.

To increase the mutation rate, dilute the results of one error-prone PCR reaction by roughly 100x and repeat the process, using the new product as template. This will increase the number of mutations per 1000 base pairs accordingly -- after three rounds, our LacI promoter mutants showed between 1 and 7 mutations per 300 bases.

Print this protocol

Nanodrop – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

- **Log in to nanodrop program.**
- **Moisten a Kim wipe and clean the pedestal.**
- **Apply 2ul H2O to pedestal and click 'OK'.**
- **Press 'Blank' button.**
- **Wipe blank from pedestal using Kim wipe.**
- **Apply 2ul of desired sample to pedestal.**
- **Click 'measure'.**
- **Print results.**

Print this protocol

Glycerol Stock – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

- **Make liquid culture of desired cell strain. Grow for 12-16hrs if using Dh5a.**
- **Mix 800ul of grown liquid culture with 800ul of 80% glycerol. Shake with vigor.**
- **Flash freeze using liquid nitrogen.**

- **Keep at -80C**
    - **Note: When streaking plates from glycerol stock, it is important to leave glycerol stock tube on dry ice.**

Print this protocol

PCR Purification – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

- **For every volume of PCR product, add 2 volumes Binding Buffer. (ex. add 100ul Binding Buffer to 50ul PCR reaction)**
- **Transfer to spin column and spin for 1 minute.**
- **Discard flowthrough.**
- **Add 500ul Wash Buffer and spin for 1 minute.**
- **Discard flowthrough.**
- **Spin for 2 minutes.**
- **Put spin column in clean 1.5ml tube and elute with desired volume.**

Print this protocol

Registry Part Distribution Rehydration – UC Davis – 2011
http://2011.igem.org/Team:UC_Davis/Protocols

Procedure

- **Add 20ul sterile H2O to desired well in distribution plate.**
- **Incubate at room temperature for ~10 minutes.**
- **Transfer resuspension to microcentrifuge tube.**

Print this protocol

Making Competent Cells – UC Davis – 2011 http://2011.igem.org/Team:UC_Davis/Protocols

Materials

- **0.5M PIPES buffer (piperazine-1,2-bis[2-ethanesulfonic acid])(pH 6.7)**
    - **Dissolve 15.1g of PIPES in 80ml of pure H2O. Adjust to pH 6.7 with 5M KOH and bring volume to 100ml with pure H2O. Filter sterilize and store at -20C**
- **Inoue transformation buffer**

- **Dissolve 10.88g McCl2.4H2O, 2.20g CaCl2.2H2O, 18.65g KCl, and 20ml PIPES in 800ml pure H2O and fill to 1L**
- **Filter sterilize and store at -20C**

- **DMSO**

Procedure

- **Starting around noon, pick a single bacterial colony (2-3mm in diameter) from a plate that has incubated for 16-20hrs at 37C. Transfer colony into 25ml of LB medium in a 250ml flask. Incubate for 6-8hrs at 37C with vigorous shaking.**
- **At about 6:00pm, use the starter culture to inoculate three 1L flasks each containing 250ml LB. The first flask receives 6ml of starter culture, the second receives 4ml, and the third receives 2ml. Incubate all three flasks at 18-22C overnight with moderate shaking.**
- **The following morning, read the OD600 of all three cultures monitoring every 45 minutes.**
- **When one of the cultures reaches an OD600 of 0.55, transfer the culture vessel to an ice-water bath for 10 minutes. Discard the other cultures unless you desire to do multiple batches at once.**
- **Harvest the cells by centrifugation at 2500g (3900rpm in a Sorvall GSA rotor) for 10 minutes at 4C.**
- **Pour off the medium and store the open centrifuge bottle on a stack of paper towels for 2 minutes. Use a vacuum aspirator to remove any drops of remaining medium adhering to walls of the centrifuge bottle or trapped in its neck.**
- **Resuspend the cells gently in 80ml of ice-cold Inoue transformation buffer.**
- **Harvest the cells by centrifugation at 2500g for 10 minutes at 4C**
- **Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 minutes. Again, use the vacuum aspirator to remove any drops.**
- **Resuspend the cells gently in 20ml of ice-cold Inoue transformation buffer.**
- **Add 1.5ml of DMSO. Mix the bacterial suspension by swirling and then store it in ice for 10 minutes.**
- **Working quickly, dispense aliquots of the suspensions into chilled, sterile microcentrifuge tubes. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen. Store the tubes at -80C until needed for transformation.**

Preparation – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

1. Inoculate a single colony into 5 mL of LB media without any antibiotics and grow overnight at 37 °C with vigorous shaking.

2. Inoculate 1 mL of the desired strain into 100 mL of fresh LB, use a 500 mL flask.
3. Incubate at 37 °C with vigorous shaking until 0.3 - 0.4 OD_{600}
4. Put the flask on ice. Pre-chill 50 mL centrifuge tubes and the centrifuge itself at 4°C.
5. Centrifuge 50 mL of the culture at 8,000 rpm for 5 minutes at 4 °C.
6. Remove the supernatant and add 10 mL of cold $CaCl_2$ 0.1 M. Vortex until the pellet is resuspended.
7. Incubate on ice for 30 minutes, shake the tube once in a while.
8. Centrifuge at 8,000 rpm for 5 minutes at 4°C. Remove the supernatant and add 2 mL of $CaCl_2$ 0.1 M. Resuspend carefully using a micropipette. Keep always on ice.
9. Mix the two preparations in a tube and store on ice, or use for transformation.

Note: The competent cells can be stored on ice up to two weeks.

Transformation of Ca^{+2} competent cells of *Escherichia coli* – UNAL Mty-Mexico – 2011
http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

1. Add 50 µL of Ca^{+2} competent cells to a pre-chilled centrifuge tube. Keep always on ice until step 4.
2. Add plasmid DNA (100 ng) or ligation (up to 5 µL) depending on DNA concentration.
3. Use 1 µL of a 1 ng/µL DNA sample as positive test in a separate tube. It is recommended to use a DNA-free negative test tube as well.
4. Chill the tube on ice for 20 - 30 minutes.
5. Expose the reaction mixture to a 42ºC 1 minute heat-shock.
6. Put the tube on ice for 2 minutes.
7. Add 200 µL of antibiotic-free LB media.
8. Incubate at 37ºC for 20 - 30 minutes.
9. Spread the appropriate quantity of cells (50-200 µL) on selective LB agar plates.
10. Incubate overnight at 37º C.
11. The positive plate must have around 1,000 colonies as an optimal ($1X10^6$ transformants per µg supercoiled DNA).

Notes:

Until heat-shock, handle the tubes from the upper part to avoid warming the cells. Low temperature is critical for successful transformation.

Avoid transforming with more than 5 µL of ligation mixture, as ligation buffer may reduce transformation efficiency.

Electrocompetent *E. coli* cells preparation – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

Preparation

1. Inoculate a single colony of *E. coli* in 5 mL of LB media. Grow overnight or for 5 hours at 37°C with shaking at 250 rpm.
2. Inoculate 2.5 mL of the previous culture in 200 mL of LB media in a 2 L flask. Grow at 37 °C shaking at 300 rpm until the culture reaches an OD of 0.5 - 0.7.
3. Chill the cells on ice for 10 - 15 minutes and then transfer the cells into a pre-chilled centrifuge bottle.
4. Centrifuge at 4,200 rpm for 10 minutes at 2 °C (Beckman J-6M).
5. Remove the supernatant and resuspend the pellet in 5 mL of cold water. Add 200 mL of cold water and mix well. Centrifuge at 4,200 rpm for 10 minutes at 2 °C.
6. Remove the supernatant and resuspend the pellet by shaking gently in the remaining liquid volume.
7. Add 200 mL of cold water, mix well and centrifuge at 4,200 rpm for 20 minutes at 2°C.
8. Add 20 mL of 10% cold glycerol and mix well. Centrifuge at 4,200 rpm for 20 minutes at 2 °C.
9. Add 10 mL of 10% cold glycerol to each tube. Resuspend and gather all the content of the tubes in a single tube, centrifuge and remove the supernatant.
10. Estimate the pellet volume and add an equal volume of 10% cold glycerol. Resuspend the cells.
11. Divide the final volume into pre chilled tubes (100 µl) and store at -80 °C.

Note: Pre-chill all the materials that will be in contact with the cells.

Transformation competent cells of *Escherichia coli* by Electroporation – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

1. Take a tube with 50 µL of electrocompetent *E. coli* cells, thaw on ice.
2. Add a volume containing 100 ng of DNA.
3. Carefully transfer the cell/DNA mix into a pre-chilled electroporation cuvette. Make sure to deposit the cells at the bottom and not to introduce any air bubbles.
4. Electroporate under the following conditions:
5. Immediately add 250 µL of SOC media to the cuvette.
6. Incubate with vigorous shaking (250 rpm) at 37 °C for 1 hour.
7. Add 750 µL of LB media and mix by pipetting up and down.

8. Spread 200 µL of cells onto a selective LB agar plate.

Note: All must be performed on ice. Electroporation cuvettes are previously chilled on ice. DNA and bacteria must be thawed on ice too.

Mini preparation of plasmid DNA – UNAL Mty-Mexico – 2011
http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

1. Pour 1.5 mL of the culture in a 1.5 mL microcentrifuge tube and centrifuge at 14,000 rpm for 30 seconds. Remove carefully the supernatant.
2. Add 200 µL of Solution I. Resuspend the pellet by using vortex briefly or by pipetting up and down. Incubate at room temperature for 5 minutes.
3. Add 200 µL of Solution II and mix gently by inverting and rotating the tube several times. Do not vortex. Incubate at room temperature for 5 minutes.
4. Add 200 µL of Solution III and mix gently by inverting and rotating the tube several times. Incubate the tube on ice for 5 minutes.
5. Centrifuge at 14,000 rpm for 5 minutes.
6. Transfer the supernatant to a fresh tube containing 1 mL of 100% ethanol.
7. Incubate at -20 ºC for 10 minutes. (Max. 2 h)
8. Centrifuge at 14,000 rpm for 10 minutes. Remove the supernatant.
9. Add 200 µL of 70% ethanol and vortex gently for 10 seconds.
10. Centrifuge at 14,000 rpm for 5 minutes. Remove the supernatant by pipetting. Aspirate off any residual supernatant.
11. Dry at 37ºC for 5 minutes.
12. Add 20 µL of H_2O + 20 µg/mL of RNase. Resuspend by using vortex briefly.
13. Run an agarose gel (0.8%) or store at 4 ºC.

Solutions for Mini preparation of Plasmid DNA – UNAL Mty-Mexico – 2011
http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

Solution I (200 mL)	**milliliters or grams**
- Tris HCl 1 M (pH 8.0)	5 mL
- EDTA 0.5 M (pH 8.0)	4 mL
- Distilled H_2O	Bring the final volume up to 200 mL
Solution II (200 mL)	

- NaOH 10N	4 mL
- SDS (powder)	2.0 gr
- Bidistilled H_20	Bring the final volume up to 200 mL
Soll III (200 mL)	
- Potassium acetate (CH_3CO_2K)	58.8 gr
- Acetic acid (CH_3-COOH)	23.0 mL
- Distilled H_20	Bring the final volume up to 200 mL

Notes:

When preparing Solution II, first add a little bidistilled water, then add NaOH and dissolve carefully SDS. Finally, bring the final volume up to 200 mL with bidistilled water.
When preparing Solution III, first add 100 mL of H_2O and then the potassium acetate. Once it has been add the acetic acid and finally bring the final volume up to 200 ml with bidistilled water.

Agarose Gel Electrophoresis Protocol – UNAL Mty-Mexico – 2011
http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

To be loaded:

- DNA molecular size marker (λ *Pst*I): 2 - 3 µL
- Plasmid DNA: 2 - 3 µL
- Enzyme restrictions: 10 µL
- PCR products: 5 µL

Procedure:

1. Prepare an agarose gel of the desired concentration (see Agarose gels section).
2. Add the necessary SB 1X buffer into the electrophoresis tank to cover the gel.
3. Load the first well with marker, and then load the DNA samples mixed with loading buffer into the wells.
4. Plug in the anode and cathode cables so that the DNA samples can move through the gel toward the anode.
5. Run the electrophoresis at 200 volts.

6. Wait approximately 20 - 30 minutes or until the bromophenol blue reaches the end of the gel and stop the electrophoresis.

Note: DNA moves toward the positive electric field (anode, usually red cable) due to the negative charges.

Agarose gel – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

Concentration for supercoiled and plasmid DNA: **0.8%**

For digestion reaction fragments over 1,000 bp: **0.8%**

For digestion reaction fragments below 500 bp: **1.5%**

DNA size marker (λ + *Pst*I): Use 2 or 3 μL per gel.

Note: Not needed when running supercoiled DNA samples, like plasmid DNA.

SB buffer 20X – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

SB (Sodium Borate) electrophoresis buffer, 20X Stock:

1. In 700 mL of distilled H2O, dissolve 8 gr of NaOH.
2. Weight 51 of Boric Acid and dissolve ¾ parts in the NaOH solution
3. Dissolve the remaining Boric Acid until the buffer reach pH 8.0.
4. Complete to 1 L with distilled H2O and store in a sterile flask.

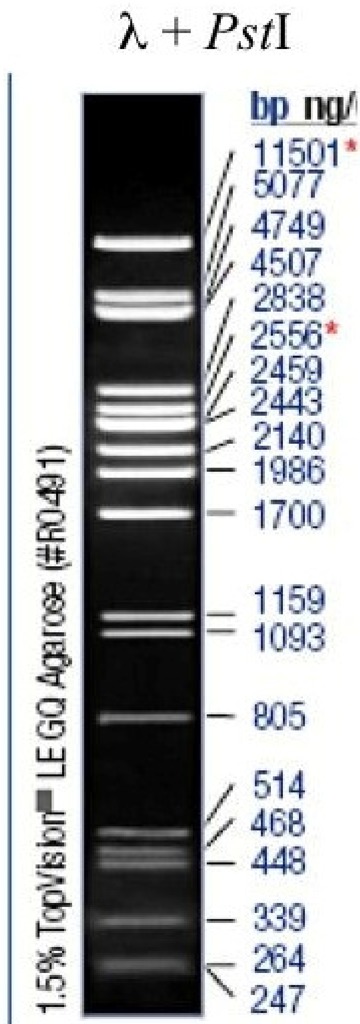
Note: Use SB 1X as buffer to run agarose gels up to 200 volts

Ethidium Bromide Gel Staining – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

1. Dilute the stock to 20 μg/mL in a special container with the gel buffer.
2. Put the gel into the container.
3. Let it stain for 3 - 5 minutes.
4. Take the gel out of the container and soak the stained gel in water for 5 minutes or more to clear background ethidium bromide from the gel.
5. View the gel under a UV light source or on a UV transilluminator.

Note: If you want to use ethidium bromide, confine its use to a small area of your laboratory. Wear gloves when staining, handle stained gels, and dispose of any waste.

Lambda/PstI Molecular Size Marker



Lambda molecular size marker.

Mix:

- phage λ DNA (500 ng/μL) 50.0 μL
- *Pst*I 2.5 μL
- Buffer 10X 6.0 μL
- H_20 1.5 μL

Procedure:

1. Mix the ingredients listed above
2. Incubate at 37ºC / 45 minutes

3. Again add 2.5 µL *Pst*I
4. Incubate 37ºC / 45 minutes
5. Add 6.0 µL Loading buffer 6X

Check on agarose gel.

Notes:

Final concentration: 0.30 µgr/µL

Final Volume: 66.0 µL

Restriction enzyme digestion of DNA – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

Mix for 1 reaction, final volume of 20 µL

Add the following to a microcentrifuge tube:

DNA	**2-3 µg**
Buffer 10x	2.0 µL
Enzyme (10 U/µL)	0.3 µL (1 enzyme unit per µg DNA)
H_2O	Until 20.0 µL

Incubate the mixture at 37 °C (it may change, check enzyme specifications) for 1 - 1.5 hours.

Note: Prepare a mix when possible to minimize enzyme handling.

PCR – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

PCR reaction mix	
DNA template	Total 100 ng (In 25 µl)
Buffer 10x	2.5 µL
Mg^{++} 50 mM	0.75 µL
dNTPs 20 mM	0.25 µL
Primer Fwd 100 ng/µL	0.50 µL

Primer Rv 100 ng/µL	0.50 µL
Taq Pol 5 U/µL	0.25 µL
H_2O	To bring the volume up to 25 µL

Procedure:

1. Add the corresponding H_2O to a sterile PCR tube.
2. Add the rest of the components but the enzyme and DNA.
3. Add the enzyme, mix gently.
4. Add the respective DNA sample and mix gently.
5. Spin the tube briefly.
6. Place the sample in the thermocycler and start your PCR program.

Notes:

Put on gloves before taking the PCR mix components out of the freezer.

DNA must be added at last because it may form complexes with Mg^{++} and inhibit the reaction.

When possible, make a mix with all the common components to minimize enzyme waste.

Antibiotics – UNAL Mty-Mexico – 2011 http://2011.igem.org/Team:UANL_Mty-Mexico/Notebook/Protocols

Antibiotic	Final concentration	Stock concentration	µL per mL
Spectinomycin (Sp)	100 µg/mL	20 µg/µL	5
Ampicillin (Amp)	50 µg/mL	10 µg/µL	1
Kanamycin (Kan)	50 µg/mL	50 µg/µL	1
Chloramphenicol (Cm)	34 µg/mL	34 µg/µL	1
Tetracycline (Tet)	10 µg/mL	5 µg/ µL	2

Notes:

Always verify stock concentration, in case of unknown assume the one indicated above.

When using more than one antibiotic simultaneously use half the concentration for each antibiotic.

Electrophoresis Agarose - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Measure 60 ml of TBE (.5 X) with the probe.

2.Take a beaker and place 60 ml of TBE.

3.Place the filter paper on the scale.

4.Weigh 600mg of agarose gel on the scale.

5.Mix 600mg of agarose gel in TBE and heat it in the microwave for time intervals of 10 seconds.

6.Place the electrophoresis comb in the tray electrophoresis.

7.Pour the solution into the electrophoresis tray, making sure the level does not reach beyond the teeth of the comb. Wait until it solidifies.

8.Remove the electrophoresis comb and place the tray electrophoresis in the electrophoresis chamber.

9.Pour TBE by the sides of the electrophoresis chamber until the level of TBE exceeds the gel.

10.Mix the DNA samples, molecular weight ladder and DNA supercoil with 12µl of loading buffer and SYBR Green 4µl.

11.Load the wells with the samples made in the previous step.

12.Connect the electrophoresis chamber to the power supply at a voltage between 60V and 70V and run it for 1.5 hours.

13.Use transilluminator to see results.

Digestion Reaction - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Perform the necessary calculations using the calculator developed by the Tec-Monterrey 2010 team. (click here)

2.Add the required amounts of buffer, plasmid DNA, nuclease free water and BSA (keep reagents in ice except the sample DNA).

3.Pour the required amount of enzyme at the end.

4.Incubate for 1 hour at 37 ° C.

DNA Extraction - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Take 1ml of transformed cells in a 1.5ml eppendorf tube.

2.Centrifuge for 1min at 10,000 rpm.

3.Discard the supernatant. Repeat step "1" 5 times.

4.Take 250µl of Cell Resuspension Solution to resuspend the pellet.

5.Take 250µl of Cell Lysis Solution. Immerse it 4 times and incubate for 4min.

6.Take 10µl of Alkaline Protease Solution and incubate for 4min.

7.Take 350µl of Neutralization Solution. Immerse it 4 times.

8.Centrifuge at 14,000 rpm for 10min.

9.Place the minicolumn in a collection tube.

10.Remove the supernatant with a point and place it in the minicolumn.

11.Centrifuge for 1min at 14,000 rpm.

12.Remove the liquid from the collection tube and replace the minicolumn.

13.Take 750µl of Column Wash Solution and place it in the minicolumn.

14.Centrifuge for 1min at 14,000 rpm.

15.Remove the liquid from the collection tube and replace the minicolumn.

16.Take 250µl of "Column Wash Solution" and place it in the minicolumn.

17.Centrifuge for 2 min at 14.000 rpm.

18.Remove the liquid from the collection tube. Take the minicolumn and place it in a 1.5ml microcentrifuge tube.

19.Take 100µl of nuclease free water and pour it in the minicolumn.

20.Centrifuge for 1min at 14,000 rpm.

21.Store at -20 ° C.

Purification - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Cut the band of interest and weigh.

2.1µl of Mem Bind Solution will be placed for each mg of gel.

3.Heat the gel with a water bath at 50 ° C to dissolve the gel.

4.Incubate at 25 ° C for 1min.

5.Place the minicolumn with its collection tube.

6.Pour the liquid incubated in the minicolumn.

7.Centrifuge at 14,000 rpm for 2min.

8.Decant the solution of the collection tube and replace the minicolumn.

9.Add 750µl of Mem Wash Solution to the minicolumn.

10.Centrifuge at 14,000 rpm for 2min.

11.Decant the solution of the collection tube and replace the column.

12.Add 500µl of Mem Wash Solution to the minicolumn.

13.Centrifuge at 14,000 rpm for 2min.

14.Decant the solution of the collection tube and replace the minicolumn.

15.Centrifuge 1 min.

16.Take the mini-column and placed in a 1.5ml eppendorf tube.

17.Pour 35µl nuclease-free water in the minicolumn.

18.Centrifuge at 14,000 rpm for 1min.

19.Store at -20 ° C.

Calciumcompetents - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Cells should have an optical density of .4 (always keep tubes on ice).

2.Centrifuge at 5,000 rpm, 2 ° C for 5min.

3.Decant supernatant.

4.Add 1 ml of CaCl2 to resuspend the pellet.

5.Add 20ml CaCl2.

6.Centrifuge 5,000 rpm, 2 ° C for 5min.

7.Decant supernatant.

8.Add 1 ml of CaCl2 to resuspend the pellet.

9.Add 20ml CaCl2.

10.Centrifuge 5,000 rpm, 2 ° C for 5min.

11.Decant supernatant.

12.Add 500µl of glycerol + CaCl2.

13.Store at -80 ° C.

Transformation using Microwave - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.You need to have calcium competent cells.

2.Take 50µl of cells and mixed with 2µl of BioBrick resuspended.

3.Place on ice for 15min.

4.Place in microwave on level 2 for 1min.

5.Place on ice for 1min.

6.Add 200µl of LB.

7.Incubate for 1 hour at 37°C with stirring.

8.Cultivate in a dish 50µl of bacteria and 150µl in another dish.

Preparation of Electrocompetents - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Streak *Escherichia coli* Top10 cells onto LB-agar plate with no antibiotics and incubate at 37°C overnight.

2.Pick one colony and place it in a 50 mL tube with 20 mL LB medium. Incubate overnight on a shaker at 37°C and 350 rpm.

3.Add 250 ml of LB medium to a flask and add the overnight culture until an OD600 of 0.1 is reached.

4.Place the flask on a shaker at 37°C, 350 rpm until an OD600 between 0.4-0.6 is reached.

5.Transfer the diluted culture to 50 mL tubes.

6.After this step, the cells must be kept at 4°C at all times. Place the cells on ice for 15 minutes.

7.Cool the centrifuge to 4°C.

8.Centrifuge the tubes for 10 min at 8000g at 4°C.

9.Remove supernatant and gently resuspend pellets with 10 mL cold sterile water by pipetting. Add the rest of the water to a total volume of 50 mL.

10.Centrifuge a second time for 10 min at 8000g at 4°C.

11.Remove supernatant and gently resuspend pellets with 10 mL cold sterile water by pipetting. Add the rest of the water to a total volume of 50 mL.

12.Centrifuge a third time for 10 min at 8000g rpm at 4°C.

13.Remove supernatant and gently resuspend pellets with the remaining water (if it's too little, add some more).

14.Calculate and add glycerol so that the final concentration is 10-15 %.

15.Resuspend the cells and aliquot 50 µL per 0.2 mL tube (tubes on ice) and store at -80°C.

Transformation via Electrophoresis - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Chill electroporation cuvettes, DNA samples and tubes on ice.

2.Once cuvettes are cold, remove electrocompetent cells from -80°C freezer and thaw on ice.

3.Turn on electroporator and set voltage to 2.5 kV.

4.Dial a micropipette to 1 or 2µL of DNA sample.

5.Dial a micropipette to 50µL of electrocompetent cells.

6.Dial a micropipette to 1000µL and pipet in SOC. Place micropipette on counter such that tip doesn't touch anything.

7.Pipet 1-2µL of DNA sample and place inside the cuvette.

8.Pipet 50µL of electrocompetent cells inside the cuvette ensuring they mix with the DNA sample. Do not pipet up and down.

9.Place cuvette back on ice to ensure it remains cold.

10.Tap the cuvette on the counter gently so that cells are at the bottom and to remove any air bubbles.

11.Wipe off excess moisture from outside of cuvette.

12.Place in chamber of electroporator so that the cuvette sits between electrodes.

13.Pulse the cells with a shock by pressing button on electroporator.

14.Remove cuvette from the chamber and immediately add SOC.

15.Transfer cuvette to 37°C incubator and shake at 350 rpm to promote aeration. Incubate for 1 hr.

16.Plate 100 µL transformation onto LB-agar plate supplemented with appropriate antibiotic.

17.Incubate plate overnight at 37°C until colonies appear.

Ligation - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Take an Eppendorf tube and add the required amounts of reagents.

2.Mix gently and centrifuge so that the contents of the tube to the bottom.

3.Incubate at room temperature for 5 min.

4.Use 2µl of the ligation reaction to transform 100µl of competent cells.

PCR - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.In a microfuge tube mix in the following order:

a)10X Amplification Buffer (5µl)

b)20mM Solution of four dNTPs pH=8.0 (1µl)

c)20µM forward primer (2.5µl)

d)20µM reverse primer (2.5µl)

e)1-5 units/µl thermostable DNA polymerase

f)H2O (28-33µl)

g)Template DNA (5-10µl)

h)Total Volume (50µl)

2.Program the thermocycler at the necessary conditions for denaturation, annealing and polymerization.

Elaboration of Polyacrylamide Gel - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

This is the recipe to prepare an Acrylamide Gel (10%):

Gel Percentage (%)	10	4
	Resolving	Stacking
Acrylamide 40% ml	4	.4
H_2O ml	7.71	2.52
Gel Buffer pH 8.8 ml	4	0
Gel Buffer pH 6.8 ml	0	1
SDS 10%	.16	.04
PSA 10% µL	120	30
TEMED µL	12	6

1. Place the resolving solution on the glass.

2. Add 1ml of water, why? Water help's the gel to no have irregularities on the surface.

3. Let polymerize for 30 minutes.

4. Dry the water on the glass with filter paper.

5. Add the stacking solution.

6. Add the comb carefully, avoid the formation of bubbles.

7. Let stand for 1 hour and a half.

8. WATCH OUT acrylamide is neurotoxic, DO NOT swallow.

Note: Unlike other protocols, PSA cannot be prepared instantly. After you prepare it, you can refrigerate it at 4°C. When using PSA, It is highly recommended to avoid sudden temperature changes. You can use a cooler to prevent the temperature changes.

Note: PSA and TEMED are polymerizing agents (MUST NOT inhaled). Once both compounds are added, must be added nimbly to the solution in the glass, because these compounds may start polymerizing once it´s added. You need abilities, if you have a Thelma in your team leave her this work.

Cell Induction - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Place the preinoculum in wildtype and transformed cells in LB liquid medium.
2.Overnight at 35°C and 250 rpm.
3.Inoculate 6ml of LB liquid medium of each preinoculum.
4.Growth for 6 hours at 35°C and 250 rpm (OD = 0.6 - 1)
5.Induce with arabinose 1mM for several time at ypur specific conditions

Lysis Cellular to Separate Soluble and Insoluble Phase | Sample Preparation for Polyacrylamide Gel - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.6 ml of transformed cell culture is harvested by centrifugation at 12,000 x g for 1 min.

2.The supernatant is removed and the pellet is dried.

3.For each 1 g of cell pellet, 20 ml of xTractor Buffer, 40 µl of DNAse 200 µl of 100X lysozyme solution is added.

4.The suspension is incubated during 10 min at room temperature.

5.The crude lysate is centrifugated at 10 – 12000 x g for 20 min and the supernatant is called as soluble fraction and the pellet as insouble fraction.

6.The insoluble fraction is sonicated with water during about 5 sec.

7.50 µl of protein fraction is mixed with 50 µl of 2x sample buffer with 2-ME and heat 10 min at 95 °C.

Filter Paper Assay for Sacharifying Cellulase - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

1.Add 1.0 ml 0.05 M Na-citrate, pH 4.8 to a test tube of 50 ml volume.

2.Add 500 micro liters of citrate buffer.

3.Make 4 dilutions:

4.500 micro liters of citrate buffer + 1.5 mL enzyme

5.500 micro liters of citrate buffer + 500 micro liters of enzyme

6.500 micro liters of citrate buffer + 500 micro liters of enzyme

7.500 micro liters of citrate buffer + 500 micro liters of enzyme

8.Temperate to 50ºC , ad done filter paper strip, mix

9.Incubate 50ºC, 60 min.

10.Add 3.0 mL DNS, mix

11.Boil for exactly 5.0 min. In a vigorously boiling water bath containing sufficient water. After boiling, transfer to a cold water bath

12.Add deionized water to reach 20 mL of volume. Miz by completely inverting the tube several times so that the solution separates from the bottom of the tube at each immersion.

13.When the pulp has settled well, after at least 20 min, the color formed is measured against the spectro zero at 540 nm.

14.Spectro Zero: 1.5 mL citrate buffer + 3.0 mL DNS

15.Enzyme blank : 1.0 mL citrate buffer + 0.5 mL enzyme + 3.0 mL DNS

Determination of Reducing Sugars by DNS - Tec-Monterrey – 2011 http://2011.igem.org/Team:Tec-Monterrey/projectprotocols

A.Preparation of the reagent DNS

1.Heat 300 ml of distilled water in a 1 L glass to a temperature of 50° C and dissolve 5 g of dinitrosalicylic acid. Shake it.

2.Add 50 ml of a 4 M NaOH solution (NaOH 8 take it to 50 ml).

3.Add 150 g of sodium and potassium tartrate and continue stirring and heating until the complete dissolution of solids.

4.Cool the dissolution to ambient temperature.

5.Transfer this solution to a flask of 500 ml and fill with distilled water.

6.The reagent will be stored in amber bottle at room temperature, avoiding that it be placed together with oxidizing or reducing agents and flammable substances.

7.The amber bottle labeled under the name of “DNS Reagent” and records the name of the project, the person responsible for the reagent and the date.

B.Calibration curve

1.Prepare 5 standard solutions of glucose with the following concentrations:

a.2.0 mM

b.1.5 mM

c.1.0 mM

d.0.5 mM

e.0.25 mM

2.The solutions prepared in point 1 will be used to perform the 5 corresponding points on the calibration curve.

3.Prepare 150 mL of stock solution of glucose 2 mM from solid D (+) - glucose

•100 ml flask requires 0.036 g of solid.

•50 ml flask requires 0.018 g of solid.

4.Separate 25 ml of this solution in small amber bottle and pour the rest into a 150 ml beaker for later use in dilutions.

5.A sample of the stock is transferred to the appropriate flask, it’s completed with distilled water, and finally, stored in a small amber vial of 25 ml properly labeled. The quantities are summarized in the following table:

Concentration (mM)	Flask dilution (ml)	Volume Stock (ml)
0.25	25	3.125
0.50	25	6.250
1.00	25	12.50
1.50	25	18.75

* Note: to measure the milliliters of stock solution employ a 10 ml pipette and to measure the tenths employ a 1000 µl micropipette.

Note: The calibration curve should be tripled and the values to correlate (absorbance vs. standard concentration) should be the average of the replications.

A.Determination of reducing sugars in the sample.

1.Take 3 ml of each solutions of the calibration curve and transfer them into 15 ml tubes. Add 1 ml of the reagent DNS.

2.Additionally, prepare a blank solution. For blank, take 3 ml of distilled water and add 1 ml of reagent DNS.

3.The tubes are immersed boiling water for 5 minutes. After this time are dipped in cold water at room temperature.

4.Determine the absorbance of the sample @ 540 nm.

Agarose Gel Electrophoresis – **Queens Canada – 2011**

http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling

- Label the file of picture of gel according to this standard:
- [#] P-dd_mm_yy
- # is the nth gel run that day in the lab
- P is the initial of the last name of the professor whose lab the gel was run in

Materials

- Loading Dye
- 100kb+ DNA Ladder
- 5X T4 DNA Ligase (4µL)
- 1% Agarose Gel (50mL 1X TBE and 0.5g Biotech Grade Agaraose)
- Gel box and power supply
- Ethidium bromide stain
- UV box/gel imager

Making Agarose Gel

1. Weigh out 0.5g of agarose and add to a 250ml Erlenmeyer flask.
2. Add 50ml 1X TBE to the flask and mix by swirling.

3. Microwave TBE agarose until the solution becomes clear and obtains a uniform consistency. (First microwave for 1min and then for 30sec intervals. DO NOT allow the solution to boil over in the microwave).
4. Use glove to remove flask from the microwave. Allow flask to cool on the lab bench for 5 min (But do not wait until the gel starts to polymerize)
5. Take out EtBr from -20°C freezer. Add 3µL to 50mL TBE agarose and swirl the flask to mix. Return EtBr to the freezer immediately after use. (Note: EtBr is a carcinogen and a mutagen. Always use glove and lab coat, if available, to handle things contaminated with EtBr.)
6. Carefully pour TBE agarose into the casting tray to avoid bubbles. Make sure the tray is placed on a flat surface. Insert comb into the TAE agarose gel.
7. Let the gel polymerize for 20min.

Sample Preparation

1. Add appropriate amount of loading dye such that the mixture of loading dye and sample contains 1X concentration of dye. If using 5X dye, use 4 parts sample and 1 part dye.
2. Use appropriate volume of ladder (depends on the ladder used).

Electrophoresis

1. Once the gel is solidified, remove the comb carefully and place the casting tray in the gel box. Make sure the wells point towards the black (negative) electrode.
2. Add 2µL of loading dye into each well.
3. Fill the gel box with TBE until the entire gel is immersed in solution.
4. Load prepared samples into the wells. Slowly pull out the pipette tip from the well before releasing the piston of the pipette. This avoids inserting bubbles into the wells, which will disturb the sample.
5. Close the lid of the gel box. Run the gel at 100V constant voltage for 1 hour.

Imaging

1. Turn off the gel box power supply.
2. Transport the gel in a plastic box to the dark room. Bring two sets of gloves if you are doing this by yourself, because you cannot touch the computer mouse with EtBr contaminated gloves. The dark room key (with attached USB key) is in the top drawer of the gel box bench.
3. Log in to the computer.
4. Open Genesnap. Click the big green button on the left to take a picture, and manipulate it with the sliders on the right.
5. Print out the gel picture, label each lane, and paste it into your lab book.
6. Save the picture (in .tif format) in the QGEM folder on the USB stick.
7. Upload the picture to Google Docs.
8. Remove the gel and dispose of it in the proper waste bin. Wipe down the imaging machine and lock the dark room door.

(Quick) Ligation **– Queens Canada – 2011**

http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling

- Store the ligation products in the -20°C freezer

- Label the product tube as “DNA (AQ)” and in accordance with the standard labelling format, as outlined at the front of your lab book and on Google Docs

Materials

- Linear vector DNA (20-100ng)
- Insert DNA (6:1 molar ration of insert:vector)
- 5X T4 DNA Ligase (4µL)
- Water, nuclease-free (15µL-vector and insert volume)
- Total volume (20µL)

Procedure

1. Prepare a mastermix that contains T4 ligase buffer and T4 DNA ligase
2. Transfer 5µl of the mastermix to each properly labeled sample tube.
3. Add corresponding insert DNA and vector DNA into the tubes. Top the volume to 20µL using ddH2O.
4. Incubate one hour at 22^{0}C (or room temperature)
5. Heat inactivate T4 DNA ligase at 65^{0}C for 10 min.
6. Use up to 5 µL of the mixture for transformation of chemically competent cells.

Digestion **– Queens Canada – 2011** http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling

- Use product immediately (if possible), or store in -20^{0}C freezer
- Product should be labelled as "DNA (AQ)," using the standard labeling technique, as outlined in the front of your lab book and on Google Docs

Materials

- Digestion Buffer (10X, 2µL)
- Enzyme(s) (0.5-1µL)
- DNA (1µL)
- ddH2O (16-16.5µL)

Procedure

1. Remove the digestion buffer and enzymes from the -20^{0}C freezer, place on ice.
2. Add 14 uL of water to an eppendorf tube.
3. Thaw the digestion buffer and the enzymes.
4. Add digestion buffer to tube.
5. Add DNA to tube.
6. Add enzymes to tube.
7. Incubate (time and temperature are variable and depend upon the enzymes used; incubate for 5-20min at 37^{0}C)

Glycerol Stock**– Queens Canada – 2011** http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling

- Store the glycerol stock product in the -80°C freezer
- Label the product tube as “EC (GL)” and in accordance with the standard labelling format, as outlined at the front of your lab book and on Google Docs

Materials

- Overnight bacterial cell culture
- Cryogenic screw-cap vials
- 30% glycerol in H2O

Procedure

1. Pipette 750μL 30% glycerol into cryogenic vials. (Note: withdraw very slowly as glycerol is very viscous)
2. Add 750μL of overnight culture to each vial.
3. Gently vortex the cryogenic vial to ensure the culture and glycerol is well-mixed.
4. Label each vial in accordance with labeling convention.
5. Store in a freeze box in -80°C freezer.

Liquid (Bacterial Cell) Culture **– Queens Canada – 2011**

http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling

- Label the product tube as “EC” and in accordance with the standard labelling format, as outlined at the front of your lab book and on Google Docs.
- Label the product plate as “EC (resistance)” and in accordance with the standard labelling format, as outlined at the front of your lab book and on Google Docs.

Materials

- Glass or plastic culture tubes
- Growth medium containing appropriate antibiotics
- Glass pipette tubes
- Parafilm

Procedure

1. Flame a glass pipette (or use a sterile plastic pipette without flaming), open the bottle of medium and flame the mouth.
2. Withdraw amount you need to fill your tubes (5ml per tube), flame the cap and recap the bottle as quickly as possible.
3. Remove the tube cap, flame the top of the culture tube, pipette in 5ml, flame the top of the tube and cap it.
4. Pick up one colony using a P20 pipette tip. Uncap the tube, flame the top, inject the tip into the tube.
5. Gently vortex the tube to ensure that the bacteria in the tip mixes into the media.
6. Incubate the tubes at 37°C overnight or until cells have reached the desired concentration. This should take between 12 and 16 hours.
7. When done, seal the transformed bacteria culture plate(s) that were used with Parafilm and store in 4°C fridge.

Heat Shock (Transformation) **– Queens Canada – 2011**
http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling
- Store the transformed cells in the 4°C fridge.
- Label the product plate as "EC (resistance)" and in accordance with the standard labelling format, as outlined at the front of your lab book and on Google Docs.

Materials
- 100µL of chemical competent cells per transformation
- Miniprep plasmid DNA
-2XTY or SOC medium
- Antibiotic plates (according to plasmid)

Procedure
1. Take out competent cells from -80°C and put them on ice immediately before they are needed.
2. Add 2µl plasmid DNA to thawed cells and mix by flicking the side of the tube.
3. Incubate on ice for 20 minutes.
4. Pre-warm antibiotic plates in 37°C incubator.
5. Heat shock for 1 min 15 sec at 42°C.
6. Place on Ice for 2 minutes.
7. Add 500ul 2XTY (or SOC) medium (kept at room temp) to each tube (medium with NO antibiotic).
8. Shake the tubes at 37°C for 1 hour on a shaking incubator.
9. Spread 100µl of each transformation tube on appropriate antibiotic plates.
-In addition, you can: Take 1µL of TOP10 and add 99µL of growth buffer, plate it. Spin remainder for 1 min at 13000, discard supernatant, re-suspend in 100µL of growth buffer
10. Incubate at 37°C overnight.

Rehydration- iGEM Standard Parts – Queens Canada – 2011
http://2011.igem.org/Team:Queens_Canada/Notebook/Protocols

Storage and Labelling
- Rehydrate primers just before they are needed for PCR
- Store primers (both concentrations) in the -20C freezer

Materials
- Dry primers
- ddH2O

Procedure

1. Ensure that you know what the primer that you are rehydrating is for so that you can label it properly.
2. Using sterile technique, add the correct volume of ddH2O to the dry primers such that they make a 100µM solution (consult the shipping invoice to see what the volume of water should be). If the volume specified by the invoice is over 900µL, add half the specified volume (to make a 200µM solution). Label the top of the tube with the concentration of the primer.
3. Briefly vortex the tube to ensure that all the DNA has dissolved (make sure there are no white flecks remaining in the bottom of the tube).
4. Let the tube sit for 1-3 minutes to ensure that the primers have completely dissolved. It is convenient to label your tubs during this time.
5. Briefly vortex again.
6. Aliquot out the correct volume of the concentrated (100 or 200µM) primer solution into an eppendorf tube and dilute it to 100µL solution at the concentration of 10µM (if your concentrated primer solution is 100µM, use 10µL or the concentrated primer and 90µL of water to make your 10µM primer solution).

Rehydration- Primers

Storage and Labelling

- Use 2µL of the DNA solution to transform bacteria (in order to amplify the part). Save the remainder of the aqueous DNA in the -20°C freezer.
- Product should be labelled as "DNA (AQ)," using the standard labeling technique, as outlined in the front of your lab book and on the Google Doc.

Materials

- Kit plate, from parts distribution
- ddH2O

Procedure

1. Ensure that you know the correct year, plate and well for your part. TRIPLE CHECK THIS. Wells are numbered and lettered as shown to the right.
2. Using sterile technique, load 10µL of diH2O into a p20 pipette.
3. Pierce the foil of the correct well on the distribution plate, firmly but carefully lower the pipette tip into the very bottom of the well.
4. Mix the DNA with the water by slowly pumping the water in and out of the pipette tip. You should see the water turn blue if you've done this correctly.
5. Leaving all of the water in the well, stretch parafilm over the plate and then replace its cover.
6. Write, "DO NOT TOUCH, KEEP LEVEL" on a piece of tape and affix the tape to the lid of the plate.
7. Ensuring that you keep the plate level, put it into a 4°C fridge, and leave it there for one hour to allow all of the DNA to dissolve.
8. Transfer the entire DNA solution from the well into an eppendorf tube.

Antibiotics – Minnesota – 2011
http://2011.igem.org/Team:Minnesota/Protocols

Prepare stock solutions of antibiotics for adding to media (1 µL per 1 mL)

- Ampicillin 100 mg/mL in water
- Chloramphenicol 50 mg/mL in ethanol
- Kanamycin 30 mg/mL in water

Media Preparation – Minnesota – 2011
http://2011.igem.org/Team:Minnesota/Protocols

LB media

  -
  - 10 g/L Tryptone
  - 5 g/L NaCl
  - 5 g/L Yeast Extract
  - 15 g/L Agar (solid media only)

- One sleeve (20 plates) can be made with 600 mL of solid media (autoclave, cool, and add antibiotics before pouring)
- One rack (72 16x100 mm tubes with 4 mL) can be made with 300 mL of liquid media (autoclave after pouring)

SOC media

  -
  - 20 g/L Tryptone
  - 5 g/L Yeast Extract
  - 0.5 g/L NaCl
  - 950 mL/L ddH_2O
- pH to 7.0, autoclave, cool, and add the following
  - 5 mL/L 2M $MgCl_2$ (filter sterilize)
  - 20 ml/L 20 mM Glucose Final Concentration* (Add 0.018 g/mL and filter sterilize)

TSS Method for Competent Cell Preparation – Minnesota – 2011 **http://2011.igem.org/Team:Minnesota/Protocols**

TSS Solution

- Use the following recipe to make 100 mL:
  - PEG 4000 15 g
  - 1 M $MgCl_2$-solution 5 mL
  - LB liquid media add to 95 mL
  - DMSO 5 mL (add after autoclaving)
- Adjust pH to 6.5 prior to autoclaving.
- After addition of DMSO aliquot TSS solution in 10 – 15 mL portions and store at –20 0C (TSS can get contaminated very quickly).

Competent Cell Preparation – Minnesota – 2011 **http://2011.igem.org/Team:Minnesota/Protocols**

- Cultivate overnight E. coli culture* (*LB, add appropriate antibiotics if competent cells containing a plasmid for co-transformation are required) to inoculate main culture* 1:100 with overnight culture.
  - Note: 50 mL culture will give 10 aliquots of competent cells, Use larger culture volumes (e.g. 100 mL) to prepare more aliquots.
- Grow main culture at 37 0C and 260 rpm to ensure rapid growth to OD 0.4 – 0.6 (typically 2 – 3 hours, fast growing cells to OD 0.4 reach highest transformation efficiencies)
- Centrifuge cells for 10 min at 4000 rpm (4 0C)
- Carefully resuspend cell pellet in cold (4 0C) TSS solution (2 mL TSS for each 50 mL culture volume).
- Incubate resuspended cells for 5 min on ice and aliquot 200 µL competent cells in 15 mL sterile tubes.
  - Note: Handle cells carefully and keep them always on ice as they get very fragile during the TSS treatment.
- Shock-freeze aliquoted cells in liquid nitrogen and store cells at –80 0C.

Restriction Digest – Minnesota – 2011 **http://2011.igem.org/Team:Minnesota/Protocols**

- Prepare the following reaction mixture for a double digest:
  - 3 µL Appropriate 10X Buffer (choose to maximize activity efficiencies)
  - 1 µL Restriction Enzymes (two, 1 µL each)
  - 10 µL Template DNA with compatible restriction sites

- 16 µL ddH2O

- Allow reaction to incubate for >2 hours or overnight at 37 0C
- Inactive restriction endonucleases by heating at 65 0C for 10 min
- Check results on agarose gel
- Isolate DNA from appropriate sized band with gel purification kit (Invitrogen or GE)

Ligation – Minnesota – 2011
http://2011.igem.org/Team:Minnesota/Protocols

- Prepare the following reaction mixture:
  - 2 µL Ligase Buffer
  - 1 µL T4 Ligase
  - 5 µL Plasmid (Cut with restriction enzymes)
  - 12 µL Insert (Flanked with restriction sits compatible with plasmid and cut with them)
- Allow reaction to incubate overnight at room temperature
- Transform reaction mixture

Primer Design – Minnesota – 2011
http://2011.igem.org/Team:Minnesota/Protocols

- Primers are the 5' ends of the sequence to be amplified by PCR
- Choose primers that have similar melting temperatures (Tm) that are between 50 0C and 65 0C
- Choose primers that have low complementation with sequence of interest
- Restriction sites are normally introduced to the 5' end of primers to aid assembly into vectors (BglII and NotI for BioBrick vectors)
- Include a G or C nucleotide at the 3' end
- Primer sequences are reported and ordered in 5' to 3' direction
- Reconstitute primer in 10 µL for every nmol reported on tube (100 pmol/µL final concentration)

Polymerase Chain Reaction – Minnesota – 2011
http://2011.igem.org/Team:Minnesota/Protocols

- Prepare reaction mixture in 200 µL PCR Tubes with following recipe:
  - 1 µL Template DNA
  - 1 µL Each primer (Forward and Reverse)
  - 5 µL 10X Thermopol Buffer

- 1 µL 10 mM dNTPs
- 2.5 µL 10 mM MgSO4
- 0.5 µL DNA Polymerase (Taq or Vent)
- 38 µL of Water to bring final volume to 50 µL

- Program themocycler with the following:
- Initial Denaturation 5 min 95 0C
- Repeat 25 times

**Denaturation 30 sec 95 0C **Annealing 30 sec >3 0C below lowest primer Tm **Extention 1 min per 1 Kb 72 0C **Final Extention 5 min 72 0C **Storage ∞ 4 0C

- Check reaction with 1% agarose gel (0.01 g/mL) in TAE buffer
- Use 2% agarose gel when checking fragments <500 bp
- If necessary, purify DNA using agarose gel purification kit

Transformation – Minnesota – 2011

http://2011.igem.org/Team:Minnesota/Protocols

- Thaw competent cells at room temperature on ice
- Add 1 µL of plasmid DNA or ligation reaction mixture to cells
- Incubate on ice for 20 min
- Heat shock at 43 0C for 40 sec
- Add 800 µL of SOC to cells
- Incubate at 37 0C for 1 hour
- Plate 50 µL of culture or for ligations, spin down, remove 900 µL media, resuspend cells, and plate on solid media with appropriate antibiotic
- Incubate at 37 0C for >18 hours
- Pick colonies and transfer to 4 mL cultures with appropriate antibiotic (add antibiotic to liquid LB before cells)
- Incubate at 37 0C for >18 hours
- Use Miniprep kit (made by Promega) to purify plasmid DNA from overnight culture

Sequencing – Minnesota – 2011

http://2011.igem.org/Team:Minnesota/Protocols

- Sequencing is conducted by the University of Minnesota Biomedical Genomic Center
- Make 1:100 dilution (1 pmol/µL) of stock primers for sequencing purposes
- Prepare the following mixture in 0.5 mL microcentrifuge tube:
    - 4 µL diluted primer

    - X µL Template DNA in vector (100 ng per Kb template, X = 100 x n Kb/DNA concentration (ng/µL))
    - Y µL Sterile water to reach final volume of 13 µL
- Name sequencing reaction with 3 letter prefix plus next highest unused number (ex. GEM01)

Flow Cytometry – Minnesota – 2011
http://2011.igem.org/Team:Minnesota/Protocols

- Inoculate single colony of freshly transformed DH5αPro or TOP10 cells in 4 ml LB medium containing 50µg/ml chloramphenicol (or appropriate antibiotic).
- Grow the culture overnight at 37 C with shaking (250 rpm).
- Next day re-inoculate the cultures into 4 ml fresh LB medium having antibiotics and varying inducer concentrations. Inducer concentrations can be varied from 0-1mM of IPTG or 0-200 ng/ml aTc.
- Collect the samples at different time intervals of 3, 6 and 9 hours.
- Monitor the growth rate by measuring optical density at 600 nm.
- Measure the fluorescence in a Becton Dickinson FACS Calibur flow cytometer equipped with a 488 nm argon laser and a 515-545 nm emission filter (FL-1) and a 585-610 nm emission filter (FL-2).
- Make sure that machine has settings for E. coli.
- To measure the fluorescence, add 3-5 µl of the growing culture in ~1 ml PBS (phosphate buffer saline, pH-7.5). Measurement should be done at low flow rate (~1000 events/second).
- For each sample, collect 50,000 events.
- Analyze the fluorescence in both FL-1 and FL-2 channel using FlowJo software (BD Biosciences).
- Determine the background fluorescence by using controls (cells having empty plasmid vector).

Silicatein Assay Reducing Agent Preparation – Minnesota – 2011 **http://2011.igem.org/Team:Minnesota/Protocols**

Metol-sulphite Solution

1. Add 6 g of anhydrous sodium sulphite to 500 mL of Milli-Q water.
2. Add 10 g of p-methylaminophenol (Metol solution).
3. Once reagents are dissolved, filter through No. 1 Whatman paper and store in a glass bottom
4. Recommended shelf life of approximately 1 month.

Oxalic Acid Solution

1. Shake 50 g of oxalic acid dihydrate in 500 mL of Milli-Q water.
2. Store in a glass bottle.
3. Solution may be stored indefinately.

Sulfuric Acid Solution

1. Add 500 mL of Milli-Q water to 250 mL of concentrated sulfuric acid solution.
2. Store in glass bottle.

Reducing Agent

1. Mix 100 mL of metol-sulphite solution with 60 mL of oxalic acid solution
2. Add 60 mL of 50% sulfuric acid solution.
3. Fill to 300 mL with Milli-Q water.
4. Prepare as needed, do not store.

Silicatein Activity Assay – Minnesota – 2011

http://2011.igem.org/Team:Minnesota/Protocols

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1. Prepare a solution of 100 mM tetramethyl orthosilicate (TMOS) in 1 mM HCl to prepare prehydrolyzed silica particles. Stir at room temperature for 15 minutes to completely hydrolyze TMOS to silica monomers.
2. Prepare two reactions:

Reaction/Reagent	**Prehydrolyzed TMOS Solution**	**Silicatein**
Assay	1 mL	200 ng
Negative Control	1 mL	0 ng

3. Incubate at NIST standard temperature and pressure (1 atm, 20 ºC) for 2 hours.

4. Centrifuge sample at 15,000 RPM for 2 minutes to precipitate polymerized silica partciles.

5. Decant supernatant containing unaggregated silica particles.

6. Wash 3 times with distilled H2O to remove free, hydrolyzed TMOS.

7. Aliquot the unreacted hydrolyzed TMOS remaining in solution after silica aggregation.

   a) Treat with 2 M NaOH for 1 hour at 80 ºC to ensure complete hydrolysis of silica particles to monomer/dimer state.

8. Remove aliquots of 0.5 µl and add to solution of 750 uL water and 75 uL of acidic solution of ammonium molybdate (20 g (NH4)6Mo7O24•4 H2O and 60 mL of concentrated Hcl [36%]).

9. Incubate at NIST standard temperature and pressure for 20 minutes. Solution should have a yellowish hue after incubation.

10. Add 400 uL of reducing agent solution (see above). Solution should turn a bluish color.

11. Record absorbance at 810 nm and compare to standard curve.

Gel Extraction (QIAquick) – ***Johns Hopkins – 2011*** **http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols**

1. Excise DNA fragment from agarose gel with clean, sharp scalpel.
2. Weigh gel slice in colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100mg ~ 100µl)
3. Incubate @ 50C for 10 min.
    - SOLUBILIZE AGAROSE COMPLETELY.
    - To help dissolve gel, vortex tube every 2-3 min during incubation.
4. After the gel has dissolved, check that the color of the mixture is yellow. If not, add 10µl 3M NaOAc. Or just add the NaOAc anyway.
5. Add 1 gel volume of isopropanol to the sample and mix
    - Do only if <500bp or >4kb
6. Place spin column in 2mL collection tube.
7. To bind DNA, apply sample to column. Centrifuge 1 min.
8. Discard flowthrough and place column back in same collection tube.
9. Recommended: Add 0.5mL of Buffer QG to column and centrifuge for 1 min.
10. To wash, add 0.75mL of Buffer PE to column and centrifuge for 1 min.
11. Discard flowthrough and centrifuge for an additional 1 min @ 17900 x g (13000rpm).
12. Place column into a clean 1.5mL microcentrifuge tube.
13. To elute DNA, add 30µL Buffer EB to center of membrane, let column stand for 1 min, then centrifuge for 1 min.

Mini-Prep (QIA) – ***Johns Hopkins – 2011*** **http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols**

1. Resuspend pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube.
2. Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
3. Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
4. Centrifuge for 10 min at 13,000 rpm in a table-top microcentrifuge.
5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
6. Centrifuge for 30-60s. Discard the flow-through.
7. Recommended: Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifuging for 30-60s. Discard flow-through.
8. Wash QIAprep spin column by adding 0.5 mL Buffer PB and centrifuging 30-60s.
9. Discard flow-through and centrifuge for an additional minute to remove residual wash bufffer. **IMPORTANT: Residual wash buffer will not be completly removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzyme reactions.**

10. Ordered List ItemPlace QIAprep column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 µL Buffer EB (10mM Tris-CL, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

PCR Reactions on Small DNA Pieces – ***Johns Hopkins – 2011***
http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols

Procedure:

1. Combine the following reagents in one tube:
   - 18uL PCR Platinum Supermix
   - .24uL Forward Primer
   - .24uL Reverse Primer
   - .4uL Target DNA
   - 1.12uL Nuclease Free Water
2. Place tube into Thermal Cycler
3. Program Thermal Cycler with desired Denaturing, Annealing and Extension Temperatures.
   - Example:

```
94C for 2 minutes
94C for 30 seconds (Denaturing)
55C for 30 seconds (Annealing)
72C for 1min/kb (Extension)
Repeat Denaturing, Annealing and Extension at least 24 times.
Hold at 4C
```

DNA Digestion – ***Johns Hopkins – 2011*** **http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols**

DNA Digestion Mixture:

- 25 µl DNA
- 3 µl Buffer 2
- 1 µl EcoRI-HF
- 1 µl PST1
- 3 µl BSA1

PCR 1.0% Agarose Gel – ***Johns Hopkins – 2011*** **http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols**

1. Add .3 grams Agarose to 30mL TAE Buffer in volumetric flask.
2. Stopper volumetric flask with Kim Wipes.
3. Microwave mixture for 1 minute to allow Agarose to dissolve.
4. Prepare BioRad Gel Machine:
   - Place gel stand.

- Add both gel blocks.
- Insert comb.

5. Allow mixture to cool down to ~40C.
6. Use micropipetter to seal edges of gel stand with the mixture.
7. Add 3uL Ethidium Bromide to remaining mixture.
8. Mix well.
9. Pour mixture onto gel stand.
10. Cover with Paper Towels and allow to harden.
11. Prepare PCR Reaction tubes:
    - For every 10uL PCR product add 1uL Blue Juice.
    - Mix well.
12. Once gel hardens remove gel blocks and comb.
    - Caution: Remove comb vertically so as not to damage wells.
13. Fill gel machine with TAE buffer till liquid reaches above gel.
14. Load 5uL DNA Ladder.
15. Load 5-10uL samples.
16. Run at 100 Volts for 20-25 minutes.
17. Turn machine off and transfer gel to imaging machine.
18. Position gel and take picture under UV.

QuickChange PCR – *Johns Hopkins – 2011* **http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols**

From Marty Taylor of the Boeke Lab:

Based on two stage protocol from - W. Wang, B. A. Malcolm, Biotechniques 26, 680 (Apr, 1999). This two-stage protocol allows formation of some of a "hybrid" between WT and mutant, reducing competition between primer and its GC

Design primer to span region to be changed. Depending on the degree of change (single point mutation vs large deletion/insertion) I usually have 15-40nt on either side with perfect match. Order primer and its reverse complement. We have had good luck using Ultramers from IDT up to ~150nt long, haven't tried anything longer. We do not PAGE purify primers for this application.

For each primer, setup a 25uL reaction (for older PCR machines with 0.5mL tubes, double everything and you may want to extend the annealing/extension times). I usually do a number of these at a time from the same template, so I make a master mix containing everything but primers, aliquot, and then add primers.

1. Template dNA (~25ng) [1 uL from miniprep]
2. 12.5pMol primer [1.25uL] of 10uM stock – be sure to use only one primer each!
3. 5x Buffer [5 uL]
4. dNTPs (2.5mM each) [2 uL]
5. Herculase II [0.5 uL]
6. H2O → 25uL

Control reaction – this is your background! Add 50uL master mix (without primers) to PCR tube and put it on the cycler, identical to the other tubes. DpnI digest transform like the others.

PCR Protocol:

1. Stage 1: setup 2 tubes as above – one for primer, one for its complement – run 2 or 3 cycles
2. Stage 2: combine the two reactions into one, mix well, run for 18 more cycles
   - 95C, 5min
   - [95C, 30sec / 55C, 30sec / 72C (1min/kB total vector+insert)] – 2-3x in stage 1, 18x in stage 2
   - 72C, 10min
   - Extension time – with the new faster enzymes, 30s/kB may be enough, but have had better luck with 1min

DpnI digest – add 0.5uL DpnI (10U/uL) to each 50uL reaction, vortex, incubate at 37°C for 1-3hrs (longer is better if time permits). Be sure to DpnI digest the control and transform and plate it equally. If you think the reaction is a hard one, you can extend the Dpn digestion to reduce background further (or add more enzyme).

Agarose Gel – WASTE OF TIME! This technique often fails to produce enough DNA to see on a gel and still works. So, basically, this step tells you nothing, since you are going to do the transformation whether or not the gel shows you something. I would do a positive control on the transformation long before I run a gel here.

PCR Cleanup – Herculase II reaction mix is incompatible with our standard competent cells (made using Mn/Ca), reducing the efficiency of transformation by at least 3 logs (perhaps due to detergent in the buffer). A quick PCR cleanup solves this problem.

Transform 5uL into a 50uL aliquot of competent cells. We make our own competent cells; for very difficult reactions, we have had success with supercompetent cells, but I haven't used these since switching to PFU-Ultra. After heat shock, I grow the cells for 1hr in 500uL SOC and plate both 50uL and 450uL. Sometimes the 450uL plate is a lawn; sometimes there are only 10-50 colonies.

Screening – if your control plate is clean (no colonies), I recommend sequencing (or otherwise screening – if your design allows) 3 clones. Usually at least 2 are positive, but sometimes only 1. Sometimes odd things happen, such as single nucleotide insertions or deletions, errors in the primer sequence itself, or part of the primer annealing with the wrong region of the template, but these results are rarely in all clones sequenced. If there are a lot of colonies on the control (25-50% of the experimental plates), you can sometimes get lucky. Consider longer DpnI digest or repeating.

Synthesis – *Johns Hopkins – 2011* **http://2011.igem.org/Team:Johns_Hopkins/Notebook/Protocols**

Overlap Extension

In order to assemble our Vitamin C genes, we used overlap extension PCR. Oligos of up to 60 bp were ordered from IDT, sequentially, in building blocks of up to 800 bp. The first oligo had a 40 base pair overlap with the next, and so on, until the end of that particular chunk of the gene, called a building block. GDP L-Galactose Phosphatase and GDP Mannose-3,5-Epimerase both are made up of two building blocks, and L-Galactose 1-phosphate phosphatase is composed of only one building block. This process is known as templateless PCR. Following this, the PCR product (which will include both incomplete building blocks and a small amount of final product) is PCR amplified using the first and last oligos. This step is called finishing PCR. These building blocks are then purified using a zymogen DNA purification column and then assembled into the final construct in the vector via a CPEC reaction.

CPEC – Johns Hopkins – 2011

(Quan 2009)

1. Measure the DNA concentration of each assembly piece
2. Assay 100ng of the linearized vector backbone and equimolar amounts of the other assembly pieces to a 25ul total volume assembly reaction mi#xture accordingly:
    - 100 ng of vector backbone
    - equimolar ammounts of each assembly piece
    - 5ul 5X HF Phusion Buffer
    - 1ul 10mM dNTPs
    - 0.75ul DMSO
    - 0.5ul 2U/ul Phusion Polymerase
    - H2O to 25ul
3. Perform the assembly reaction in a thermocycler as follows:

Temperature	Time	Cycles
98C	3 min	1
98C	30 sec	15 *
55C	30 sec	15 *
72C	total length(kb) * 15 sec	15 *
72C	10 min	1

- Note: the number of repeated cycles should exceed the number of assembly pieces

4. Transform 5ul of the assembly reaction into 100ul of competent E. coli and/or run a diagnostic agarose gel to check for successful assembly.

E. coli Calcium Chloride competent cell protocol – ITESM Mexico – 2011
http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Materials**

Equipment

- **Centrifuge**
- **Pipette tips**
- **Micropipettes**
- **Sterile tubes**

- **Racks**

Reagents

- **0.1M CaCl2**
- **Glycerol**

1. **Inoculate a single colony (diameter: 2-3mm) into 100mL Lb in a falcon tube. Shake @ 37°C for 3hrs, 150-200rpm**

a.From an ON culture (single colony into 5 mL LB in 50 mL falcon 37°C, 250 rpm), inoculate 1 mL into 100 mL LB (0.25 mL from ON culture into 25 mL LB in 50 mL falcon)

1. **When the O.D. 600=0.35 put the cells on new tubes on ice for 10-15 mins (keep cold form now on).**
2. **Collect the cells by centrifugation 2700g (4100rpm) for 3 min at 4ºC**
3. **Decant supernatant (be careful not to dump out pellet, and drain tube on paper towel).**
4. **Gently resuspend on 10 mL cold 0.1M CaCl2 (cells are susceptible to mechanical disruption, so treat them nicely).**
5. **Incubate on ice for 15 min**
6. **Repeat 3 and 4.**
7. **Gently resuspend pellet on 2mL cold 0.1MCaCl2/15%Glycerol (2ml 0.1MCaCl2/50ml bacterial culture)**
8. **Dispense in microtubes. Freeze in -80°C.**

REHYDRATION – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Materials**

Equipment

- **Pipette tips**
- **Micropipettes**
- **Sterile tubes**
- **Racks**

Reagents

- **Distilled water**

Biological agents

- **Resuspended DNA. DNA kit plate**

To use the DNA in the Distribution Kit you may follow these instructions:

1. **With a pipette tip, punch a hole through the foil cover into the corresponding well of the Biobrick™-standard part that you want. Make sure you have properly oriented the plate. We recommend that you do not remove the foil cover, as it could lead to cross contamination between the wells. But it is important to take care of the plate orientation after punching the foil.**
2. **Pipette 10uL of dH2O (distilled water) into the well. Pipette up and down a few times and let sit for 5 minutes to make sure the dried DNA is fully resuspended. We recommend that you do not use TE to resuspend the dried DNA.**
3. **Transform 2µL of the resuspended DNA into your desired competent cells, plate your transformation with the appropriate antibiotic and grow overnight.**

TRANSFORMATION – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Materials**

Equipment

- **Incubator**
- **Water bath**
- **Petri dishes (with LB agar and appropriate antibiotic/ two per a transformation)**
- **Pipette tips**
- **Micropipettes**
- **Sterile tubes**
- **Sterile loops**
- **Racks**

Reagents

- **LB agar**
- **LB broth**
- **Distilled water**
- **Antibiotics (according to each BioBrick)**

Biological agents

- **Resuspended DNA**

- **Competent cells**

1. **Start thawing the competent cells on crushed ice.**
2. **Add 100 µL of thawed competent cells and then 2 µL of the resuspended DNA to the labelled tubes. Make sure to keep the competent cells on ice.**
3. **Incubate the cells on ice for 30 minutes.**
4. **Heat shock the cells by immersion in a pre-heated water bath at 42ºC for 2min. A water bath improves heat transfer to the cells.**
5. **Incubate the cells on ice for 5 minutes.**
6. **Add 200 µl of LB broth (make sure that the broth does not contain antibiotics and is not contaminated)**
7. **Incubate the cells at 37ºC for 2 hours while the tubes are rotating or shaking. Important: 2 hour recovery time helps in transformation efficiency, especially for plasmids with antibiotic resistance other than ampicillin.**
8. **Label two petri dishes with LB agar and the appropriate antibiotic(s) with the part number, plasmid, and antibiotic resistance. Plate 20 µl and 200 µl of the transformation onto the dishes, and spread. This helps ensure that you will be able to pick out a single colony.**
9. **Incubate the plate at 37ºC for 16 hours, making sure the agar side of the plate is up. If incubated for too long the antibiotics start to break down and un-transformed cells will begin to grow. This is especially true for ampicillin - because the resistance enzyme is excreted by the bacteria, and inactivate the antibiotic outside of the bacteria.**
10. **Pick a single colony and inoculate broth (with the correct antibiotic) and grow for 16 hours.**
11. **Use the resulting culture to miniprep.**

Glycerol stock – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Materials**

Equipment

- **Pipette tips**
- **Micropipettes**
- **Sterile tubes**
- **Racks**

Reagents

- **60ml glycerol: 4ml $CaCl_2$: 36 ml de H_2O**
- **1ml glycerol/ 1 ml bacteria**

1. **Add 1 ml of 60% glycerol in H_2O to a falcon tube.**
2. **Add 1 ml sample from the culture of bacteria to be stored.**
3. **Gently vortex the cryogenic vial to ensure the culture and glycerol is well-mixed.**

a. Alternatively, pipet to mix.

1. **Use a tough spot to put the name of the strain or some useful identifier on the top of the vial.**
2. **On the side of the vial list all relevant information - part, vector, strain, date, researcher, etc.**
3. **Store in a freezer box in a -80ºC freezer. Remember to record where the vial is stored for fast retrieval later.**

***Miniprep Plasmid DNA Isolation* – ITESM Mexico – 2011**
http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Materials**

Equipment

- **Centrifuge**
- **Pipette tips**
- **Micropipettes**
- **Sterile tubes**
- **Racks**

Reagents

- **Solutions:**

- **Solution 1:**

- **1.8g→ 50 mM glucose**
- **0.6057g→ 25 mM Tris-HCl pH 8.0:**
- **0.744g→ 10 mM EDTA pH 8.0:**
- **Add H_2O to 200 ml.**

- **Solution 2:**

- **2g→ 1% SDS. Add H_2O to 198 ml.**
- **Add 1.6g→ 0.2 N NaOH**

- **Add H_2O to 200 ml.**

- **Solution 3:**

- **98.14g→ 5 M Potassium Acetate**
- **Add glacial acetic acid to 200 ml.**

- **TE:**

- **0.2428g → 10 mM Tris-HCl pH 8.0**
- **0.744g→ 1 mM EDTA**
- **Add H_2O to 200 ml.**

- **Optional: RNAse can be added to TE at final concentration of 20 µg/ml.**

***Biological agents* – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

Bacterial culture grown

1. **Fill a centrifuge tube with saturated bacterial culture grown in LB broth + antibiotic. Spin tube in centrifuge for 1 minute, and make sure tubes are balanced in centrifuge. Dump supernatant and drain tube briefly on paper towel.**
2. **Repeat step 1 in the same tube, filling the tube again with more bacterial culture. The purpose of this step is to increase the starting volume of cells so that more plasmid DNA can be isolated per prep. Spin tube in microcentrifuge for 1 minute. Pour off supernatant and drain tube on paper towel. If necessary, repeat this step (depending on the amount of pellet)**
3. **Add 2 ml ice-cold Solution 1 (GTE) to cell pellet and resuspend cells as much as possible using disposable transfer pipet.**

a.Solution 1 contains glucose, Tris, and EDTA. Glucose is added to increase the osmotic pressure outside the cells. Tris is a buffering agent used to maintain a constant pH (8.0). EDTA protects the DNA from degradative enzymes (called DNAses); EDTA binds divalent cations that are necessary for DNAse activity.

1. **Add 4 ml Solution 2 (NAOH + SDS 1%), cap tubes and invert five times gently. Let tubes sit at room temperature for 5 minutes.**
2. **Solution 2 contains NaOH and SDS (a detergent). The alkaline mixtures ruptures the cells, and the detergent breaks apart the lipid membrane and solubilizes cellular proteins. NaOH also denatures the DNA into single strands.**
3. **Add 3 ml ice-cold Solution 3 (Potassium acetate), cap tubes and invert five times gently. Incubate tubes on ice for 10 minutes.**

a.Solution 3 contains a mixture of acetic acid and potassium acetate. The acetic acid neutralizes the pH, allowing the DNA strands to renature. The potassium acetate also precipitates the SDS from solution, along

with the cellular debris. The E. coli chromosomal DNA, a partially renatured tangle at this step, is also trapped in the precipitate. The plasmid DNA remains in solution.

1. **Centrifuge tubes for 5 minutes. Transfer supernatant to fresh centrifuge tube using clean disposable transfer pipet. Try to avoid taking any white precipitate during the transfer. It is okay to leave a little supernatant behind to avoid accidentally taking the precipitate.**
2. **This fractionation step separates the plasmid DNA from the cellular debris and chromosomal DNA in the pellet.**
3. **Fill remainder of centrifuge tube with isopropanol. Let tube sit at room temperature for 2 minutes.**
4. **Isopropanol effectively precipitates nucleic acids, but is much less effective with proteins. A quick precipitation can therefore purify DNA from protein contaminants.**
5. **Centrifuge tubes for 10 minutes (13.4krpm max). A milky pellet should be at the bottom of the tube. Pour off supernatant without dumping out the pellet. Drain tube on paper towel.**
6. **This fractionation step further purifies the plasmid DNA from contaminants. This is also a good place to stop if class time is running out. Cap tubes and store in freezer until next class period.**
7. **Add 10 ml of ice-cold absolute ethanol. Cap tube and mix by inverting several times. Spin tubes for 1 minute. Pour off supernatant (be careful not to dump out pellet) and drain tube on paper towel.**
8. **Ethanol helps to remove the remaining salts and SDS from the preparation.**
9. **Allow tube to dry for ~5 minutes. Add 500 ul TE to tube. If needed, centrifuge tube briefly to pool TE at bottom of tube. DNA is ready for use and can be stored indefinitely in the freezer.**

Preparing Plates, Solutions, and Bacterial Starter Plate **– ITESM Mexico – 2011**
http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Follow the kit directions to prepare and pour the agar plates, and to rehydrate the provided lyophilized materials such as E. coli bacteria, antibiotics, DNA, etc.**

1. **Prepare and pour the agar plates—LB only (LB) and LB plus ampicillin (LB:AMP).**

a. Label the plates with permanent marker: LB and LB:AMP.

b. After the agar solidifies, cover the plates, put them in their original plastic bags, and store them in a lab refrigerator stacked upside down. Store plates wrapped up in their original plastic wrappings. Storing upside down will ensure condensation does not wet the surface of the agar.

c. Note that the pGlo Transformation kit also allows for visualization of the transformation. In addition to the acquisition of Ampicillin resistance, the transformed bacteria can also express another gene on the pGLO plasmid which causes the bacteria to glow a brilliant green color. In order to see this, prepare the LB:AMP:ARA agar plates as specified in the pGLO transformation kit product insert. After transformation on day 2, plate the transformed cells on the LB:AMP:ARA plates as well. The arabinose in the agar will induce expression of the green fluorescent protein and the bacteria will glow green. While this step is cool to see it is not required for you to determine transformation efficiency. The Bio-Rad pGLO transformation kit comes with one UV penlight. This should be sufficient to visualize the glowing bacteria. However if you have

access to a laboratory with a long wave UV lamp, that will be great. Caution - Do not shine UV light directly into the eyes, use a UV-protective face shield or goggles, and limit exposure to UV light.

1. **Rehydrate bacteria and streak LB starter plates.**
2. **Incubate starter plates overnight at 37°C (or 2 to 3 days at room temperature until colonies are clearly visible). 5 mL Overnight**

What you will need:

- **10 mL culture tube Use 16mm x 160mm or 16mm x 125 mm**
- **5 mL LB**
- **5uL 1000X antibiotics**
- **Single colonies on a plate Best not to start O/N from glycerol stocks**

Procedure

1. **Pipet 5uL 1000X antibiotic into culture tube.**

1. **Add 5 mL non-contaminated LB. Do this first, then add antibiotic**
2. **Select single colony using sterile toothpick or a flamed loop that has been cooled**
3. **Place toothpick or loop in culture tube, stir.**
4. **Remove toothpick or loop and place culture tube in incubator at 37 C overnight shaking vigorously (250 rpm).**

Annexes

***SOC media preparation* – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

Materials

- **SOB media**
- **20 mM glucose**

Protocol

1. **Follow directions to make 1 liter of SOB media**
2. **After cooling medium to less than 50°C, add 20 ml filter sterilized 20% glucose solution**

***SOB media preparation* – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

Materials

- **0.5% (w/v) yeast extract**
- **2% (w/v) tryptone**
- **10 mM NaCl**
- **2.5 mM KCl**
- **20 mM MgSO4**

Per liter:

- **5 g yeast extract**
- **20 g tryptone**
- **0.584 g NaCl**
- **0.186 g KCl**
- **2.4 g MgSO4**

Note: Some formulations of SOB use 10 mM MgCl2 and 10 mM MgSO4 instead of 20 mM MgSO4. SOB medium is also available dry premixed from Difco, 0443-17. Important: Adjust to pH 7.5 prior to use. This requires approximately 25 ml of 1M NaOH per liter.

***EDTA preparation* – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **0.5 M EDTA stock**
- **18.61 g EDTA (Sodium Salt)**
- **dH2O to 90 ml**
- **adjust pH to 7.0**
- **adjust volume to 100 ml**

***Agarose Gel Electrophoresis* – ITESM Mexico – 2011**
http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Material:**

- **1X TAE**
- **Graduated cylinder**
- **125 mL flask**
- **Agarose**
- **Gel pouring tray**
- **Tape**
- **Gel rig**

- **Ethidium bromide**

Procedure:

1. **Dilute stock of 10X TAE to 1X with ddH2O.**
2. **Measure 40 mL of buffer.**
3. **Transfer buffer to 125 mL flask**
4. **Weigh out enough agarose to make 1% gel. (1% of 40mL is 0.40 g)**
5. **Transfer agarose to 125 mL flask.**
6. **Melt agarose in microwave, stirring ever 15-20 seconds. This should take about 2 min.**
7. **Allow agarose to cool.**
8. **While agarose is cooling, assemble gel pouring apparatus by inserting gate into slots.**
9. **Use a pastuer pipet to run a bead of molton agarose along the edges of the gates to seal the box and prevent leaks.**
10. **Allow gel to cool until flask can be handled comfortably.**
11. **Place comb in the gel rig.**
12. **Pour agarose into gel tray.**
13. **Allow to solidify. While the gel is solidifying, prepare the samples. Add your sample and 2uL of OG loading dye to a tube, then make the total volume of the tube up to 20 uL.**
14. **Pour 1X TBE over gel so that gel is covered by 3-5 mm of buffer.**
15. **Load samples into lane. Do not forget to load 1kb+ ladder into one of the lanes.**
16. **Hook electrodes to gel apparatus. Nucleic acids are negatively charged, so they will run to the positive (red) terminal.**
17. **Pipette 10 uL ethidium bromide into the buffer at the bottom of the gel. Mix well**
18. **Turn on the gel. Run for 60 min @ 90V. Check with handheld UV Source.**
19. **Place gel in plastic wrap.**
20. **Carry to g311.**
21. **With bare hands log in as Gen 420 with password Molecular1.**
22. **Double click on the Genesnap from Syngene icon.**
23. **Click on the Green Button to start live image.**
24. **Put one glove on your left hand and place gel on transilluminator. Now do not touch anything with your left hand.**
25. **With your right hand slide the door down completely.**
26. **The transilluminator image on the screen should turn purple.**
27. **Use the arrows on the exposure button to increase the exposure time until the gel and bands are clearly visible.**

Butanerds Protocols 8

1. **If necessary use the zoom arrows to increase or decrease the size of the gel.**
2. **Reposition the gel if necessary – open the door with your right hand and move the gel with your gloved left hand.**
3. **To fine focus the image use the eye arrows.**
4. **When the image is sized and focused properly capture it by clicking the red button.**
5. **Print a photograph by clicking the printer icon in the tool bar at the top.**
6. **Record your use on the sheet. Supervisor=iGEM and your initials.**
7. **Log off.**
8. **Remove your gel and clean the transilluminator with water and dry with paper towels.**
9. **Take the gel back to the lab.**

Biological agents **– ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

• Bacterial culture grown

1. **Fill a centrifuge tube with saturated bacterial culture grown in LB broth + antibiotic. Spin tube in centrifuge for 1 minute, and make sure tubes are balanced in centrifuge. Dump supernatant and drain tube briefly on paper towel.**
2. **Repeat step 1 in the same tube, filling the tube again with more bacterial culture. The purpose of this step is to increase the starting volume of cells so that more plasmid DNA can be isolated per prep. Spin tube in microcentrifuge for 1 minute. Pour off supernatant and drain tube on paper towel. If necessary, repeat this step (depending on the amount of pellet)**
3. **Add 2 ml ice-cold Solution 1 (GTE) to cell pellet and resuspend cells as much as possible using disposable transfer pipet.**

a. Solution 1 contains glucose, Tris, and EDTA. Glucose is added to increase the osmotic pressure outside the cells. Tris is a buffering agent used to maintain a constant pH (8.0). EDTA protects the DNA from degradative enzymes (called DNAses); EDTA binds divalent cations that are necessary for DNAse activity.

1. **Add 4 ml Solution 2 (NAOH + SDS 1%), cap tubes and invert five times gently. Let tubes sit at room temperature for 5 minutes.**

a. Solution 2 contains NaOH and SDS (a detergent). The alkaline mixtures ruptures the cells, and the detergent breaks apart the lipid membrane and solubilizes cellular proteins. NaOH also denatures the DNA into single strands.

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a. Solution 3 contains a mixture of acetic acid and potassium acetate. The acetic acid neutralizes the pH, allowing the DNA strands to renature. The potassium acetate also precipitates the SDS from solution, along with the cellular debris. The E. coli chromosomal DNA, a partially renatured tangle at this step, is also trapped in the precipitate. The plasmid DNA remains in solution.

1. **Centrifuge tubes for 5 minutes. Transfer supernatant to fresh centrifuge tube using clean disposable transfer pipet. Try to avoid taking any white precipitate during the transfer. It is okay to leave a little supernatant behind to avoid accidentally taking the precipitate.**
2. **This fractionation step separates the plasmid DNA from the cellular debris and chromosomal DNA in the pellet.**
3. **Fill remainder of centrifuge tube with isopropanol. Let tube sit at room temperature for 2 minutes**
4. **Isopropanol effectively precipitates nucleic acids, but is much less effective with proteins. A quick precipitation can therefore purify DNA from protein contaminants.**
5. **Centrifuge tubes for 10 minutes (13.4krpm max). A milky pellet should be at the bottom of the tube. Pour off supernatant without dumping out the pellet. Drain tube on paper towel.**
6. **This fractionation step further purifies the plasmid DNA from contaminants. This is also a good place to stop if class time is running out. Cap tubes and store in freezer until next class period.**
7. **Add 10 ml of ice-cold absolute ethanol. Cap tube and mix by inverting several times. Spin tubes for 1 minute. Pour off supernatant (be careful not to dump out pellet) and drain tube on paper towel.**
8. **Ethanol helps to remove the remaining salts and SDS from the preparation.**
9. **Allow tube to dry for ~5 minutes. Add 500 ul TE to tube. If needed, centrifuge tube briefly to pool TE at bottom of tube. DNA is ready for use and can be stored indefinitely in the freezer.**

Backbone protocol **– ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

• Digestion Enzyme Master Mix for Plasmid Backbone (25ul total, for 6 rxns)

- **5 ul NEB Buffer 2**
- **0.5 ul BSA**
- **0.5 ul EcoRI-HF**
- **0.5 ul PstI**
- **0.5 ul DpnI (Used to digest any template DNA from production)**
- **18 ul dH20**

Procedure:

- **Digest Plasmid Backbone**
- **Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)**
- **Add 4 ul of Enzyme Master Mix**
- **Digest 37C/30 min, heat kill 80C/20 min**

• Ligation – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Add 2ul of digested plasmid backbone (25 ng)**
- **Add equimolar amount of EcoRI-HF SpeI digested fragment (< 3 ul)**
- **Add equimolar amount of XbaI PstI digested fragment (< 3 ul)**
- **Add 1 ul T4 DNA ligase buffer. Note: Do not use quick ligase**
- **Add 0.5 ul T4 DNA ligase**
- **Add water to 10 ul**
- **Ligate 16C/30 min, heat kill 80C/20 min**
- **Transform with 1-2 ul of product**

Note: For linearized plasmid backbones provided by iGEM HQ, a plasmid backbone with an insert of BBa_J04450 was used as template. As a result any red colonies that appear during your ligation may be due to the template as a background. Digesting with Dpn1 before use should reduce this occurrence.

• PCR mix – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **100 ul PCR Supermix High Fidelity**
- **0.7 ul of SB-prep-3P-1**
- **0.7 ul of SB-prep-2Ea**
- **0.5 ul template DNA at 10 ng/ul**

Notes: Do not use a sample of linearized plasmid backbones (PCRed) as a template, The Registry uses BBa_J04450 as a template

• PCR program – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **94C/2min**
- **94C/30s**
- **55C/30s**
- **68C/3min**
- **Repeat cycle (steps 2 to 4, 35 more times)**
- **68C/10min**
- **Digest with DpnI enzyme: 2ul in 100ul reaction, incubate 37C/hour; heat kill 80C/20min**

• PCR cleanup – ITESM Mexico – 2011 http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **QIAquick PCR Purification**
- **Add 500 ul Qiagen buffer PB**
- **Spin through a column twice, discard flowthrough**

- **Wash 1x with 700 ul buffer PB**
- **Wash 2x with 760 ul buffer PE**
- **Discard liquid, spin dry at 17000g for 3 min**
- **Elute into a new tube twice with 50 ul of TE (100 ul total)**

• **Quality Control – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **We recommend QCing constructed linearized plasmid backbones, to test success of PCR, ligation efficiency, and background.**
- **Run unpurified PCR product (1 ul) on a gel to verify the correct band and concentration and lack of side products.**
- **Test concentration of purified PCR product. Note: Expected yield should be 40ng/ul or higher. Adjust to 25ng/ul with TE.**
- **Run a digest and ligation test with purified PCR product to determine EcoRI and PstI cutting and ligation efficiency.**

• **Digest – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Digest Master Mix (10rxns)**
- **15 ul NEB Buffer 2**
- **1.5 ul BSA**
- **90 ul dH20**
- **Run Digest**
- **4 ul of plasmid backbone (approximately 100 ng)**
- **10.5 ul of Digest Master Mix**
- **0.5 ul either EcoRI-HF or PstI enzyme (not both!)**
- **Digest 37C/30min; 80C/20 min**
- **Proceed directly to ligation**

• **Ligation – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Ligation Master Mix (10rxns)**
- **20 ul T4 DNA ligase buffer**
- **5 ul T4 DNA ligase**
- **25 ul water**
- **Ligation Test**
- **Add 5 ul of ligation master mix to digested product**
- **Ligate 16C/30min; 80C/20 min**

- **Run all 20 ul on a gel**
- **Compare intensity of the single and double length bands. More efficient ligations will show stronger double length bands than single.**

• **Transformation – ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **Transform 1 ul of the diluted final product into highly competent cells**
- **Control transform 10 pg of pUC19**
- **Plate on the appropriate antibiotic**
- **Observe few colonies. Any colonies represent background to the three antibiotic assembly process**
- **Quantify the effective amount of remaining circular DNA able to transform**

1. **Miniprep your two parts.**
2. **Digest your two parts and construction plasmid backbonedestination vector with the following enzymes**

- **Left part with EcoRI and SpeI**
- **Right part with XbaI and PstI**
- **Construction plasmid backbone with EcoRI and PstI. Also digest the construction plasmid backbone with DpnI if possible to eliminate any plasmid remaining from the PCR.**

1. **Combine 1 ul of each restriction digest reaction with 1 ul of ligase in a 25 ul reaction.**
2. **Transform the ligation product.**
3. **If the input parts are good, almost all colonies will be correct.**
4. **If desired analyze the transformation with single colony PCR followed by agarose gel electrophoresis.**

- **In rolling, large scale assembly, this step is often omitted.**

1. **Miniprep clones that generated a band of the appropriate size.**
2. **Sequence the clone.**
3. **Record the sequence information in the Registry.**

Some other protocols **– ITESM Mexico – 2011** http://2011.igem.org/Team:ITESM_Mexico/Protocols

- **PureLink PCR Purification kit ·k3-100-01**

- **Biobrick TM Assembly Kit**

- **Aurum Plasmid minikit 100 preps #732-6400**

- **Extraction of DNA from Filter Paper**

- **Purelink Quick Gel Extraction Kit #k2100-12**

- **Pureyield plasmid miniprep system #a1223**

- **Plasmid Purification Protoco**

Competent Cells – Grinnell – 2011 http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Inoculate 500mL LB with 2mL overnight culture. Incubate with shaking to early log phase (~5 x 10^8 cells/mL, OD_{600} = 0.2-0.4).**
2. **Chill cells on ice for 15-120min (generally ~30min).**
3. **Pellet cells in a prechilled sterile centrifuge tube by centrifugation at 5-8krpm for 5min at 4°C. Discard supernatant.**
4. **Fill centrifuge tube about two thirds full with cold 100mM $CaCl_2$ (+10% glycerol) and gently but completely resuspend cells; incubate on ice for 3hr or overnight.**
5. **Harvest cells by cetrifugation as before. Discard supernatant.**
6. **Gently resuspend cells in 5mL cold 100mM $CaCl_2$ (+10% glycerol). Incubate on ice for at least 1hr. Aliquot into 100µL aliquots, then flash freeze and store at -80°C.**

Plasmid Transformation by Heat Shock – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Thaw 100µL aliquots of competent cells on ice.**
2. **Add 10µL DNA to cells.**
3. **Incubate tubes on ice for 30min.**
4. **Heat shock tubes at 42° C for 90sec.**

5. **Incubate tubes on ice for 2min.**
6. **Add 300µL LB to cells and incubate shaking at 37° C for 1-2hrs.**
7. **Spread 200µL cells on selective media.**
8. **Incubate plates overnight at 37° C.**

Isolation of DNA for Colony PCR using GeneReleaser – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

GeneReleaser is a proprietary reagent that releases DNA from cells while sequestering cell lysis products that might inhibit DNA polymerases.

1. **Resuspend the GeneReleaser through inversion, not vortexing. Add 20µL GeneReleaser to each PCR tube.**
2. **Add cells from plates with a sterile pipette tip OR 10µL from overnight liquid culture.**
3. **Run PCR tubes on following thermal cycle program:**

Temperature (°C)	Time (sec)
65	30
8	30
65	90
97	180
8	60
65	180
97	60
65	60
80	hold

4. **DNA will be in the clear liquid above the white precipitate at bottom of tube.**

Agarose Gel Electrophoresis – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **To make a 0.7% agarose content gel first add 0.21g agarose and then 30mL 1 X TBE buffer to a 250mL Erlenmeyer flask.**
2. **Microwave until the solution boils, about 45-60sec. Let boil for 5sec, then check for agarose that has not gone into solution. If there is undissolved agarose, boil for 5sec at a time until solution is homogeneous.**
3. **Let solution sit until it is cool enough to touch and then add 2µL ethidium bromide using caution and swirl mixture.**
4. **Set up gel tray and combs and pour gel until it is solidified, about 30min.**

5. **Place gel in chamber oriented with positive electrode at the bottom of the gel and cover with 1X TBE.**
6. **Add 5µL water, 5µL DNA, and 2µL 6X loading dye.**
7. **Remove the comb and load each sample along with 5µL of selected ladder. Run at ~100V (lower voltage for clearer bands but slower run time).**
8. **When loading dye has run about two thirds of the gel, remove gel and image with UV.**

Colony PCR – Grinnell – 2011 http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Prepare primers as follows:**
    1. **Spin down at 13300 rpm for 50sec.**
    2. **Add appropriate amount of nuclease free water to make a 100µM stock solution, from which a 20µM working solution is made.**
2. **Make the solution for PCR according to the following recipe**
    - **The resulting mixture we got from DNA isolation (either add directly to GeneReleaser product w/o disturbing the precipitate OR use 8µL offreeze-thaw product).**
    - **7.65µL nuclease free water (9.65µL if using freeze-thaw product)**
    - **5µL Phusion HF or GC Buffer**
    - **0.5µL dNTP (10µM)**
    - **0.5µL left primer (20µM)**
    - **0.5µL right primer (20µM)**
    - **0.6µL DMSO**
    - **0.25µL Phusion DNA polymerase**
    - **For a final volume of 25µL**
3. **Run PCR tubes on following thermal cycle program**

Step	Temperature (°C)	Time (sec)
1	98	60
2	98	10
3	3°C above the T_m of the primer (without prefix/suffix) that has the lower T_m of the two	30
4	72	extention rate at 30 sec/kb
	repeat steps 2 through 4 for 5 iterations	
5	98	10
6	3°C above the T_m of the primer (with prefix/suffix) that has the lower T_m of the two	30
7	72	extention rate at 30 sec/kb

	repeat steps 5 through 7 for 25 iterations	
8	72	300
9	4	hold

DNA sample	Temperature used in step 3(°C)	Temperature used in step 6(°C)	Time used for extention steps(°C)
rsaA	60	72.1	30
esp	41.2	68.4	45
P_{rsaA}	61.6	73.4	30
P_{xyl}	49.2	71.4	30

4. Take amplified DNA from the clear liquid layer on the top (if used GeneReleaser for DNA isolation).

5. Occasionally, we used colony PCR to check if transformation cells have the target gene in them. The recipe for PCR is the same as the aforementioned one but a different thermal cycle program is used.
6.

Step	Temperature (°C)	Time (sec)
1	98	30
2	98	10
3	Use 47 for pMR10 plasmid and 55.3 for pSB1C3 plasmid	30
4	72	extention rate at 30 sec/kb
	repeat steps 2 through 4 for 30 iterations	
8	72	300
9	4	hold

Colony PCR with GoTaq® Green Master Mix – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

We also used GoTaq® Green Master Mix, a product of Promega Corporation.

1. Thaw Master Mix (MM) to rt, vortex, and centrifuge.
2. Prepare rxn mixture on ice:
   - 12.5µL 2X MM
   - 0.25-2.5µL each 10µM primer
   - 1-5µL template DNA (<250ng)

- **Nuclease-free H_2O to 25µL**

3. **Add mineral oil (1-2 drops) if your thermocycler does not have a heated lid.**
4. **Perform PCR (see guidelines below).**

This is the generalized PCR cycling protocol for use with GoTaq®

Step	Temp (°C)	Time (sec)
1	95	120
2	95	30 to 60
3	T_M - 2	30 to 60
4	72 to 74	60/kb
Repeat steps 2 through 4 25 to 40 times		
5	72 to 74	300
6	4	hold

Purification of DNA > 300bp by Centrifugation

We used the Wizard® SV Gel and PCR Clean-Up System Technical Bulletin from Promega to clean out PCR products >300bp in length. The protocol is below.

1. **Make an SV Minicolumn assembly by placing a minicolumn in a collection tube.**
2. **Transfer impure DNA solution to minicolumn assembly and incubate at rt for 1min.**
3. **Centrifuge assembly for 1min at 16,000 x g (14krpm). Remove minicolumn from collection tube and discard liquid in collection tube. Reassemble assembly.**
4. **Wash minicolumn by adding 700µL Membrane Wash Solution, previously diluted with 95% EtOH, to minicolumn and centrifuging as in step 3. Discard liquid in collection tube.**
5. **Wash again with 500µL of wash solution, this time centrifuging for 5min at 16,000 x g.**
6. **Discard liquid in collection tube. Centrifuge for 1min with microcentrifuge lid off or open to allow any remaining EtOH to evaporate. (This step generally also works with the lid on.)**
7. **Transfer minicolumn to a clean 1.5mL microcentrifuge tube and add 50µL nuclease-free H_2O to column membrane without touching the membrane with the pipette tip. Incubate at rt for 1min, then centrifuge as in step 3.**
8. **Discard the minicolumn and store the microcentrifuge tube that contains the eluted DNA at -20°C.**

Gel extraction – Grinnell – 2011 http://2011.igem.org/Team:Grinnell/Notebook/Protocols

We used the same kit from Promega as we used for DNA > 300bp purification. The protocol is slightly different.

1. **Find the desired bands on gel under UV and cut out the gel containing these bands. (Smaller slices are better. Also, as the UV tends to damage the DNA, minimize the exposure to UV.)**
2. **Weigh the gel slice.**
3. **Save the gel slice in a 1.5ml microcentrifuge tube.**
4. **Add 1µL Membrane Binding Solution per mg of gel slice.**
5. **Vortex and incubate at 55°C until gel slice is completely dissolved.**
6. **Treat the gel mixture the same as PCR product, follow the DNA > 300bp purification protocol to finish the rest of the gel extraction.**

Gel extraction 2 – Grinnell – 2011 http://2011.igem.org/Team:Grinnell/Notebook/Protocols

Alternatively, we used Zymoclean Gel DNA Recovery Kit from Zymo Research.The protocol is slightly different from the Promega kit.

1. **Pre-weigh the 1.5ml microcentrifuge tube. – Grinnell – 2011** http://2011.igem.org/Team:Grinnell/Notebook/Protocols
2. **Find the desired bands on gel under UV and cut out the gel containing these bands. (Smaller slices are better. Also, as the UV tends to damage the DNA, minimize the exposure to UV.)**
3. **Save the gel slice in a 1.5ml microcentrifuge tube.**
4. **Weigh the microcentrifuge tube and calculate out the mass of the gel slice.**
5. **Add 300µL ADB per 100mg of gel slice.**
6. **incubate at 55°C for about 10min until gel slice is completely dissolved.**
7. **Transfer the melted agarose solution to a column in a collection tube.**
8. **Add 200µL of Wash Buffer to the column and centrifuge at 16000g for 30 seconds. Discard the flow-through and repeat the wash step.**
9. **Add >=6µL water directly to the column matrix. Place column into a 1.5ml tube and centrifuge at 16,000g for 30sec to elute DNA.**
10. **Pure DNA in water now is ready for use.**

Purification of DNA < 300bp by Centrifugation – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Estimate the volume of DNA solution.**
2. **Adjust the concentration of monovalent cations by addition of sodium acetate (0.3M).**
3. **Mix well. Add 2 volumes ice cold ethanol and mix well. Store in -20°C freezer for 30 minutes.**
4. **Centrifuge at 0°C taking care of the orientation of the tubes because the DNA pellet will be invisible.**
5. **Remove supernatant.**
6. **Fill tube halfway with 70% ethanol and centrifuge at maximum speed for 2 minutes at 4°C.**

7. **Remove supernatant.**
8. **Store tube open in a heat block to evaporate any remaining fluid off.**
9. **Dissolve DNA pellet with buffer from Miniprep kit.**

Miniprep to Obtain Plasmid DNA from Overnight Culture – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

We used the PureYield™ Plasmid Miniprep System from Promega to obtain Plasmid from overnight cultures. The protocol is below.

1. **Add 600µL of bacterial culture to a 1.5mL microcentrifuge tube.**
2. **Add 100µL of Cell Lysis Buffer and mix by inverting tube 6 times.**
3. **Add 350µL of cold Neutralization Solution, and mix by inversion.**
4. **Centrifuge at maximum speed for 3 minutes.**
5. **Transfer supernatant to PureYield Minicolumn and Collection Tube and centrifuge at maximum speed for 15 seconds. Discard the flow-through.**
6. **Add 200µL Endotoxin Removal Wash and centrifuge at maximum speed for 15 seconds.**
7. **Add 400µL Column Wash Solution and centrifuge at maximum speed for 30 seconds.**
8. **Add 30µL nuclease free water to column and let stand for 1 minute before centrifuging into a 1.5mL centrifuge tube.**

Alternate Miniprep Protocol – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

We also used Promega's Wizard® ***Plus*** **SV Minipreps kit. The original procedure can be found at Promega's website.**

1. **Pellet overnight culture for 5min. at rt (use 3mL overnight culture if plasmid is low copy, 1.5mL otherwise).**
2. **Resuspend in 250µL of the provided cell resuspension sol'n.**
3. **Add 250µL cell lysis sol'n, invert 4 times to mix.**
4. **Add 10µL alkaline protease sol'n, invert 4 times to mix, let stand at rt 5min.**
5. **Add 350µL neutralization sol'n, invert 4 times to mix.**
6. **Centrifuge at top speed 10min at rt.**
7. **Insert column into collection tube and decant lysate into the column.**
8. **Centrifuge at top speed for 1min, discard flowthrough and reinsert column into collection tube.**
9. **Add 750µL column wash sol'n (w/EtOH), centrifuge at top speed 1min, discard flowthrough and reinsert column into collection tube.**
10. **Repeat previous step with 250µL wash sol'n.**

11. **Centrifuge at top speed 2min at rt.**
12. **Transfer to a new 1.5mL microcentrifuge tube, being careful to avoid leaving any wash sol'n on the column.**
13. **Add 50µL nuclease free H_2O and centrifuge at top speed for 1min at rt.**
14. **Discard column and store flowthrough at -20°C or below.**

Freeze-Thaw Cell Lyse

As a preparation of template DNA for colony PCR – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Add 10µL nuclease-free water to a PCR tube.**
2. **Inoculate tubes with some cells (generally from plate cultures).**
3. **Freeze cells at -20°C or -80°C for 10 to 20 minutes. (Depending on container, this may take 30 to 40min.)**
4. **Transfer cells to hot block or thermocycler set at 95°C for 10 minutes.**

This method has a lower success rate than using GeneReleaser, but is well suited to doing larger numbers of samples simultaneously.

Conjugation: Transfer desired plamid from *E. coli* to *Caulobacter* – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

After obtaining pMR10 plasmid (can replicate in both *E. coli* and *Caulobacter*) that contains promotor and desired protein gene in *E. coli*, we will need to transfer the plasmid from *E. coli* to *Caulobacter* through conjugation.

1. **Prepare liquid overnight cultures of recipient (e.g. *Caulobacter*), donor (e.g. *E. coli* with pMR10 plasmid) and helper strains (e.g. *E. coli*KR2515).**
2. **Add 600µL of recipient culture and 80µL of both helper and donor strains in a 1.5mL microcentrifuge tube.**
3. **Spin at 7000 RPM for 1 min and then remove the supernatant.**
4. **Gently suspend the cells in 1mL PYE (no vortexing).**
5. **Spin again as above, remove supernatant and resuspend in 25µL PYE.**
6. **Pipette all concentrate cell culture on a plain PYE plate (w/o spreading) and incubate at 30°C for 5h to overnight.**
7. **Streak some of the big colony growth from the plain PYE plate out on a PYE plate containing nalidixic acid and kanamycin.**
8. **Nalidixic acid will kill remaining *E. coli* but leave the *Caulobacter*, and kanamycin will select for those cells that have pMR10 plasmid in them.**

Preparing samples for and running a polyacrylamide gel – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Inoculate 3mL of broth and let grow overnight.**
2. **Spin down 1mL of overnight culture.**
3. **Collect supernatant (~900µL) and perform protein precipitation protocol.**
4. **Resuspend pellet in 100µL broth.**
5. **Add 50µL 3X Sample Buffer (aliqiots are 850µL, add 150µL β-mercaptoethanol).**
6. **Heat tubes of pelleted cells and supernatant with Sample Buffer at 95°C for 3-5 minutes. They can now be frozen at 20°C until further use or used immediately.**
7. **Load 20µL of sample into each well of a polyacrylamide gel and let gel run for 1 minute.**
8. **Then load 15-20µL more sample, or however much will fit in the well.**
9. **Run at 125-200V for 1-2 hours. Make sure the buffer is always covering the wells.**
10. **When the dye has reached the bottom of the gel, remove from gel rig, open plastic gel container using well comb or a coin, and place in bin.**

Staining a Protein Gel with Coomassie Blue – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Rinse with deionized water once and then let it sit in deionized water for 5 minutes then pour water off then let sit in water again for 5 minutes. Pour off water.**
2. **Prefix the gel in "fixing" solution (50% MeOH, 10% HoAC, and 40% water) for 30 minutes or overnight shaking slowly.**
3. **Then stain the gel with Coomassie stain ("fixing" solution with 0.25% Coomassie Blue R-250 added) for 2-4 hours, until the gel is a uniform blue color. Staining is complete when the gel is no longer visible in the dye solution.**
4. **Destain for 4-24 hours (5% MeOH, 7.5% HoAC, 87.5% water). Bands will begin to appear in 1-2 hours. Destain until background is clear.**

Precipitating proteins out of supernatant in preparation for running a protein gel – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Add 110µL/mL sample of 100% TCA to each tube and vortex for 10 seconds.**
2. **Incubate tubes on ice for at least 15 minutes (lengthen time for more precipitation).**
3. **Centrifuge tubes at full speed for 5 minutes in a chilled rotor.**
4. **Discard supernatant by decanting. Pellet will remain at the bottom of the tube. It is important to remove all of the supernatant. Blot the lip of the inverted tube with a Kim Wipe. Spin down again for 5 seconds and use a micropipettor to remove any remaining supernatant.**
5. **Resuspend the pellet in 40µL of 0.5 M Tris base or PYE and then add 20µL 3x Sample buffer.**

Biofilm Assay to test the biofilm inhibition activity of Esp/DspB (pre-formed Biofilm) – Grinnell – 2011
http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Inoculate *S. aureus* in 3ml L-Broth and incubate at 37°C overnight.**
2. **Dilute culture by adding 1ml of overnight *S. aureus* culture to 50ml L-Broth.**
3. **Shake for 1h or longer (up to overnight).**
4. **Add 100µL of the diluted *S. aureus* culture to each cell of the 96-well plate.**
5. **Cover the plate and parafilm the 96-well plates to prevent significant evaporation.**
6. **Incubate at 37°C for several days.**
7. **Inoculate *Caulobacter* strains in M2g-kan Broth and incubate at 30°C overnight.**
8. **Take Optical Density of each overnight *Caulobacter* culture and equate the OD by diluting the more concentrated ones with plain M2g broth.**
9. **The resulting cultures can be used directly for biofilm assay, otherwise incubate cultures at 30°C for another couple of hours.**
10. **Dump off the remaining L-Broth that is in the 96-well plates to a bucket that has bleach in it.**
11. **Add 10µL of *Caulobacter* culture to corresponding well and add 10µL 2% xylose if necessary.**
12. **Add plain M2g broth to the wells to make a total of 100µL mixture.**
13. **Incubate at 30°C for 16-24h.**

Biofilm Assay Harvest – Grinnell – 2011 http://2011.igem.org/Team:Grinnell/Notebook/Protocols

1. **Remove the culture from each well (pour into bucket of 1% bleach).**
2. **Rinse out the plate 3 times with water.**
3. **Add 125µL of 0.1% crystal violet to each well.**
4. **Allow the plate to sit for 10-15 minutes.**
5. **Rinse out the plate 3 times with water.**
6. **Allow the plate to dry for at least overnight.**
7. **Add 150µL ethanol to each well and incubate for 10 minutes.**
8. **Measure the absorbance at 550 nm.**

PCR for 16s (10 µl) – Colombia – 2011
http://2011.igem.org/Team:Colombia/Notebook/Protocols

Reactives	µl	µl

Buffer	2.5	1
MgCl2	2	0.8
DNTP´s	0.5	0.2
P1492R	0.5	0.2
P27F	0.5	0.2
Taq	0.25	0.1
DNA	2	0.8
H2O	17.75	6.8
Final Vol	26	10

PCR Pfx – Colombia – 2011 http://2011.igem.org/Team:Colombia/Notebook/Protocols

Reactives	ul
H2O	17.2
Buffer PFX	3
MgSO4	0.6
dNTP´s	0.9
FW	0.9
RV	0.9
Enhancer	3
Taq PFX	0.5
ADN	3
Total Mix	30

PCR CBP – Colombia – 2011 http://2011.igem.org/Team:Colombia/Notebook/Protocols

Reactives	1X µl	3X µl
H2O	18.8	56.4
Buffer PFX	2.5	7.5
MgSO4	0.5	1.5
dNTP´s	0.75	2.25
FW (Pr3)	0.75	2.25
RV (Pr4)	0.75	2.25
PFX	0.2	0.6

ADN	0.75	2.25
Mix Volume	25	75

PCR Sensor – Colombia – 2011
http://2011.igem.org/Team:Colombia/Notebook/Protocols

Reactives	µl
H2O	17.3
Buffer PFX	3
MgSO4	0.6
dNTP´s	0.9
FW (1)	0.9
RV (2)	0.9
PFX	0.4
Enhancer	3
ADN	3
Total Mix	30

Biobrick Assembly Restriction Digest – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

For a double digest:
1. To a pcr tube, add 10 ul of miniprepped plasmid or purified PCR product
2. Also add 6 ul H2O.
3. Add 2 ul of the appropriate buffer (look at NEB's Restriction Enzyme Activity chart)
4. Add 1ul of each enzyme.
5. Incubate at 37˚C for 1 hour.
6. Add CIP to the backbone digest. Incubate at 37˚C for 1 hour
7. PCR purify the reactions, unless you need to select out your part through gel extraction. Avoid these when possible, but if your insert is coming from a plasmid rather than a PCR, you need to select the insert you want and avoid having any of the leftover backbone in your ligation reaction. Also, you may need to do a triple digest to be able to size select for your insert. Use Geneious to find restriction sites.

Biobrick Assembly Ligation – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

1. In a PCR tube, add restriction digested and PCR purified insert to backbone in a 3-5:1 molar ratio, usually 1 ul backbone with 2 ul insert works.
2. Add 5.5 ul H20 (or other, so that the total volume of the ligation is 10 ul)
3. Add 1ul T4 ligase buffer
4. Add 0.5 ul T4 ligase
5. Create a negative control replacing the insert with water
6. Add reactions to thermal cycler. Incubate at 22˚C for 30 minutes, at 65˚C for 10 minutes to heat inactivate, and if not being used right away, leave at 4˚C.
7. Use 2 ul of the ligation reactions to transform cells.

Electrocompetent cells – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

1. Centrifuge 1 mL of the overnight E. coli culture to be transformed.
2. Pour off the supernatant in liquid waste container.
3. Add 1 mL of cold 10% glycerol.
4. Vortex the cells to resuspend them in the cold 10% glycerol.
5. Centrifuge the culture again and go to #2. Repeat 4-5 times. This step washes the cells with cold 10% glycerol to remove the LB.
6. Centrifuge the cells one final time, pour off the supernatant, add 50 microliters of cold 10% glycerol and vortex to resuspend.
7. Let the cells chill on ice for ~30 minutes.

Electroporation – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

1. Take 1 50ul aliquot of DH5a electrocompetent cells from -80˚C freezer.
2. Add 50 ul COLD 10% glycerol to dilute the cells, as ours are too concentrated. Divide into 2 50ul aliquots
3. Put electrocuvettes in freezer to cool. Keep on ice at all times before shocking
4. Add 1-2 ul of DNA (depends on source of DNA, use less for plasmids/biobricks, more for ligations etc.) to each 50 ul aliquot of cells.
5. Leave on ice for 2-5 minutes
6. Electroporate at 2.5 kV, 25 uF, 200 ohms by sliding cuvette into the machine and pressing the two buttons until you hear a beep
7. Immediately dilute with 250 ul LB or SOC. 8. Incubate in a shaker at 37˚C for 1 hour. 9. Plate using 50ul, otherwise you might get way too many colonies.

Enrichment cultures – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

- For BPA (since soluble)

1. Set up 16 tubes: 8 tubes with vitamin media vs. 8 tubes with media (no vitamin), 4 tubes for each of the four locations.
2. Place 8 test tubes in 30°C shaker and 8 test tubes in room temperature shaker.

- For 17α-estradiol, DDT, and nonylphenol (since non-soluble)

1. Set up two flasks: one with vitamin media, one without vitamin.
2. Add small amounts (around 50mL or 50mg) of the ten LA river samples into each flask.

For both: culture initially for 3 days, then reculture for 7 days. Then test for DNA and continue cultures.

CopyControl Fosmid kit – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

http://www.epibio.com/pdftechlit/171pl1010.pdf
alternate link: http://arb-ls.com/products/copycontrol_fosmid_library_production_kit/171.pdf

Gibson Assembly (Adapted from Cambridge 2010) – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

0a. PCR DNA strands (50uL rxn)
0b. DpnI digest and purify products (elute w/ 20-30uL EB or H_2O);(Nanodrop)-> normally 50-120ng/nL
1. Mix DNA
2. To 3uL of DNA, add 7.5uL of Gibson Mix
3. Incubate @ 50C for 30-60 minutes with heated lid
4. Cool, then transform into chemically competent cells, or dilute 1:3 and use 1-3 ul of that to transform electrocompetent cells.

Mobio PowerMax Soil kit – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

http://www.mobio.com/images/custom/file/protocol/12988-10.pdf

p450 binding assay, organic extraction for analysis by HPLC – Caltech – 2011 **http://2011.igem.org/Team:Caltech/Protocols**

1. Obtain a ~80mM solution of the chemicals in DMSO, 1mL total.
2. Form a 200uL solution of the diluted substrate, p450, $NADP^+$, glucose, glucose dehydrogenase, and buffer. (see Mixes)
3. Leave overnight for reaction.
4. Add 200uL water and 180uL DCM; vortex thoroughly.
5. Centrifuge briefly and pipette out the organic (bottom) layer into a new tube.
6. Repeat steps 3 and 4.
7. Evaporate liquid from the new tube on 40°C plate.
8. Add 50uL of .5x diluted ACN, then centrifuge at 12,000 RPM for 5 min.
9. Place the 50uL samples into HPLC tube and bottle.
10. Run HPLC for 10 min per sample.

p450 binding assay, organic extraction for analysis by GCMS – Caltech – 2011 http://2011.igem.org/Team:Caltech/Protocols

1. Obtain a ~80mM solution of the chemicals in DMSO, 1mL total.
2. Form a 200uL solution of the diluted substrate, p450, $NADP^+$, glucose, glucose dehydrogenase, and buffer. (see Mixes)
3. Leave 4 hours for reaction.
4. Add 300uL buffer and 180uL DCM; vortex thoroughly.
5. Centrifuge briefly and pipette out the organic (bottom) layer into a new tube.
6. Repeat steps 3 and 4, but with 500uL DCM (to dilute sample for GCMS).
7. Run GCMS.

Pulse Gel Field Electrophoresis – Caltech – 2011 http://2011.igem.org/Team:Caltech/Protocols

PFGE separation of 0.5 µg of Lambda Mono Cut Mix, 0.1% agarose gel, 0.5X TBE
Parameters: 6 V/cm, 15°C for 20 hours.
Switch times ramped from 0.5-1.5 seconds.

Phusion PCR – Caltech – 2011 http://2011.igem.org/Team:Caltech/Protocols

Thermocycling conditions:
Initial Denaturation: 98°C for 30 seconds
25-35 cycles:

- 98°C for 10 seconds
- 55°C, 60°C, 65°C for 15 seconds

- 72°C for 15 seconds

Final Extension: 72°C for 5 minutes

Qiagen Miniprep kit – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

www.qiagen.com/hb/qiaprepminiprep

Transforming DNA from Distribution Plates: – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

1. Thaw competent cells on ice.
2. Add 10 microliters of pure water to each well of DNA from plates, pipette up and down.
3. Transfer into storage tube.
4. Pipette 1-2 microliters of the DNA into the competent cell tubes.
5. Stir with pipette tip, gently flick tube.
6. Leave on ice for 30 minutes.
7. Heat Shock for 45 sec by using a water bath set to 42°C and then chill on ice for 2 min.
8. Pipette 500 micro Liters of S.O.C. (LB + glucose) into 14ml culture tubes; transfer the competent cells into these tubes and incubate in a 37 degree shaker for 0-60 minutes before plating.
9. For source plate DNA, plate 100 microliters.

Taq PCR (16s insert) – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

Initial denaturation: 94°C for 1:30 min
35 cycles of:

- 94°C for 0:30 min
- 54°C for 1:00 min
- 72°C for 2:00 min

Final extension: 72°C for 6:00 min

Colony PCR (for ~.7kb insert) – Caltech – 2011
http://2011.igem.org/Team:Caltech/Protocols

Suspend colonies in 10 ul H20
Lyse with 98°C incubation for 10 minutes
Use 1 ul of this suspension as template
Set up tubes with 7 ul H20, 1 ul template, 1 ul forward primer, 1 ul reverse primer, 10 ul Phusion

master mix
Cycle:
2 minutes at 98°C
Run 30 cycles of:

- 10 seconds at 98°C
- 30 seconds at 55°C-65°C
- 40 seconds at 72°C

Final extension: 5 minutes at 72°C

X-Gal Plates – Caltech – 2011 http://2011.igem.org/Team:Caltech/Protocols

1 Dissolve X-Gal in DMSO at concentration of 20mg/ml (50x). Store at -20˚C. (There is a stock as of 8/24)
2 For typical 20 ml plate (with antibiotic already added in if needed), add 40 ul to the top of the plate and spread immediately with an L-shaped spreader to form an even coat.
3 Wait 30+ minutes until the X-Gal layer is dry.
4 Plate as usual. Colonies expressing beta-galactosidase (lacZ) will be blue.

PCR – BYU Provo – 2011
http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

There are two types of PCR polymerases used in the lab, Taq and high fidelity polymerases (such as Phusion). Taq has an error rate of about 1 bp changer per 1 kb, while many high fidelity polymerases have an error rate that is 60 times lower. Taq is cheaper and will give an abundance of product, so use when accuracy is not an issue.

PCR (Phusion Polymerase - 50 µl reaction) – BYU Provo – 2011
http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

- ~35 µl ddH2O
- 10 µl 5x Phusion Buffer
- 1.5 µl 10 mM dNTP's
- 1 µl of each primer
- 1 µl appropriate diluted template DNA
- 0.5 µl Phusion Polymerase

PCR (Taq Polymerase - 50 µl reaction) – BYU Provo – 2011

http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

- 40 µl ddH2O
- 5 µl 10x Thermopol Buffer
- 1.5 µl 10 mM dNTP's
- 1 µl of each primer
- 1 µl appropriate diluted template DNA
- 0.5 µl Taq Polymerase

Colony PCR (25 µl reaction) – BYU Provo – 2011

http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

- 19 µl H2O
- 2.5 µl 10x reaction buffer
- 0.5 µl 10 mM dNTP's
- 0.5 µl each primer
- 0.5 µl Taq DNA polymerase (*add last to master mix)
- add 2 µl of boiled colony sample

Mix (vortex or flick) tubes well before adding to the reaction. Set up reactions on ice and keep them on ice until placing them on the PCR machine which has been pre-warmed to 94˚C (this is called a "hot start"). Extension times vary according to target size. Taq requires 1 min per kb of product, and Phusion requires 2 min per kb of product. Annealing temperature depends on primer Tm values. For typical primers, 55˚C is a good guess. Sample program for amplifying a 1kb target with Taq polymerase:

1. 94˚C, 2:00
2. 94˚C, :30
3. 55˚C, :30
4. 72˚C, 1:00
5. Repeate (steps 2-4) 35 times
6. 72˚C, 2:00
7. 4˚C forever

Standard Agarose Gel – BYU Provo – 2011

http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

For a standard 1% agarose gel, use 50 mL of 1xTAE and 0.5 grams of agarose (regular agarose, NOT low-melt). Microwave for about 1 minute or until the agarose is completely disolved. Pulsing the microwave may be necessary to avoid boiling over. Add 8 µl ethidium bromide and swirl to mix. BE SURE TO WEAR GLOVES AND TO HANDLE ETHIDIUM BROMIDE CAREFULLY ... IT IS A CARCINOGEN.

Allow the flask to cool so that the glass feels warm/hot not burn/hot. Pour the liquid into the gel bed and let it cool. Insert the appropriate sample comb.

To run the gel:

Add loading dye to each sample. Move the gel into the proper orientation in the gel box and cover with 1x TAE buffer. Load all your samples into the wells (4-5 µl into each well should be sufficient). ADD DNA LADDER AS REFERENCE. Put lid on, and set power supply to between 130 and 170 volts. It will take between 15 and 30 minutes to run, depending on the desired resolution.

Visualize gel on imager, print of results and paste into your notebook.

Restriction Digest of insert and/or vector (50 µl reaction) – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

- ~14 µl H_2O
- 5 µl 10x NEB buffer (check online for double enzyme digest chart) [1]
- 0.5 µl 100x BSA
- 30 µl DNA sample
- 1-2 µl of each restriction enzyme
- **Always mix reagents well before adding enzyme as the final reagent**

(incubate this reaction at 37˚C for at least 2.5 hours)

Note: If a non-directional ligation will be performed (single restriction site), then you must dephosphorylate the vector by adding 0.5 µl of calf intestinal phosphatase (CIP) to the restriction digest for the last hour prior to low-melt gel purification.

Low-melt gel purification – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

Low-melt agarose gel electrophoresis is similar to regular gel, with some exceptions. The low-melt agarose dissolves faster, and boles over more easily during microwaving, so microwave in brief pulses. Add the normal amount of ethidium bromide (~160 ng/ml) to the molten agarose before casting. Cast your gel in the fridge to accelerate solidification. Use a large-tooth comb to form the wells that will accomidate ~40-50 µl of sample. Use TAE buffer to make and run the gel. Run gel at 90 volts. It will take about 45 - 60 minutes to run.

When the gel has run, use a portable UV lamp and carefully remove the DNA bands from the gel with a razor blade. Place the DNA samples in clearly labeled 1.5 ml tubes. Also, cut out a slice of gel that has no DNA for use as a vector-only control sample (see ligation section).

Ligation (15 µl reaction) – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

- 6.5 µl H_2O
- 1.5 µl 10x ligase buffer (includes ATP)
- 1 µl T4 DNA ligase
- 3 µl vector

- 3 µl insert

(incubate this reaction at room temperature for at least 30 minutes)

Compatible sticky ends will bring vector and insert together only transiently. DNA ligase forms phosphodiester bonds between vector and insert. Set up two reactions: vector + insert and the vector-only control. The gel slices should be heated to 65˚C to melt them. Once melted, the samples will remain melted for a few minutes.

Transformation – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

1. Typically, E. coli strain DH5α is made chemically competent. Thaw DH5α chemically competent cells on ice. Meanwhile, melt the ligations for a few minutes at 65˚C. Also, be sure you have a 42˚C heating block ready, as well as LB-agar plates with the proper antibiotic.
2. When the DH5α is thawed, quickly add 10 µl of the ligation mix to ~25 µl of competent cells. Flick or vortex briefly and put them back in the ice for 5 - 15 minute (it is important that the DH5α cells be kept as cold as possible during this process).
3. Heat shock at 42˚C for 60 seconds. Immediately place the tubes back on ice.
4. Add 500 µl of plain LB to the reactions and incubate at 37˚C for 30-60 minutes (30 minutes if the selection is ampicillin, 60 minutes for everything else).
5. Plate 100 µl of cells and incubate at 37˚C overnight.

Sequencing (submitting to BYU DNA sequencing center) – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

- 5 µl H2O
- 5 µl template
- 1.5 µl primer (ONLY ONE PRIMER)

Submit sample online and label tubes with sample number. Take to sequencing center and place on "cycle sequencing ready" shelf.

Freezing strains in -80˚C freezer – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

200 µl DMSO 1.3 mL overnight culture Place in sterile 1.5 ml tube. Vortex and quickly freeze at -80˚C.

Electroporation – BYU Provo – 2011 **http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols**

Cell preparation:

1. Fill a 1.5 mL tube with 500 µl ddH2O
2. Add approximately 3 mg cells (3 generous swipes across a patch ... don't include any agar since it inhibits transformation)

3. Centrifuge at 14000 rmp for 1 minute. Remove supernatant and suspend cells in 500 µl ddH2O.
4. Centrifuge again at 14000 for 1 minute. Remove supernatant and suspend cells in 40 µl ddH2O. Keep on ice.
5. Cells are now ready for electroporation.

Electroporation:

1. Add 5 - 50 ng of plasmid DNA and mix gently to ensure homogenous suspension.
2. Transfer DNA/cell suspensions to electroporation cuvettes and keep on ice.
3. After pulsing, immediately add ice-cold LB to each cuvette.
4. Transfer to culture tubes and incubate for 1 hour at 37˚C.
5. Spread on selective plates and incubate overnight at 37˚C.

Mutagenic PCR (from paper by RC Cadwell and GF Joyce in Genome Research, 1994) – BYU Provo – 2011

http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

1. Prepare a 10x mutagenix PCR buffer containing 70 nM MgCl2, 500 mM KCl, 100 mM tris (pH 8.3 at 25˚C), and 0.1% (wt/vol) gelatin.
2. Prepare a 10x dNTP mix containing 2 mM dGTP, 2 mM dATP, 10 mM dCTP, and 10 mM TTP.
3. Prepare a solution of 5 mM MnCl2. DO NOT combine with the 10x PCR buffer, which would result in formation of a precipitate that disrupts PCR amplification.
4. Combine 10 µl of 10x mutagenic PCR buffer, 10 µl of 10x dNTP mix, 30 pmoles of each primer, 20 fmoles of input DNA, and an amount of H2O that brings the total volume to 88 µl. Mix well.
5. Add 10 µl of 5 mM MnCl2. Mix well and confirm that a precipitate has not formed.
6. Add 5 units (2 µl) of Taq polymerase, bringing the final volume to 100 µl. Mix gently. Cover with mineral oil or a wax bead if desired.
7. Incubate for 30 cycles of 94˚C for 1 minute, 45˚C for 1 minute, and 72˚C for 1 minute. Do not employ a "hot start" procedure or a prolonged extension time at the end of the last cycle.
8. Purify the reaction products and run a small portion on an agarose gel.

"Plate Reader Experiment" Protocol – BYU Provo – 2011

http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

1. Start overnight cultures on the day before the experiment. Use appropriate antibiotic in the media, and label tubes well. Place on shaker at 37˚C overnight, on shaker.
2. Measure 1/10 optical density (OD) of each overnight culture by diluting 100 µl of saturated overnight culture into 900 µl of plain LB. Place the resulting mL of diluted culture into a cuvette and measure 1/10 OD. Multiple resulting measurement by 10 for actual OD of culture.
3. Dilute each culture to an OD of 0.025 into overnight culture tubes. An example calculation:
   - 1/10 OD = 0.62
   - Actual OD = 6.2
   - 6.2 / 0.025 = 248x (The overnight culture is 248 times more concentrated than our desired dilution)

- 2000 µl / 248 = 8.06 µl
  - Thus we must add ~8 µl to 2 mL of plain LB to reach our desired OD of 0.025.
  - Perform this calculation for each culture, and make the dilutions.

4. Incubate at 37˚C for 1.5 hours, on shaker.
5. Add H2O2 (hydrogen peroxide) to the 2 mL dilutions so that desired concentrations of H2O2 are in the desired range (similar concentration calculations as in step 3).
6. Incubate at 37˚C for 3 hours, on shaker.
7. Add 100 µl of each H2O2-induced culture, in order, into the wells of a black plate, and identically into a clear plate. The clear plate is for measuring the OD of each sample, and the black plate for measuring fluorescence.
8. Use iGEM protocols in lab machine. For OD, use 600 nm, and for fluorescence, use excitation 485 nm and emission 528 nm. Set fluorescence reader sensitivity to 45.
9. Export results to spreadsheet. Prepare data by dividing fluorescence readings by corresponding OD measurement. Graph or analyze appropriately.

Beta-Galactosidase Assay – BYU Provo – 2011
http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

1. Grow cells overnight in minimal media.
2. Inoculate 3 - 1.5 mL of assay media with 20 uL fresh overnight culture. Grow at 30˚C, 35˚C and 37˚C until culture reaches OD600 of 0.2 to 0.7.
3. Chill culture on ice for 20 minutes. Record cell density by measure OD600.
4. Add 500 uL cells to 550 uL ZS-buffer.
5. Add 100 uL chloroform to the tubes. Incubate for 2 minutes at 30˚C.
6. Add 200 uL 4mg/mL o-NPG to start the reaction. (o-NPG made fresh daily 56mg o-NPG in 14 mL sterile water). Note the time of addition precisely.
7. Incubate the reaction at 37˚C until sufficient yellow color has developed.
8. Stop the reaction by addition of 500 uL 1M Sodium Carbonate. Incubate for 5 minutes at 30˚C. Note the time of addition precisely.
9. Centrifuge to precipitate cell debris and transfer 1 mL of supernatant to a cuvette.
10. Record OD405 and OD550 for each tube. OD550 is for cell debris control and should be low.
11. Calculate Miller Units with the equation: (1000)*[OD405-1.75*OD550]/[t*v*OD600] where t is reaction time in minutes and v is volume of culture used in mL.

ZS Buffer – BYU Provo – 2011
http://2011.igem.org/Team:BYU_Provo/Protocols#Protocols

- 16.1 g Na2HPO4.7H2O
- 5.5 g NaH2PO4.H2O
- 10.0 mL 1 M KCL
- 1.0 mL 1 M MgSO4

- 2.7 mL beta-Mercaptoethanol
- 985 mL water
- 100 mL 0.1% SDS

Mix all ingredients together except the SDS first, then add in the SDS and gently mix to prevent it from bubbling.

Yeast Blue Stain Fungi Co-culture Experiment **– British Columbia – 2011 http://2011.igem.org/Team:British_Columbia/Protocols/Sdm**

1) Place mycelium plug of BSF in centre of OMEA or YPD plate. Streak out appropriate yeast in a ring around the fungus.

2) Allow to grow for several days.

Beetle Transfer Experiment **– British Columbia – 2011 http://2011.igem.org/Team:British_Columbia/Protocols/Sdm**

In this experiment, we wanted to find out whether the beetles could serve as a vector for our genetically modified yeast. To do this, we followed the following procedure.

1) Obtain beetles

2) Place beetles using small sterile forceps on plates with appropriately labeled yeast (GFP or Clonat resistance). Allow beetles to walk on the yeast for 1 minute.

3) Place the beetles on empty plates for various amounts of time (0 hours, 10 hours, 24 hours, 36 hours)

4) Place beetles on new, clean YPD plates. Allow the beetle to walk on the clean plate for one hour.

5) Remove and properly dispose of the beetle. Allow the plates to grow for 2-3 days.

6) All plates should have yeast colonies, even the control. Streak out the colonies on appropriate plates (Note auxotrophy and antibiotic resistance). Allow to grow 1-2 days.

7) Check the plates. The control should not have grown and the clonat plates should have colonies only from the appropriate plates. Follow the protocol for GFP fixation and FACS on this page to check for GFP activity.

GC-MS Sample Preparation Protocol for yeast **– British Columbia – 2011 http://2011.igem.org/Team:British_Columbia/Protocols/Sdm**

Supplies Needed:

1. Erlenmeyer Flasks
2. Appropriate Media [Glucose (GPD) or Galactose (GAL)]
3. Spectrophotometer
4. sdH2O
5. Falcon Tubes
6. GLASS test tubes (large and small)
7. Pentane
8. Glass Beads
9. Sodium Sulphate (anhydrous)
10. GC Vials
11. 5mL Pasteur Pipets and Bulbs

Procedures:

Part 1: Preparation for Extraction

1. Make 5 mL overnight cultures of yeasts to be sampled (18-20 hours @ 30 degrees). The media used here must be SD-Leu (glucose).
2. Measure OD 600 of the cultures using the spectrophotometer. The OD 600 should be around 2.
3. Add the remaining cultures to Erlenmeyer flasks and dilute with 50 mL of of SD-Leu.
4. Grow up the cultures for about 2-3 hours (until OD 600 is 0.6-0.8).
5. Transfer all of the cultures into falcon tubes and spin down the cells (5 min @ 2500g).
6. Pour out media and resuspend the yeast pellets with about 47mL of SD-Leu (GPD) or SG-Leu (GAL).
7. Transfer the cultures to Erlenmeyer flasks and grow up overnight (18-20 hours@ 30 degrees).
8. Harvest cells (step 5).
9. Weigh the large glass tubes and record values.
10. Keep 1 mL of the media and transfer to large glass tubes and discard the rest of the media.
11. Wash the yeast pellet with 5 mL of sdH2O.
12. Transfer the 5mL of yeast to the glass tubes.
13. Spin down the cells (5 min @ 2500 x g).
14. Pour out water and weigh the glass tubes again to obtain the weight of the yeast pellet.
15. Proceed to Extraction.

Part 2: Extraction (all of the following steps should be done in the fumehood in Bohlmann lab)

1. Pipet in 2 mL of pentane to each glass tube.
2. Add 0.5-1 mL of glass beads to each tube.
3. Vortex as fast as possible without spilling for about 20 seconds.

4. Pour solvent into smaller glass tube. Pipet the solvent out for the media samples using the pasteur pipets. Avoid glass beads!
5. Add about 200 µL of Sodium Sulphate to the small glass tubes.
6. Make one blank (4 mL of pentane and Sodium Sulphate).
7. Repeat 1-4.
8. Bring down the volume to about 1 mL by applying a stream of nitrogen.
9. Place GC vials in a rack and label them.
10. Pipet out solvent and transfer to vials.
11. Apply nitrogen to vials and bring down the volume to about 0.4 mL.
12. Add pentane to 0.5 mL line.

GC-MS Sample Preparation Protocol for bacteria

Supplies Needed:

1. Erlenmeyer Flasks
2. Appropriate Media [LB Broth and Terrific Broth)
3. Spectrophotometer
4. sdH2O
5. Falcon Tubes
6. GLASS test tubes (large and small)
7. GC Vials
8. 5mL Pasteur Pipets and Bulbs
9. His-tag protein purification column
10. A chem stand with a clamp
11. Large centrifuge tubes
12. Nanodrop

Reagents Needed:

1. Lysis Buffer
2. Wash Buffer
3. Elution Buffer
4. 0.5M NaOH
5. GPP (geranyl pyrophosphate)
6. Enzyme Assay Buffer
7. Pentane
8. DTT (final conc of 5 mM)
9. Protein Inhibitory Cocktail (PIC)

Procedures:

Part 1: Preparation for Extraction

1. Make 50 mL overnight cultures of selected colony to be sampled (18-20 hours @ 37 degrees). The media used here must be LB broth with the selectable antibiotics.

2. Transfer 1 mL of overnight culture (in LB broth) to 250 mL of Terrific broth with the selectable antibiotics in a 1 L or 2 L Erlenmeyer flask.
3. Grow up the cultures for about 4-5 hours @ 37 degrees (until OD600 is 0.8).
4. Induce cells with 250 µL of 1M IPTG. Incubate culture for 16-18 hours at 16 degrees, shaking at 205 rpm.
5. Pour the culture into the centrifuge tubes.
6. Spin cells at 3000 rpm at 4 degrees for 20 minutes.
7. Transfer the cultures to Erlenmeyer flasks and grow up overnight (18-20 hours @ 30 degrees).
8. Carefully pour out supernatent and keep the pellet
9. Proceed to extraction or keep pellet at -80 degrees

Part 2: Extraction

1. Set up stand and clamp with the his-tag purification column. (Note: His-tag Column must never run dry. Therefore, it is advantageous to keep the column wet with wash buffer)
2. Add 1 tablet of protease inhibitor cocktail and DTT (final conc of 5 mM) to 50 mL of Lysis Buffer
3. Weigh out 1-1.5 g of cell pellet into falcon tube
4. Add 5 mL of Lysis Buffer (containing protease inhibitor and TPP) to the cell pellet. Pipet up and down until the pellet is dissolved and the cell lysate is homogeneous.
5. Sonicate cells (ready when there's a change in color; usually a lighter colour than the cell pellet)
6. Centrifuge sonicated cells at max speed for > 20 min (until all cells are pelleted)
7. Collect supernatent (should be around 5 mL).
8. Transfer supernatent into the his-tag column. Adjust flow such that the drop of supernatent passing the column is about 1-2 drops per second. Supernatent can be passed through column twice.
9. Add 10 mL of wash buffer. Collect drops slightly after the start of wash buffer flow through the column. Nanodrop to find out the absorbance and concentration of proteins passing through the column. Once this 10 mL of wash buffer has passed through, add another 10 mL of wash buffer to wash the column again to collect any residue proteins not binding to the beads of the column. Take a nanodrop reading at the end of the washing steps (last few drops of the wash buffer). Nanodrop reading should read 0 mg/mL at the end of second wash. If proteins are still present, continue with another wash until no protein can be found in the wash buffer.
10. Elute with 5mL of elution buffer and collect into a falcon tube.

Part 3: GPP assay

1. Pipet in 1 mL of purified protein to 4mL of enzyme assay buffer in a test tube
2. Add 7 µL of GPP
3. Add 1-2 mL of pentane (less is better in terms of getting high concentration yield; but may need large volume of pentane to avoid emulsion formation)
4. Incubate in water bath for 1 hour at 30 degrees celsius
5. Place GC vial in a rack and label it.
6. Pipet out solvent (the less dense pentane layer) and transfer to vials to 0.5 mL line.
7. Prepare a control without GPP added
8. Freeze both samples
9. Send for GC-MS

Yeast Transformation **– British Columbia – 2011 http://2011.igem.org/Team:British_Columbia/Protocols/Sdm**

1. Grow overnight culture; rotating/shaking at 30 degrees Celsius
2. The next day, dilute culture 1/10 and grow at 30 degrees Celsius for 3-4 hours
3. Pellet and wash once with 0.1 M Lithium Acetate (LiAc)
4. Pellet and discard supernatant
5. Add in order: 240 µL 50% Polyethylene Glycol (PEG), 36 µL 1M LiAC, 10 µL Boiled Herring Sperm DNA, 50 µL of your desired DNA construct (~10 ng/µL)
6. Vortex until uniform and incubate at 30 degrees Celsius for 30 min
7. Heat-shock at 42 degrees Celsius for 20 min
8. Pellet and discard supernatant
9. Resuspend with 200 µL sterile water and spread on selective media plate
10. Incubate plate at 30 degrees Celsius for 3-4 days and you should see colonies!

Yeast Crude Extract for SDS-PAGE

1. Grow overnight culture; rotating/shaking at 30 degrees Celsius
2. The next day, dilute culture 1/10 and grow at 30 degrees Celsius for 3-4 hours
3. Pellet and wash once with sterile water
4. Pellet and discard supernatant
5. Resuspend in lysis buffer and transfer to capped O-ring tubes
6. Add 200uL of tiny glass beads
7. Place in lytic beater at 4 degrees Celsius for 7 min
8. Centrifuge at 4 degrees Celsius for 10min at 13000 rpm
9. Carefully take the supernatant and transfer to a new microcentrifuge tube
10. Add appropriate amount of 5x SDS-PAGE loading buffer
11. Store at -20 degrees Celsius

GFP Fixation for microscopy and fluorescence activated cell sorting (FACS) (from Koshland Website)

1. Pellet yeast cells and discard supernatant
2. Add 100 µl of 4% paraformaldehyde, 3.4% sucrose and vortex
3. Incubate at RT for 15 min
4. Pellet yeast cells and discard supernatant
5. Wash once in KPO4/sorbitol (60 ml 2M sorbitol, 10 ml 1M potassium phosphate, 30 ml water)
6. resuspend in small volume of KPO4/sorbitol

7. Sonicate cells for 3 seconds on setting 3

Competent Cell Preparation (Small Scale) – British Columbia – 2011

http://2011.igem.org/Team:British_Columbia/Protocols/Sdm

Protocol generously donated by Jeanette (Beatty lab manager).

Supplies needed:

- DH5(alpha)
- LB broth--about 50mL per culture (each culture makes about 25-30 * 100 µL aliquots); about 5 mL for the initial overnight culture (inoculates multiple cultures)
- 0.1M CaCl2 (filter sterilized and chilled)--about 40-50 mL per culture
- Ice water bath
- 60% glycerol--about 1 mL per culture
- Spectrophotometer
- 100-250 mL flasks, centrifuge tubes (50 mL Falcon or equivalent), microcentrifuge tubes

Note: When working with cultures (that you will use later), always work aseptically. That is, work near a flame and be sure to turn off the flame at the end and follow aseptic procedures.

Steps: 1. Inoculate 5mL overnight culture (see overnight culture protocol) and grow at 30°C. You can use a rotating incubator. Approximately 30°C (can be a little higher or lower) seems to be fine. Otherwise, shaking speed ~200 rpm.

Note:A possible source of DH5(alpha) are frozen aliquots of DH5(alpha) competent cells. You can use the whole aliquot.

2. Dilute 0.5mL overnight culture into 50mL LB and incubate at 30°C, shaking vigorously. Label with name, date, strain, flask # (when making more than 1 culture), etc. Shaking speed ~200 rpm.

- Note: You can inoculate and use several 50 mL LB cultures at the same time. A 125 mL or 250 mL Erlenmeyer flask works well as a culturing container. Make sure to not rip or contaminate the aluminum foil cover. When you're done using the flasks, rinse with 10% bleach, then with water and put into dirty basket (ask Tony Lam for help if needed).

3. Harvest at Abs600nm = 0.401. Higher than this seems to work well anyway. Pipet the cultures into 50 mL Falcon tubes (don't overfill).

Using a spectrophotometer: Take 1 mL from the culture and 1 mL from a LB stock and put into cuvettes. Turn on the spectrophotometer (on Antonio's bench; make sure to ask if he needs to use it and ask him if you need help). Wipe the cuvette with Kimwipe (to clean the part where light passes through). Press the OD600 button. Put the 1 mL LB cuvette into the slot and press the Blank button. Replace the 1 mL LB cuvette with 1 mL culture cuvette and press sample. Rinse the cuvettes twice with 70% ethanol and twice with sdH20. Turn off spectrophotometer.

Note: You don't have to work aseptically when using the spectrophotometer. However, as always, transferring cultures that you want to use later on means you should work aseptically.

4. Centrifuge at 1600 x g for 7 minutes (4°C).

5. Pour out the supernatant aseptically (also flame mouth and cap before and after). Wash gently in ~20mL COLD filter sterilized 0.1M CaCl2 (i.e. put the tube on ice or put in cooler). Swirl the tube using your wrist until the pellet disappears.

6. Filter sterilization: Use a syringe to suck up some CaCl2 (from a beaker probably). Attach a 0.4 micron filter unit to the tip. Press the plunger. Whether any part above the filter is sterile is unimportant; anything that passes through the filter is sterile. Make sure the filtrate enters a sterile container (e.g. Falcon tube). You can re-use the filter: remove the filter, use the syringe to suck up more CaCl2 and repeat. The original packaging material can hold the filter while you do this. Make sure the first few drops of filtrate does not go into the container with your previously sterilized CaCl2. You can drop it back into the unsterilized CaCl2. Label finished product.

7. Spin down gently: 1100g, 5 minutes, 4°C. See 4 about using the centrifuge.

8. Pour out the supernatant aseptically (flame mouth and cap before and after too). Resuspend in 12.5mL cold 0.1M CaCl2. Wrist.

9. Keep on ice 40 minutes. 10. Spin down: 1100g, 5 minutes, 4°C. See 4 about using the centrifuge.

11. Pour out the supernatant asepticall (flame mouth and cap before and after too). Resuspend in 2-2.5mL cold 0.1M CaCl2. Wrist.

12. Store over night at 4°C

13. Add glycerol to 15% and divide into 100μL aliquots, store at -80°C in Lagally Lab freezer; label with name, date, strain, flask where it came from, etc. Adding about 1 mL 60% glycerol to 2.5 mL of the final resuspended cells yields a ~15% glycerol solution.

14. Throw away your used tubes, pipets, filters, syringes, etc.

Bacterial Transformation

1. Thaw 50 μL competent cells on ice for 15min
2. Add 1-2 μL of your plasmid or ligation mix
3. Keep on ice for 45min
4. Heat shock at 42 degrees Celsius for 45 seconds
5. Keep on ice for 2min
6. Add 250 μL of LB or SOC media
7. Incubate (while rotating/shaking) for 1 hour at 37 degrees Celsius
8. Spread 50 μL and 200 μL on selective media plates
9. Incubate plates at 37 degrees Celsius overnight and you should see colonies!

Protein expression of C41 DE3 cells with a lac promotor

Note: This protocol was done specifically for a lac promotor, the limonene synthase composite part

1. Pick a colony, grow in 50 ml LB in a 250 ml erlenmeyer flask with the appropriate antibiotics, overnight

2. Take 1 ml of the solution and put it into a 500 ml terrific broth solution in a 2 litre flask with appropriate antibiotics
3. Grow the cells until = 0.8
4. Add IPTG to a final concentration of 1 mM
5. Express cells at 20 degrees (or 16 degrees) at 205 rpm overnight
6. Spin down cells
7. Store pellet in -80 for use

Site Directed Mutagenesis – British Columbia – 2011

http://2011.igem.org/Team:British_Columbia/Protocols/Sdm

Procedures:

1) Prepare reaction master mix for n+1 reactions, where n is the number of samples on which you wish to use SDM. For a single reaction, use the following reactants in the quantity given. For greater than 10 reactions, make 1 extra sample for every 10 extra reactions (e.g. prepare n+2 master mix reactions for 20 samples, n+3 for 30, etc.)

Reagents	**1x Reaction Volume**
ddH2O	36 µL
10X Rxn Buffer	5 µL
dNTP (25 mM)	1 µL
Forward primer(125 ng)	1 µL
Reverse primer(125 ng)	1 µL
Pfu polymerase (0.5x)	2 µL
DNA template (50 ng/uL)	1 µL
DMSO	2 µL
MgCl2	1 µL

2) Aliquot 48 uL master mix into n+1 PCR tubes. Add 50 ng template (diluted to 25 ng/uL, 2 uL/reaction used) per sample tube. Add 2 uL dH2O for water control. Be sure to clearly label the PCR tubes!

3) Place PCR tubes in thermocycler, and follow the following cycling conditions.

Temperature	**Time**	**Cycles**
95°C	30 sec	1
95°C	1 min	15

55°C	1 min	
68°C	1 min/kb	
4°C	Hold	

4) Add 0.5 uL DPNI enzyme to each sample and treat at 37° for 60 minutes or overnight to get rid of the template. Run samples on gel to confirm SDM worked.

Troubleshooting notes:

- 1 µL Pfu was initially used, but 2 µL gave a brighter band on the gel
- 10 mM dNTPs were originally used, but there was no product until we increased the concentration to 20 mM.
- First reactions did not use MgCl2 or DMSO, but we found that their addition gave a more consistent, brighter band on the gel.
- Pfu was used due to exonuclease activity. Only 16 cycles in total were used to prevent non-specific amplification.
- 1 µL of 10 µM/µL primers were initially used, but the reaction did not work until we added the suggested mass.

Cell Cultures – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

Liquid Culture:

- Pick single colony from plate with pipet tip.
- Drop tip in tube of ~7 mL LB media (with selected antibiotic if necessary).
- Shake overnight at 37C.

Plating Cells:

From solution/liquid culture:

- Apply 100-200 µl cell solution to LB agar plate containing appropriate antibiotic.
- Place spreader in 95% ethanol, remove and sterilize in flame.
- Using sterilized spreader, gently spread solution around plate.
- Store upside down at 37C overnight to grow cells.
- Store at 4C long-term.

Streak plating:

- Place inoculation loop in 95% ethanol, remove and sterilize in flame.

- Cool inoculation loop by pressing into a section of agar in used plate.
- Pick single colony of cells from original plate.
- Streak colony across new plate.
- Store upside down at 37C overnight to grow cells.
- Store at 4C long-term.

Glycerol Stock:

- Prepare solution of 40% glycerol by volume.
- Add 750 µl cell solution and 750 µl glycerol solution (or 1:1 mix) to Eppendorf tube.
- Store at -80C.

Transformation – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

NEB (10-beta and Turbo)

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1. Thaw a tube of NEB Turbo/NEB 10-beta Competent E.coli cells until the last ice crystals disappear. Mix gently and carefully pipette 50 uL of cells into a transformation tube on ice.
2. Add 1-5 uL containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42 C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 uL of room temperature SOC into the mixture.
7. Place at 37 C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plate to 37 C.
9. Spread 50-100 uL of each well-mixed dilution onto a selection plate and incubate 8-12 hours to overnight at 37 C. Alternatively, incubate at 30 C for 16 hours or 25 C for 24 hours.

TSS

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1. Thaw 50ul prepared competent TSS cells on ice.
2. Add approximately 1 ul prepped plasmid to the thawed cells. Make sure the culture is thoroughly mixed.
3. Incubate for 30 minutes on ice.
4. Incubate for 30 seconds at 42C (in a water bath).
5. Incubate for 2 minutes on ice.

6. Add 1 ml room temperature SOC.
7. Incubate for 1 hour at 37C with shaking.
8. Plate with appropriate antibiotic.
9. Grow overnight at 37C.

Ligation – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

50 uL Ligation (from OpenWetWare):

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1. Add 22.5 uL deionized H20 to sterile eppendorf tube
2. Add 5 uL of ligation buffer to the tube

   Vortexing buffer before pipetting helps ensure that it is well mixed

3. Add 15 uL of insert to the tube
4. Add 5 uL of vector to the tube
5. Add 2.5 uL of ligase

   Pipetting up and down before adding to tube helps ensure that it is well-mixed

   (vortexing the ligase may be inappropriate due to the sensitivity of the enzyme)

6. Let 50 uL solution sit at 22.5 C (room temperature) for at least 30 mins
7. Heat inactivate ligase at 65 C for 10 mins
8. Store at -20 C or proceed to transformation

Restriction Digests – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

Linearized Plasmid Backbone Restriction

- Preparation of Master mix:

  - 5 uL NEB Buffer 4
  - 0.5 uL BSA
  - 0.5 uL EcoRI

    - 0.5 uL PstI
    - 0.5 uL DpnI
    - 18 uL dH2O

- 4 uL linearized plasmid backbone + 4uL master mix in tube.
- Digest at 37C for 30 minutes. Heat shock at 80C for 20 minutes.

Open WetWare Restriction

- Master mix (good for 8 restrictions)

    - 40 uL Buffer 2
    - 0.5 uL BSA
    - 296 uL ddH2O

        - 42.5 uL master mix + 300ng DNA + 1 uL of each restriction enzyme in tube.
        - Digest at 37C for 1 hour (or longer). Heat shock at 80C for 20 minutes.

Media – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

SOB / SOC

"Super optimal broth" (SOB) and "super optimal broth with catabolite repression" (SOC) are nutrient-rich media. Use extreme caution (and if possible make only small amounts) when manipulating, as they are very easy to contaminate.

For 1 L SOB, combine:

- 20 g Tryptone
- 5 g Yeast extract
- 0.5 g NaCl (8.56 mM)
- 1.86 g KCl (2.5 mM)
- ddH_2O to 1 L

Autoclave.

SOC: Add to SOB:

- 0.952 g MgCl2 (10 mM) or 2.408 g MgSO4 (20 mM)
- 3.603 g glucose (20 mM)

Do not autoclave SOC, as the glucose will react with the tryptone. Either autoclave glucose separately or filter sterilize the SOC.

LB agar

- Combine 3.7 g LB agar with 100 ml H_2O. Autoclave or microwave. Makes ~4 plates.

LB broth

- For 1 L, add to 800ml water:
    - 10 g tryptone
    - 5 g yeast extreact
    - 10 g NaCl
- Adjust ph to 7.5
- Adjust volume to 1 L
- Autoclave

Trypticase soy broth

Trypticase soy agar

- For 1 L:
    - 30 g soy broth
    - 15 g agar
    - H_2O to 1 L.
        - Autoclave

Visualization – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

50 mL Gel Electrophoresis

1. Mix 1x TAE Buffer with 0.50g agarose in 250 mL Erlenmeyer flask. Swirl well.

2. Microwave solution until it begins to boil. Gently swirl the flask and repeat the microwaving process until the agarose is completely dissolved and the solution is clear.
3. Add 5 µL dye (SYBR green or ethidium bromide) and gently swirl the mixture. Let the solution sit for 5 minutes.
4. Carefully pour the solution into the 50 mL gel tray and add in the desired comb. Let sit for 30-45 minutes until gel solidifies.
5. Place gel tray in the -4C refrigerator for 10-15 minutes.
6. Remove gel tray from refrigerator, remove rubber ends, and place the gel in electrophoresis chamber with the comb towards the negative end.
7. Add in enough 1x TAE Buffer in the chamber to just barely create a thin film of buffer over the gel.
8. Add 2 µL of each DNA sample to a strip of parafilm. Add 8 µL of loading buffer to each of those 2 µL samples.
9. Add the necessary DNA ladder to the first well in the gel.
10. Add each 10 µL mix of buffer and DNA into individual wells in the gel.
11. Attach the electrophoresis chamber lid onto the chamber and plug into the power source.
12. Set the power source to 100-110 V for fast visualization (or 80V for optimal separation and resolution) and let sit for approximately 30-45 minutes or until the dye marks are near the end of the gel.
13. Turn off the power source, remove the gel, and use ultraviolet light to visualize the DNA bands.

Extraction – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

Ethanol precipitation

This protocol purifies / concentrates a DNA sample.

1. Measure out 2-3 volumes 100% ethanol into sample.
2. Mix solution by pipetting up and down or by inverting the tube several times.
3. Incubate at -20C for 12 hour, or for 20 minutes to 1 hour at -80C.
4. Centrifuge at maximum speed, at 4C, for 30 minutes. Discard supernatant.
5. Airdry for 15 minutes to remove excess ethanol.
6. Resuspend pellet in a 1.5 ml microcentrifuge tube. Be careful of the pH of the water used, which can vary widely. The nucleic acid should be suspended at a neutral pH.

Gel extraction

Qiagen Miniprep:

The following protocol is applicable to the QIAprep Spin Miniprep Kit, using micro-centrifuge:

1. Add 1.5 mL of liquid culture to microcentrifuge tube and centrifuge at 13,000 rpm for 1 minute. Discard supernatant.
2. Resuspend pelleted bacterial cells in 250 µL Buffer P1 and vortex.
3. Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
4. Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 10 min at 13,000g.
6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pippetting.
7. Centrifuge for 30-60 seconds. Discard the flow-through.
8. Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30-60 seconds. Discard the flow-through.
9. Wash QIAprep column by adding 0.75 ml Buffer PE and centrifuging for 30-60 seconds.
10. Discard the flow through, and centrifuge for an additional 1 minute to remove residual wash buffer.
11. To elute DNA, place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 50 µL PCR water (if sample is to be sequenced) or 50 µL elution solution to the center of each QIAprep spin column, let stand for 1 minute, and centrifuge for 1minute.

Sigma Aldrich miniprep

The following protocol is applicable to the GenElute™ Plasmid Miniprep Kit, using micro-centrifuge:

1. Add 1.5 mL of liquid culture to microcentrifuge tube and centrifuge at 13,000 rpm for 1 minute. Discard supernatant.
2. Resuspend pelleted bacterial cells in 200 µL resuspension solution. Vortex or pipette up and down to completely resuspend cells.
3. Lyse cells with 200 µL lysis buffer. Mix by gentle inversion- do not vortex.
4. Add 350 µL neutralization buffer to precipitate cell debris. Mix by gentle inversion.
5. Prep provided binding column by adding 500 µL column preparation solution and centrifuging at 12,000g for 1 minute.
6. Transfer the cell material from step 4 to the column and centrifuge at 12,000g for 1 minute. Discard flow through.
7. Add 500 µL wash solution 1. Centrifuge at 12,000g for 1 minute. Discard flow through.
8. Add 750 µL wash solution 2. Centrifuge at 12,000g for 1 minute. Discard flow through.

9. Transfer the column to a new collection tube.
10. Add 100 µL PCR water (if sample is to be sequenced) or 100 µL elution solution to the center of each column. Centrifuge at 12,000g for 1 minute. The DNA is now collected in the flow through liquid.

Genome prep

We extract bacterial DNA to use for PCR and sequencing. The protocol used throughout our project used the GenElute™ Bacterial Genomic DNA Kit.

Poly-X Ligation – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

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The goal of this protocol is to produce a polymer of DNA starting from a single unit with the Biobrick prefix and suffix. Cutting with XbaI and SpeI produces sites that can ligate into XbaI-XbaI, XbaI-SpeI, or SpeI-SpeI. Finally, the ligation product can be cut with XbaI and SpeI a final time. XbaI-SpeI scars are not cut, while any incorrectly oriented inserts are. This allows the DNA to form chains of various lengths. See results for further characterization of this process.

File:Arizona State poly-x

Procedure

1. Run a PCR of your BioBrick (ideally this PCR should not produce extra bands, as these may eventually interfere with the ligation).
2. Following the PCR, use the solution as the DNA sample for restriction digest.

   Restriction reaction mixture:

   50µL PCR product

   7µL 10x NEB4 buffer

   8µL $H_2 0$

   1µL BSA (10mg/ml)

   2µL XbaI

   2µL SpeI

3. Incubate at 37C for at least 1 hour.
4. Column purify the 70µl restriction reaction by following Sigma-Aldrich column purification protocol. For the elution step use 50µl elution solution.
5. From the column purified product: use 25µl for ligation and keep 25µl for a diagnostic gel and/or future use.

   Ligation reaction mixture:

25µl column prepped DNA

17µl $H_2$0

5µl 10x T4 ligase buffer

3µl T4 DNA ligase

Continue ligation for at least 1 hour at room temperature. I recommend 12-16 hours at 16C when larger linear products are desired.

6. If orientation of the product is a concern, run a final XbaI + SpeI restriction to eliminate X-X and S-S sites.
7. Following the previous step a small portion (<10µl ligation reaction) should be visualized by a 1 or 2% agarose gel. About 5µl of column preparation product and/or restriction product should be run in the adjacent lane to the ligation product.
8. Run the gel and assay the poly-X product created (e.g. 1x, 2x, 3x etc.). I don't recommend gel extraction, it may help in isolating a specific polymer length, so you have to determine if you have a high enough concentration to extract (GEL EXTRACT AT YOUR OWN RISK).
9. Take an aliquot of ligation product, and run in a new ligation reaction with pSB1A3, or 1K3 etc. Cut plasmid with XbaI and SpeI (or whatever restriction enzymes necessary) and dephosphorylate using the standard protocols. (I recommend using higher concentrations of backbone than the protocol usually calls for). I recommend running a ligation of linearized plasmid only, this will serve as a negative control, where if you get results either the plasmid wasn't completely cut or dephosphorylated.

NOTE: For mini-prepped plasmids it is always a commendable effort to run cut (linearized) and uncut (unlinearized) on an agarose gel to determine the purity of the sample. Simply looking at nano-drop results and 'scoring high' is meaningless because you may have contamination, however when using the nanodrop to good things to note are 230:260:280 which should be 1: 1.8: 1 respectively. Jagged peaks mean you need to re-blank the instrument and clean the pedestal.

10. Ligate the poly-X sample with the linearized vector using the basic ligation protocol (Consider what ratios would be best for the ligation). Also consider elongated ligation time (to long of ligation can reduce transformation efficiency due to long linear products).
11. Transform the product with the necessary controls.

Competency – ASU – 2011 http://2011.igem.org/Team:Arizona_State/Lab/Protocols

NEB cells

These cells can be ordered from New England Biolabs and should be stored at -80C. To transform, follow theNEB cell transformation protocol.

TSS cells

Materials:

- Cells (plated)
- TSS buffer
- LB broth

Preparation:

1. Grow a 5ml overnight liquid culture of cells in LB broth. After 12 hours, dilute this culture back into 25-50ml of fresh LB broth in a conical flask, diluting the overnight culture by at least 1/100.
2. Grow the diluted culture to an OD_{600} of 0.2 - 0.5 (1-2 hours).
3. Chill X (where X is the volume in ml of your culture) eppendorf tubes so that they are cold when cells are aliquoted into them later. Chill TSS buffer.
4. Split the culture into two 50ml falcon tubes and incubate on ice for 10 min.

All subsequent steps should be carried out at 4C and the cells should be kept on ice wherever possible

1. Centrifuge for 10 minutes at 3000 rpm and 4C.
2. Pour / pipette off supernatant.
3. Fully resuspend in chilled TSS buffer, using 10% of the culture volume that you spun down.
4. Add 100 µl aliquots to your chilled eppendorfs and store at -80C.

Growth in Liquid Culture – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Into a 500 mL baffled flask, under a flame, add 100 mL of autoclaved media (PD, VSuTB, or etc)
2. Add approximately 6 x 10^6 cells to the each flask.
3. Incubate the cultures in a rotating incubator at 37 °C and a speed of 200 rpm. Grow until saturation.

Solid Culture Growth – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Add 15g/L of Agar to any liquid media recipe, before autoclaving.
2. Autoclave to sterilize.
3. While still hot and in liquid form, pour the media into the desired container. 25 mL in a 100 mL conical flask is sufficient.

4. When the media has polymerized, inoculate the media with a loop of conidia (bright orange in color) from a previous culture or a small amount of conidia suspended in water. Seal the top of the tube with a sponge top.
5. Incubate the flask at 37 °C for 2 days, then transfer to a well lit room and let the culture conidiate for 5 days at room temperature. It should produce a layer of bright orange conidia, which are ready for harvest.
6. For growth experiments, harvest this conidia within one or two days to ensure maximum viability. For propagation of a strain, the solid culture can be stored at room temperature for up to 1 month

Harvesting Conidia from Solid Cultures – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Add 50 mL of ddH_2O to the 100 mL of flask of conidia to be harvested
2. Swirl the flask a few times to allow for most of the conidia to be suspend. Transfer the suspension of conidia to a 50 mL plastic tube.
3. Centrifuge the tube at 6000 rpm for 2 min.
4. Decant the supernatant. Wash the conidia with 40 mL of ddH_2O.
5. Centrifuge the tube again at 6000 rpm for 2 min.
6. Decent the supernatant. Resuspend the pellet with 10 ml of ddH_2O.
7. Using a hemocytometer and serial dilutions when necessary, count the conidia and estimate the concentration of the tube.
8. Dilute the conidia to the desired concentration.

Potato Dextrose Media (PD) – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Take 300 g of Potato (Baking Potatoes) and dice into 0.5 cm cubes.
2. Add 1.0 L of water and boil for 30 min.
3. Strain out the potato chunks and add 20 g of dextrose (D-glucose)
4. Autoclave solution
5. For solid PD agar, add 15 g of agar per 1 L of media.

VSuTB (Vogel's Sucrose Trace Elements and Biotin) Media – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

The following protocol was given to us from the Nargang Lab at the University of Alberta. It is based off the protocol listed on www.fgsc.net/methods/vogels.html.

VSuTB	50 mL	100 mL	500 mL	1000 mL

50x Vogel's Salts	1 mL	2 mL	10 mL	20 mL
15g/L Table Sugar	0.75 g	1.5 g	7.5 g	15 g
100mg/L Trace Elements	50 µL	100 µL	0.5 mL	1 mL
100mg/L Biotin	50 µL	100 µL	0.5 mL	1 mL
15g/L Agar	0.75 g	1.5 g	7.5 g	15 g
dH_2O	48.9 mL	97.8 mL	489 mL	978 mL

- For liquid VSuTB media, exclude the agar.
- Mix all the ingredients in a proper container. Autoclave to sterilize.
- The composition of the 50x Vogel's Salts, Trace Elements and Biotin solutions are listed onwww.fgsc.net/methods/vogels.html. The above protocol deviates for 50x Vogel's Salts; the Trace Element and Biotin solutions are added separately as individual components to make the final Vogel's media.

Coffee Grounds Media (10% Wet Weight) – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Take 50 g of Coffee Ground from Starbucks
2. Add 500 mL of dH_2O and autoclave to sterilize

Sawdust Media (5% dry weight) – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Take 25 g of sawdust (source: RONA woodshop)
2. Add 500 mL of dH_2O and autoclave to sterilize

Fertilizer solution – Alberta – 2011 http://2011.igem.org/Team:Alberta/Methodology/Protocols

- Prepared 10X concentrated as per the dilution recommend by the supplier.

1st Wheat Straw Media – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. 10 g wheat straw cut into ~2 cm pieces

2. Add 500 mL of dH_2O and boiled for 30 mins
3. Strained 250 mL with a kitchen strainer and left the other 250 mL unstrained.
4. Autoclaved both to sterilize.

1st Grass Media – Alberta – 2011 <u>http://2011.igem.org/Team:Alberta/Methodology/Protocols</u>

- Same as 1st wheat straw media, except grass was dried on a hot plate first (10 g grass)

Wheat Straw with 1% w/v NaOH treatment – Alberta – 2011 <u>http://2011.igem.org/Team:Alberta/Methodology/Protocols</u>

1. 1 g of ground wheat straw (using food processor until it passed through the pores of a kitchen strainer)
2. Add 100 mL of 1% NaOH, autoclaved
3. Washed the wheat grounds on filter paper with dH_2O until pH 7.0.
4. Add 100 mL of dH_2O to the neutralized grounds and autoclaved to sterilize.

10X MgCa solution – Alberta – 2011 <u>http://2011.igem.org/Team:Alberta/Methodology/Protocols</u>

1. Dissolve 16.6 g of $MgCl_2$* 6 H_2O and 10.0 g of $CaCl_2$ * 2 H_2O in 100 mL of dH_2O.
2. Mix under totally dissolved
3. Autoclave to sterilize.

Baked Sawdust – Alberta – 2011 <u>http://2011.igem.org/Team:Alberta/Methodology/Protocols</u>

1. Baked sawdust in an oven at 400^oF for 1 hr.
2. Take 2 g of the baked sawdust, and add 200 mL of dH_2O.
3. Boil for 30 min. Strained out the sawdust for half the solution; leave the other half in a separate bottle.
4. Autoclaved both bottles.

Race Tube – Alberta – 2011 <u>http://2011.igem.org/Team:Alberta/Methodology/Protocols</u>

- Race tubes were used to show amount of growth by hypheal extension.
- Race tubes were custom-made by the University of Alberta Glass Shop.
- Our race tubes were 1 meter in length with an interior diameter of 25 mm.

1. For each of media, stopper one tube with a rubber stopper.

2. After autoclaving, while it is still hot (and liquid), pour the media in the tube. And stopper the other end with a rubber stopper.
3. Allow the media inside the tube to cool and the agar to polymerize.
4. When the agar is solid, remove a rubber stopper at ONE end of the tube and immediately replace with a sponge stopper.

WS + FBT	3 g wheat straw, 100 µL trace elements, 100 µL biotin, 10 mL MgCa solution 10 mL fertilizer, 1.5 g agar, 80 mL dH_2O.
WS + F	3 g wheat straw, 10 mL MgCa solution, 10 mL fertilizer, 1.5 g agar, 80 mL dH_2O.
GS + FBT	3 g grass clippings, 100 µL trace elements, 100 µL biotin, 10 mL MgCa solution 10 mL fertilizer, 1.5 g agar, 80 mL d H_2O.
GS + F	3 g grass clippings, 10 mL MgCa solution, 10 mL fertilizer, 1.5 g agar, 80 mL dH_2O.
Vogel's media	prepared as listed above and add a few drops of food colouring to add contrast from*Neurospora crassa* in race tube experiments

PCR Clean-up Protocol – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Add 5X the volume of buffer PB, and ~10µL of 3M sodium acetate and mix by inverting.
2. Apply the mixture to filter-columns that have been placed on the vacuum manifold.
3. Wash the columns by adding 750 uL Buffer PE and applying vacuum. Wait until all liquid is gone and then turn vacuum off.
4. Place each column in the 2 mL rounded collection tubes and centrifuge at 13000 rpm for 1 min
5. Discard the 2 mL collection tubes and place your columns into labelled 1.5 mL eppendorf tubes.
6. Apply 30 uL of Sterile TE Buffer (10 mM EDTA) to the CENTER of each column
7. Let stand for AT LEAST 10 MINUTES
8. Centrifuge for 1 min at 13000 rpm.
9. Discard the column into the discarded column beaker.

Miniprep Protocol – Alberta – 2011 http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Pellet your overnight culture tubes by adding ~1800 uL of each culture to separate 2mL eppendorf tubes, centrifuging at 13000 rpm for 7 minutes, discarding the supernatant (just pour it out), and adding more culture. Centrifuge again, discard the supernatant, and if there's any culture left, add it and centrifuge again. Discard the final supernatant.

2. Resuspend each pellet in 250 uL of Buffer P1. Vortex to ensure complete resuspension
3. Add 250 uL Buffer P2, and immediately mix thoroughly by inverting ~7 times. Do NOT leave this mixture stand for more than 5 minutes. Preferably, move on to step 3 within 2 minutes or so.
4. Quickly add 350 uL Buffer N3. Mix a few times via inversion.
5. Centrifuge all tubes for 10 min at 13000 rpm
6. Apply the supernatant from each tube to 4 LABELLED filter-columns that have been placed on the vacuum manifold. Once all samples have been applied to the column, apply the vacuum to the manifold. Let the samples drain through until no liquid is left.
7. Wash the columns by adding 750 uL Buffer PE and applying vacuum. Wait until all liquid is gone and then turn vacuum off.
8. Place each column in the 2 mL rounded collection tubes and centrifuge at 13000 rpm for 1 min
9. Discard the 2 mL collection tubes and place your columns into labelled 1.5 mL eppendorf tubes.
10. Apply 50 uL of Sterile TE Buffer (10 mM EDTA) to the CENTER of each column
11. Let stand for AT LEAST 10 MINUTES
12. Centrifuge for 1 min at 13000 rpm.
13. Discard the column into the discarded column beaker.

Creating Parts Plasmids – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

1. Ligate together the correct prefix, (suffix, if needed), cut part, and cut plasmid backbone.
    - 2 uL of cut 0.025 pmol/uL part
    - 2 uL of cut 0.025 pmol/uL plasmid
    - 2 uL of 0.025 pmol/uL prefix
    - (2 uL of 0.025 pmol/uL suffix)
    - 2 uL of 5X T4 DNA Ligase Buffer (in PEG)
    - 1 uL T4 DNA Ligase.
    - Add Mq'd H_2O to 10 uL (or just add none)

    Ligate anywhere between an hour and overnight

2. Transform the entire 10 uL of ligation reaction into 100 uL of competent cells per the usual transformation protocol. Plate on chlor-inoculated plates.
    - Chlor plates have 4.0 mL chloramphenicol/L of LB agar
3. The next day, pick 4 individual white colonies and inoculate into sterile culture tubes with 4 mL LB medium and chlor at 4 mL/L. Shake at 37oC for ~16 hours (overnight).
4. Miniprep the overnights to get 4 concentrated tubes of YOUR particular parts plasmid.
5. Check the concentration of your minipreps. If they look ok (i.e. high), then go ahead and digest some of MP#1 with NotI, and some of MP#1 with BsaI.

- NotI Digestion protocol:
    1. Add 1 ug of your DNA to a 1.5 mL eppendorf tube (will be a variable volume)
    2. Add 5 uL of 10X NEBuffer 3
    3. Add 5 uL of 10X BSA
    4. Add 1 uL of NotI
    5. Add Mq'd H_2O to a total volume of 50 uL (be sure to mix)
    6. Incubate at 37°C for at least 1 hour. Can incubate overnight.
    7. PCR clean-up, and elute in 30 uL TE.
- BsaI Digestion Protocol:
    1. Add 1 ug of your DNA to a 1.5 mL eppendorf tube
    2. Add 5 uL of 10X NEBuffer 4
    3. Add 5uL of 10X BSA
    4. Add 1 uL of BsaI-HF
    5. Add Mq'd H_2O to a total volume of 50 uL.
    6. Incubate at 37°C overnight.
    7. PCR clean-up, and elute in 30 uL TE

6. Run on a gel the following samples:
    - Lane 2: The pure Miniprep that you took some of to digest with (MP #1)
    - Lane 3: MW Ladder
    - Lane 4: The results of your NotI digestion
    - Lane 5: The results of your BsaI digestion

Extraction and Esterification – Alberta – 2011

http://2011.igem.org/Team:Alberta/Methodology/Protocols

Sample Preparation for GC Analysis:

i. wash clean glass tubes with a 2:1 chloroform:methanol solution; drain solution and let evaporate in fume hood
ii. set water bath to 70 degrees Celsius
iii. weigh out day 5 *N. crassa* samples (~0.1-0.3g)
iv. transfer samples to clean class tubes; add 2 mL of methanolic HCl to each sample
v. incubate samples in glass tubes in a 70 degree water bath for one hour
vi. add 0.9% NaCl to each rxn tube and mix (rxn is stopped)
vii. add 2 mL hexane_IS (0.5 mg/ml C15) to each glass tube, vortex 2 min @ max speed
viii. spin @ 3000 rpm for 5 mins

ix. carefully take the tubes out and transfer the top phase (hexane_IS) into a fresh glass tube;**IMPORTANT: do not disturb the intermediate layer**

(Hexane_IS NU-CHEK Prep Inc. LOT# A-615-J30-)

Production of raw fuel: – Alberta – 2011
http://2011.igem.org/Team:Alberta/Methodology/Protocols

i. wash clean glass tubes with a 2:1 chloroform:methanol solution; drain solution and let evaporate in fume hood
ii. set water bath to 70 degrees Celsius
iii. weigh out day 5 *N. crassa* samples (~10-30g)
iv. dried out large mass of *N. crassa* and crushed with a motor and pestle
v. add the *N. crassa* to a large screw top jar, and add methanol and methanolic HCL
vi. use 10x ml of liquid for mass of fungus. ¾ of liquid volume should is methanol, ¼ is 3M methanolic HCL.
vii. incubate samples in glass tubes in a 70 degree water bath for two hours
viii. add ½ the volume of the reaction of dd H_2O
ix. add ½ the volume of hexane, and shake vigorously
x. Let settle and pipette off the top hexane layer, being careful not to take any of the bottom layer.
xi. repeat steps ix-x, and add to the rest of the hexane
xii. remove hexane under negative pressure.

2010

MiniPrep – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison
Alkaline Lysis
Alkaline Lysis is for screening of plasmids

1. Pellet the overnight culture(s) in a 1.5 ml or 2ml eppendorf tube. (I usually do 10,000 rpm, 3 minutes) 1 minute works fine. I usually use 3 ml culture per prep.
2. Resuspend each pellet in 200 µl Alkaline Lysis Sol I, RnaseA added (final RNase A concentration should be 100 µg/mL). Make sure there are no lumps.
3. Add 400 µl Alkaline Lysis Sol II. Invert 4-6 times to mix. Do not allow reaction to lyse for more than 5 min. Sample should clarify.
4. Add 300 µl Alkaline Lysis Sol III. Invert 4-6 times to mix. Sample should have a white precipitate.
5. Add 100 µl chloroform. Do this in a fume hood. Invert 4-6 times to mix.
6. Rest on ice for 5-10 minutes. This step is so that the chloroform does not get too hot in the centrifuges and leak out of the tubes. If you want to skip this step you might consider using less chloroform. I put the tubes at -20 for a couple of minutes.

7. Centrifuge at max. speed (14,000 rpm) for 10 minutes.
8. Pipet 750µl of supernatant/aqueous layer into a fresh tube. I do up to 800 uL
9. Add 1/10 volume (75µl) 3M NaOAc, pH 5.2. Vortex/flick to mix. 80 uL
10. Add 0.7-1.0 Volume COLD isopropanol. Vortex/flick to mix. If in a hurry go straight to step 11, otherwise rest on ice for 10-30 minutes. I have even let it precipitate overnight at 4°C if convenient. 600 uL isopropanol. Then I put it at -20 for 5 minutes up to over the weekend if needed.
11. Centrifuge at max. speed for 25 min. Most miniprep protocols say to do this at 4°C, but I have not noticed decreased yield by centrifuging at room temp.
12. Remove and discard the supernatant. Don't disturb the pellet. Sometimes I can't see a pellet, and more often than not I still have DNA.
13. Add 1ml of 70% EtOH (at room temp.). Invert 4-6 times to rinse the tube.
14. Centrifuge at max speed for ~5 minutes. Room temp. is fine. Remove and discard the EtOH.
15. Repeat steps 14 and 15 to remove all traces of isopropanol. Pulse spin after removing bulk of final EtOH wash and pipet off remaining EtOH.
16. Air dry the pellet for ~15 minutes (pellet will change from white to clear as it dries). Resuspend in desired volume of H2O or T10E1, depending on downstream applications. If you pipet off the EtOH well, then I have done this for as little as 2 minutes before. For fosmids, I usually resuspend the pellet in 20 uL water.

Kit

Use the kit when you need very clean DNA. Ex cloning, sequencing

1. Refer to kit instructions

Digestion – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

Screening

For screening, you only need a small amount to run on a gel - 10uL rxn

Check enzyme compatibility, what buffer is needed, and whether BSA is necessary

- DNA - 2uL *(usually fine)*
- Buffer(10x) - 1uL
- BSA(10x) - 1uL
- Enzyme - 0.4uL each *(ADD LAST and no more than 10% of rxn volume)*
- Water - fill to 10uL

1-2 hours in 37C waterbath (check NEB if you want quicker time)

- Add 2uL of 6x Dye
- Load 6uL in gel

Cloning **– UW Madison – 2010** http://2010.igem.org/Team:Wisconsin-Madison

During cloning, you will need to digest more DNA for gel extraction - 50uL rxn

Check enzyme compatibility, what buffer is needed, and whether BSA is necessary

- DNA - 2ug
- Buffer(10x) - 5uL
- BSA(100x) - 0.5uL
- Enzyme - 2uL each *(ADD LAST and no more than 10% of rxn volume)*
- Water - fill to 50uL

1-2 hours in 37C waterbath *(check NEB if you want quicker time)*

- Add 10uL of 6x Dye
- Load 60uL in gel

Template Destruction **– UW Madison – 2010** http://2010.igem.org/Team:Wisconsin-Madison
If your product for digestion came directly from PCR you can destroy the original template by preforming a DpnI digestion. DpnI will digest methylated DNA. PCR product is unmethylated. If needed, do this step before cloning digestion.

- 1uL DpnI/50uL rxn
- incubate in 37C waterbath for 1 hour

Gel Extraction – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison
Use the kit and refer to kit instructions

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.** Minimize the size of the gel slice by removing extra agarose."
2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).** For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.
3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.** IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.
4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).** If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
5. **Add 1 gel volume of isopropanol to the sample and mix.** For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.
6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.** The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.** Collection tubes are re-used to reduce plastic waste.
9. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.** Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
10. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x g (~13,000 rpm).** IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
11. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
12. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alter-natively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.** IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure

that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Ligation – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

1. measure the concentration of the inserts and the vectors.
2. use In-Fusion® Molar Ratio Calculator from Clontech to calculate the mixing ratio of the inserts and vectors. Insert/Vector Ratio: 3-5
3. once the amount of inserts and vectors has been calculated, make a calculation for a 10ul total volume reaction
4. After the calculation has been done, get a small tube and label it
5. place the T4-buffer on ice to let it dissolve
6. Add the insert and vector into the tube, mix (Add water to compensate if needed)
7. Add 1ul T4-buffer into the mixing solution
8. Add 1ul Ligase into the mixing solution
9. **Either** leave it on the bench for a bench-top ligation for 1-2 hours **OR** put into the thermocycle at 16C for overnight

Transformation – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

1. Clean the cuvette and UV for 15mins.
2. Locate Electroporator source and cuvette holder.
3. Thaw required number of frozen cell aliquots on ice
4. Thaw required DNA on ice
5. Place the clean cuvette on ice
6. mix 0.5ul to 1.0ul DNA with 40ul competent cells
7. let it sit on ice for 2-5mins.
8. transfer the DNA-Cell mixture into the cuvette
9. Place the cuvette in the holder
10. Have 960ml of LB broth ready
11. Press the button to "shock" the cells
12. Immediately put 960ml LB into the cuvette and mix well
13. Transfer the cells into a 1.5ml centrifuge tube
14. Place tubes in 37C shaker 1-1.5hour.

Plating – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

1. Place the plate with the correct antibiotic in the 37C incubator 1 hour before the plating for pre-warming
2. Get the transformed cells and centrifuge at 2500rpm for 5 mins.
3. Discard about 850ul of supernatant, and re suspend the rest of the cells.
4. Put the liquid culture (about 150ul) onto the LB agar plate
5. Use a glass sticks to spread out the cells
6. Place the plate in the 37C incubator with the bottom facing upward

PCR clean-up – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison
Use the kit and refer to the kit instructions as follows

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.** For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. **Place a QIAquick spin column in a provided 2 ml collection tube.**
3. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
4. **Discard flow-through. Place the QIAquick column back into the same tube.** Collection tubes are re-used to reduce plastic waste.
5. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
6. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min at maximum speed.** IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
8. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. (Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.)

Colony PCR – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

1. Lyse a single colony in 50uL water
2. Vortex
3. Plate 2uL
4. Use 5uL for DNA template (see components below)

For 10uL total Rxn

- 2.9 uL water
- 5 uL 5x GoTaq Master Mix
- 0.05uL Forward Primer
- 0.05uL Reverse Primer
- 2uL DNA Template

Electro-competent Cells – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

The process consists of growing cells to mid-log stage, harvesting, and performing multiple washes with sterile 10% glycerol to remove salts which interfere with electroporation.

General Considerations:

- Keep everything cold, on ice
- Glycerol pellets are not firm; try to remove as much supernate as possible, but be careful not to lose the pellet
- All containers that come in contact with cells should be sterile
- Keep centrifuge bottles dedicated for making Electrocompetent cells
- Have 1 liter of 10% sterile glycerol chilled on ice, to less than 4C... or in a cold box overnight.

- Keep manipulation of cells to a minimum, be gentle.
- Resuspend pelleted cells using a sterile plastic pipette. Work quickly.
- Harvest cells at 0.6 – 0.75 O.D. (A600nm)

Fermentation:

- Inoculum:
  - Streak for single colony from -70C glycerol stock
  - Start 50 ml, No Salt LB inoculum, 37C, overnight
- Fermentation
  - Use 25 ml of the above Inoculum per liter of No Salt LB media (prewarm media to 37C)
  - Grow at 37C, shake at approximately 200 rpm
  - Grow to 0.6 – 0.75 O.D. (A600nm)......transfer to ice immediately to chill

Processing

1. Spin the chilled culture at 8,000 rpm, 10 minutes, 2 degrees C (use four 250 ml centrifuge bottles). Remove the supernate carefully. Save the pellets.
2. Resuspend all four pellets in a total volume of 200 ml cold 10% glycerol. Combine all resuspended pellets in one 250 ml centrifuge bottle.
3. Spin at 8,000 rpm, 10 minutes. Remove the supernate carefully.
4. Resuspend pellet in 150 ml cold 10% glycerol.
5. Spin at 8,000 rpm, 10 minutes. Remove the supernate carefully.
6. Resuspend pellet in 100 ml cold 10% glycerol.
7. Spin at 8,000 rpm, 10 minutes. Remove the supernate carefully.
8. To the pellet, add 2 ml 10% glycerol. Resuspend carefully with a 1 ml Pipetteman.
9. Transfer 110 ul of resuspended cells into cold***(-70C) 1.5 ml microcentrifuge tubes.
10. Transfer immediately to a -70C freezer (Do not use liquid nitrogen).
11. Freeze overnight before using cells.

Freeze Stock – UW Madison – 2010 http://2010.igem.org/Team:Wisconsin-Madison

Mix in cryotube. Make three stocks for each sample; place one in the working box and two in the backup box. Document placement of samples of lab site.

- 750uL overnight culture
- 250uL sterile 60% glycerol

GFP EXPRESSION ASSAY – **Washington – 2010**

http://2010.igem.org/Team:Washington/Protocols/KunkelCapD

Day 1: OVERNIGHTS

- Prepare a 96 deep well plate with 1ml of LB + antibiotic
- Inoculate well by taking at scraping from glycerol stock
- Shake at 37deg 16-24hrs

Day 2: EXPRESSION

- Prepare 96 deep well plate with 1ml of TB + antibiotic,
- Inoculate well by taking 20ul of the overnight and place it in 1mL TB, do all inducible twice so to allow for induced verse uninducible expression
- Grow on shaker at 37C for 3 hours
- Induce all inducible constructs with 50microL of 10mM IPTG
- Allow to grow for 18 hour at room temperature on a shaker

Day 3: DATA

- Take overnights plates and spin on plate centrifuge for 20 minutes at 4000rpm
- Pour of broth and resuspend in 1ml PBS 7.5 pH on plate shaker
- Spin down PBS suspension for 20 minutes at 4000rpm
- Pour of supernatant
- Resuspend in 1ml PBS 7.5 pH on plate shaker
- Take 100ul of PBS suspension and place into clear bottom plate reader plate
- Take 100ul of PBS suspension and place into black plate reader plate
- Read cell density by measuring absorbance at 600nm, and GFP fluorescence by exciting at 485 and reading emission at 525nm.

Gibson Assembly – **Washington – 2010** **http://2010.igem.org/Team:Washington/Protocols/KunkelCapD**

The cloning process is often the most time-intensive task for iGEM teams. Methods to streamline the assembly and tuning of gene circuits can increase productivity and lead to more successful iGEM projects. Traditionally, restriction enzymes and their DNA recognition sequences are used to assemble promoters, ribosome binding sites, and gene coding sequences into gene networks.

To create plasmids with more freeform inserts than possible with traditional BioBrick restriction/ligation cloning, we used a method described inNature Protocols 2009 in which parts are extracted from standard biobricks with primers that have homologies introduced on their 5' ends that overlap the parts that they will be next to. Small parts less than 250 bp are then stitched together using "overlap extension" PCR to create large enough fragments (roughly 500bp) that they can be assembled in a one step reaction as described by Gibson. This procedure allows the construction of plasmids that have no seams between parts and allows for arbitrary numbers of parts to be combined quickly and efficiently.

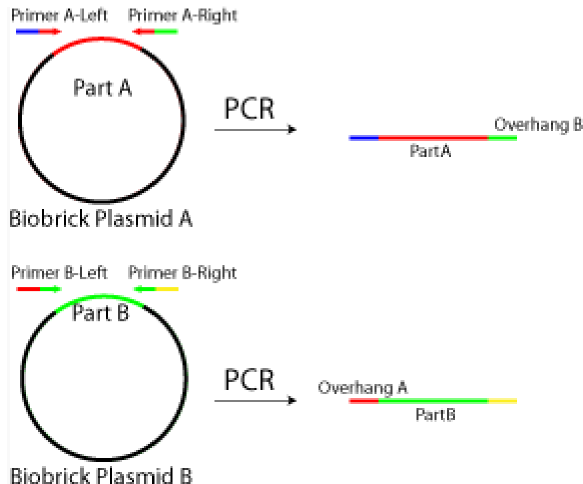
Utility

Gibson assembly is particularly useful for the following tasks

- Extracting Biobrick parts from multiple source plasmids and assembling them arbitrarily into one plasmid using one cloning step
- Modifying short sequences of promoters, ribosome binding sites, and gene coding sequences - from point mutations to inserting or replacing moderately large tracts (20-60 bp) of DNA
- Adding ssrA degradation tags to genes
- Creating fusion proteins and operons

- Flipping transcription direction of operons

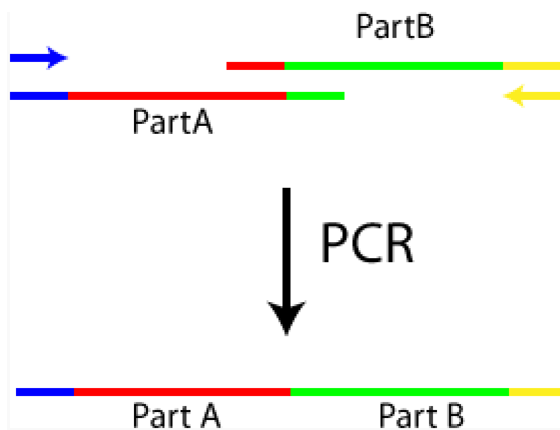
Part Extraction



Extraction Protocol

The first step is to design primers to extract the parts out of standard biobricks. The primers should be designed as usual (i.e. design for whatever Tm is desired over the ends of the part to be extracted. We aimed for around 60C). Then an extra sequence is added to the 5' end of the primers that is homologous to whatever part will be next to it in the final construct. These "overlaps" must have a Tm above 50C for efficient plasmid assembly. In some cases, new parts (i.e. promoters, ribosomal binding sites, ssrA degradation tags, and point mutations) can be introduced in these overlaps, provided they are short enough.

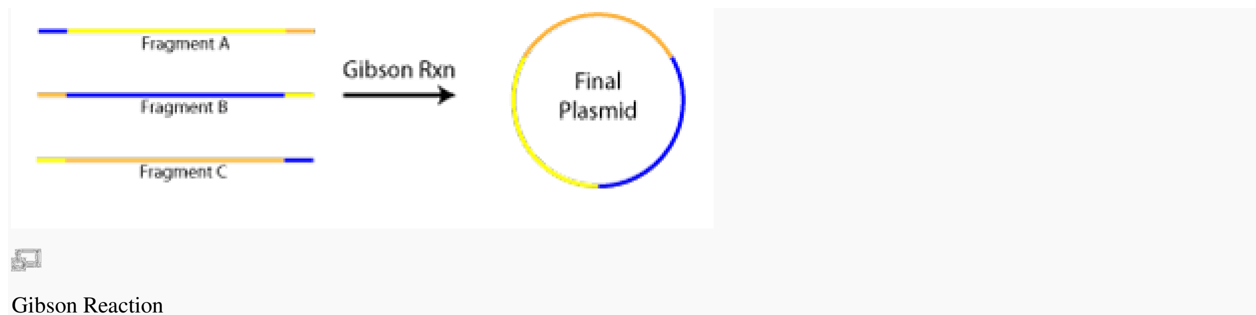
Overlap Extension for Short Amplicons



Overlap Extension PCR

After two adjacent parts have been extracted as above, if one of them is short (<250 bp) they are put into a standard PCR reaction with two primers that match the added homology on the outside of the desired construct. This results in linear DNA that is the result of the two parts stuck end-to-end with no seam and homologies to parts that will be adjacent in the final plasmid. This process can repeated to build DNA fragments roughly 500bp in length.

Gibson Assembly Reaction



Gibson Reaction

The ~500bp pieces of the final plasmid are then all fused together in one reaction with T5 exonuclease, heat-stable *Taq* ligase, Phusion polymerase, and free nucleotides with buffer. The exonuclease chews back the 5' ends of the strands, leaving "sticky ends." Due to the introduced homologies, the complementary single-stranded DNA anneal to each other in the desired order. The polymerase repairs any extra nucleotides chewed back by the exonuclease, and - finally - the ligase repairs nicks in the DNA. This process results in a circular plasmid that can be transformed efficiently.

Issues and Solutions

This type of procedure relies heavily on introduced homologies on the ends of all the parts involved. Initially we used the standard bioBrick prefix and suffix as the homologous region with which to insert our construct into standard backbones, but this proved problematic because of the NotI site which lies between the E/X and S/P restriction sites in bioBrick backbones, as it is long, consists mostly of Gs and Cs, and is palindromic. This leads to possible mispriming, and an ambiguity in the final configuration of our plasmid (the insert could end up forward, backward, or circular. The backbone could also recircularize without taking up the insert at all) and in turn, a very low yield of our desired construct.

To remedy this, we designed a new prefix and suffix, based on the BglBrick standard which allows for the elimination of the NotI sites. We developed the prefix gaattcctgctgcggagatct and the suffix ggatccaacagggttctcgag by aiming for roughly 50% GC content and a Tm around 68 as calculated by Finnzymes Tm calc. We then modified Psb1A3 and Psb3K3 with these prefixes and suffixes, and had much greater success with our cloning.

We have found that with inserts of less than 500 bp, one often gets many colonies containing insertless plasmid that has been circularized by DNA ligase. In order to determine if this is an issue in a given gibson reaction, for shorter inserts, we often conducted two reactions at once, one including the vector and the insert, and one containing only the vector, with the difference in volume made up with water. The reaction mixes were transformed into E. coli, and each tranformant mixture was plated on LB agarose plates with selective media. The number of colonies on the control plate (the plate with transformants of the reaction without any insert) corresponds roughly to the number of colonies on the the cloning plate (the plate with transformants of the vector/insert reaction). If the ratio of colonies on the cloning plate to colonies on the control is 1:1, it would mean that the vast majority of the colonies on the cloning plate are due to insertless, circularized vector. We were able to obtain correct plasmids when the cloning colony:control colony ratio was as low as 2:1, but this required double restriction digest screening of a large number of colonies (around 24) in order to obtain vector with insert.

References

Daniel Gibson, One-step enzymatic assembly of DNA molecules up to several hundred kilobases in size

Nature Protocols 2009

Gibson, D.G., *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases

Nature Methods 2009

galK Recombineering – **Washington – 2010**
http://2010.igem.org/Team:Washington/Protocols/KunkelCapD

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1. Design *galK* primers with 50 bp homology to an area flanking the desired site to be modified. The 3' end of these primers bind to the *galK* cassette. The primers should look as follows:

Forward:

5'------------50bp_homology-------CCTGTTGACAATTAATCATCGGCA-3'

Reverse:

5'----50bp_homology_compl._strand----TCAGCACTGTCCTGCTCCTT-3'

4. Transform a well-characterized BAC (or fosmid, in our case) into electrocompetent SW102 cells. Recover for 1 hour at 32°C, and plate on LB plates with 12.5 mg/ml chloramphenicol. (See steps 9-13 if you don't know how to prepare electrocompetent E. coli).

5. PCR amplify the *galK* cassette using the primers designed in step 1 and a proof-reading Taq-mix (we used Invitrogen's Platinum HiFi Taq mix). Use 1-2 ng template (the pgalK plasmid). 94°C 15 sec., 60°C 30 sec., 72°C 1 min., for 30 cycles. Add 1-2 ml *DpnI* per 25 ml reaction, mix, and incubate at 37°C for 1 hour. This step serves to remove any plasmid template; plasmid is methylated, PCR products are not. The digest was gel purified, then the PCR repeated using the *DpnI*-digested PCR product as template, and undergoing a second *DpnI* digestion on the result. This greatly cuts down on the non-recombinant background. From a strong PCR band, purified, and eluted in 30 ml ddH2O, we use 2.5 ul for a transformation (approx. 10-30 ng).

6. Inoculate an overnight culture of SW102 cells containing the fosmid in 5 ml low-salt LB + chloramphenicol (12.5 mg/ml). Incubate at 32°C.

7. Next day, turn on two shaking waterbaths: One at 32°C, the other at 42°C. Make an ice/water slurry and put a 50 ml tube of ddH2O in there to make sure it's ice-cold (see later). Also ice three 15 ml **round**-bottomed Falcon tubes and three micorcentrifuge tubes (1.5-2 ml). Dilute 600 ml of the overnight SW102 culture containing the target BAC in 30 ml low salt LB with chloramphenicol (12.5 mg/ml) in a 50 ml baffled conical flask and incubate at 32°C in a shaking waterbath to an OD600 of approx. 0.6 (0.55-0.6). This usually takes 3-4 hours.

8. Transfer 10 ml each to baffled 50 ml conical flasks and heat shock at 42°C for exactly 15 min. in a shaking waterbath. The remaining 10 ml is left at 32°C as the uninduced control.

9. After 15 min, the three samples are briefly (~5 min) cooled in an ice/waterbath slurry and then transferred to three 15 ml Falcon tubes and pelleted using 5000 RPM at 0°C for 5 min. (We had good results spinning at 4150-our max rotor speed- but 5000 rpm is recommended.) It's important to keep the bacteria as close to 0°C as possible in order to get good competent cells.

10. Pour off all of the supernatant and resuspend the pellet in 1 ml ice-cold ddH2O by gently swirling the tubes in the ice/waterbath slurry. No pipetting. This step may take a while. When resuspended, add another 9 ml ice-cold ddH2O and pellet the samples again.

11. Repeat step 10.

12. After the second washing and centrifugation step, remove 9 ml of supernatant and resuspend by swirling the pellet in the remaining 1 ml water. Transfer the cell suspension to the chilled microcentrifuge tubes, and spin again at 5000 rpm, 0 C, 5 min. Remove supernatant and resuspend in 50 ul of ice-cold water.

13. Transform the now electrocompetent SW102 cells. We use 25 ul cells for each electroporation in a 0.1 cm cuvette (BioRad) at 25 mF, 1.75 kV, and 200 ohms. After electroporation of the PCR product, the bacteria are recovered in 1 ml LB for 1 hour at 32°C. Transform the 32 C and one of the 42 C cultures with the *galK* casette; transform the other 42 C culture without adding DNA to control for any background *galK*metabolism in your parent culture.

14. After the recovery period the bacteria are washed twice in 1xM9 salts as follows: 1 ml culture is pelleted in an eppendorf tube at 13,200 RPM for 15 sec. and the supernatant removed with a pipette. The pellet is resuspended in 1 ml 1xM9 salts, and pelleted again. This washing step is repeated once more. After the second wash, the supernatant is removed and the pellet is resuspended in 100 ul 1xM9 salts before plating onto M63 minimal media plates with galactose, leucine, biotin, and chloramphenicol. Washing in M9 salts is necessary to remove any rich media from the bacteria prior to selection on minimal media. We plated all 100 ul onto a single plate per sample.

15. Incubate 3 days at 32°C in a cabinet-type incubator.

16. Streak a few colonies onto MacConkey + galactose + chloramphenicol indicator plates. The colonies appearing after the 3 days of incubation should be Gal+, but in order to get rid of any Gal- contaminants (hitch-hikers), it is important to obtain single, bright red colonies before proceeding to the second step. Gal- colonies will be white/colorless and the Gal+ bacteria will be bright red/pink due to a pH change resulting from fermented galactose after an overnight incubation at 32°C.

17. Pick a single, bright red (Gal+) colony and inoculate a 5 ml LB + chloramphenicol overnight culture. Incubate at 32°C. It is useful to confirm the *galK* insertion via PCR at this point.

18. Repeat steps 7 through 12 above to obtain electrocompetent SW102 cells (now ready for a *galK* <> mutation substitution).

19. Transform the bacteria (25 ml of heat-shocked, 25 ml of uninduced control, and 25 ml without PCR product) with 200 ng double-stranded oligo, a PCR product, or anything containing a mutation and with homology to the area flanking the *galK* cassette. Recover in 10 ml LB in a 50 ml baffled conical flask by incubating in a 32°C shaking waterbath for 4.5 hours. This long recovery period serves to obtain bacteria, by "dilution", that only contains the desired recombined BAC, and thus have lost any BAC still containing the *galK* cassette.

20. As in step 14, pellet 1 ml culture and wash twice in 1xM9 salts, and resuspend in 100 ul 1xM9 salts after the second wash before plating on M63 minimal media plates with glycerol, leucine, biotin, 2-deoxygalactose (DOG), and chloramphenicol. The DOG creates a toxic product if the cell is able to metabolize galactose but is harmless to GalK- recombinants.

21. Incubate at 32°C for three days.

22. There may be a number of background colonies on your control plates; however, you will still be able to find true recombinants with a high frequency. Analyze, say, 10-12 colonies by digestion, PCR, or sequencing.

Protocol is adapted from NCI-Frederick [1]

Michaelis-Menten Assay – Washington – 2010
http://2010.igem.org/Team:Washington/Protocols/KunkelCapD

Prepare a 10x Master Mix with these concentrations (10uL/rxn)

Make one for each enzyme

- HEPES 7.4 pH (250mM)
- 1% Tween
- Purified Enzyme (50nM)
  - *Dilute enzyme in 1x HEPES/Tween buffer*

Note: Make one without enzyme for blank

Prepare Substrate

- Prepare 10x substrate (10uM) in one well of a 12-well strip tube
- Do half concentration serial dilutions (equal parts diH2O and substrate) until 11th well.
- Leave the 12th well with no substrate

Final Concentrations of Substrate (Left to Right in uM):

10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625, 0.01953125, 0.009765625

1.1x Master Mix

- Put equal parts 10x Master Mix and 10x L-Glu eppendorf tube (Referred to as L-Glu Master Mix)
- Put equal parts 10x Master Mix and diH2O in an eppendorf tube (Referred to as diH2O Master Mix)
- Fill each tube with diH2O (70uL/rxn)

Prepare a 96-well plate for transpeptidation

- Pipette 90uL of 1.1x L-Glu Master Mix for one each enzyme across a row for the transpeptidation reaction
- Repeat for each enzyme
- Make one row of blank (1.1x L-Glu Master Mix without enzyme)

Prepare a 96-well plate for hydrolysis

- Pipette 90uL of 1.1 diH2O Master Mix for each different enzyme in each new row
- Repeat for each enzyme
- Make one row of blank (1.1x diH2O Master Mix without enzyme)

Prepare Spectramax plate reader with desired settings

- Pipette 10uL of Substrate into each well. (Column 1 from well 1 of strip tube, Column 2 from well 2 of strip tube, etc.)
- Immediately place into Spectramax plate reader and begin reading

Final Concentrations

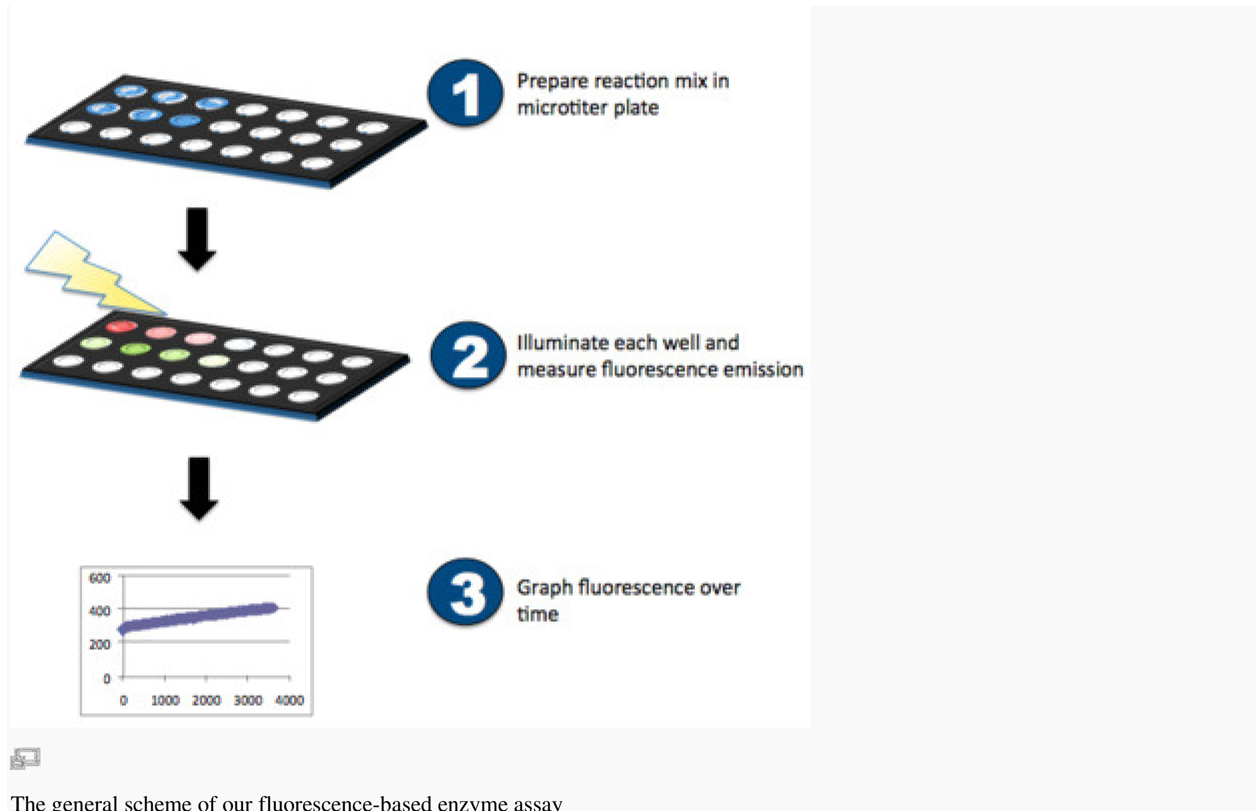
- Enzyme (CapD) - 5nM (0.00025mg/mL)
- Amino Acid (L-Glutamate) - 0mM or 5mM
- Substrate - Variable Concentration
- HEPES (7.4pH) - 25mM
- 0.1% Tween

Final Reaction Volume 100uL

Substrate is 5-FAM-(D-gamma-Glu)5-K(QXL520-NH2 from AnaSpec

Product produced from reaction is 5-FAM-(D-gamma-Glu)5-NH2 from AnaSpec

Enzyme Assay – Washington – 2010 http://2010.igem.org/Team:Washington/Protocols/KunkelCapD



The general scheme of our fluorescence-based enzyme assay

After we have the CapD_CP mutants, we tested our mutants for their catalytic activity using our fluorescence-based enzyme assay scheme. Fluorescence-based enzyme assay measures the rate at which fluorescence in the testing media is released and the amount fluorescence depends on the rate at which fluorophore-quencher linkage is disrupted. Our substrate PDGA contains a linked fluorophore-quencher component. The faster fluorophore-quencher component is cleaved, the higher the amount of fluorescence is released and thus the greater the enzymatic activity observed.

Run one transpeptidase reaction and one hydrolysis reaction per enzyme (always run control for each set)

Prepare 10x Master Mix with these concentrations. (10uL/rxn)

- HEPES 7.4 pH (250mM)
- 1% Tween
- Substrate (500nM)

Dilute Enzymes

- Measure enzyme concentrations by protein gel band intensity or with spectrophotometer (A280)
- Dilute enzymes to 50nM (~0.0025mg/mL) in strip tubes
    - *Make sure you have at least 30uL of your final diluted enzyme*

Prepare a 96-well plate

- Pipette diH2O into each well (Two per enzyme)
    - Transpeptidase: 70uL
    - Hydrolysis: 80uL
- Pipette 10uL of 10x Master Mix into each well.
- Pipette 10uL of 50mM L-Glu into each **transpeptidation** reaction well.

Prepare Spectramax plate reader with desired settings and desired emission and excitation wavelengths

- Pipette 10uL of your enzyme into each well
- Immediately place into Spectramax plate reader and begin reading

Final Concentrations

- Enzyme (CapD) 5nM (0.00025mg/mL)
- Amino Acid (L-Glutamate)0mM or 5mM
- Substrate 50nM
- HEPES (7.4pH) 25mM
- 0.1% Tween
- Final Reaction Volume 100uL

Substrate is 5-FAM-(D-gamma-Glu)5-K(QXL520-NH2 from AnaSpec

Product produced from reaction is 5-FAM-(D-gamma-Glu)5-NH2 from AnaSpec

BioRad Micro Column Protein Prep Protocol - **Washington – 2010** **http://2010.igem.org/Team:Washington/Protocols/KunkelCapD**

Day 1: OVERNIGHTS

- Pick a single colony from plate and inoculate 2mL TB/LB +Kan in a 14mL culture tube
- Shake at 37deg 16-24hrs

Day 2: EXPRESSION

- Inoculate a STERILE 250mL flask that contains 50mL of TB+Kan media with 1mL (or 500µL of overnight if you used TB) of the overnight.
- Grow at 37 degrees until OD600 reaches 0.3-0.6 (generally 2-4 hrs)
- Add IPTG for final concentration of 0.5mM.
- Transfer to desired expression temperature and let express for the appropriate amount of time:
    - 22C = 16-24hrs
    - 30C = 8-12hrs
    - 37C = 4-6hrs

Day 3: STORE

- Spin down cells at 4000rpm for 20min in 50mL falcon tubes
- Pour off supernatant
- Store cells at -20C until ready for purification

Day 3/4: PURIFICATION

- Lysis (~1hr) **KEEP ON ICE OR IN COLD ROOM AS MUCH AS POSSIBLE**
    - Add 500uL of wash buffer and vortex to resuspend cells
    - Add 1mL of lysis buffer and gently pippette up and down (minimizing bubbles)
    - Transfer to a 2mL Eppendorf tube.
    - Continue to incubate at room temp for 20min, mixing with plate mixer.
        - *If worried about protein stability then incubate in the cold room for 1hr on rocker.*
        - Save 50uL of lysis if want to run on gel.
    - Spin the lysis for 30-60min at 15000rpm
        - *Spin longer if supernatant is not clear enough, but one hour should really be sufficient!*
    - Transfer supernatant to a fresh tube (~1800uL) by decanting or careful pipetting

- Purification **KEEP ON ICE OR IN COLD ROOM AS MUCH AS POSSIBLE**
    - Bind Protein (~1/2hr)
        - Add 200uL of TALON/NiNTA Agarose 50% slurry to the column and place in 2mL centrifuge tube
            - *Use a 1000uL tip otherwise the beads get stuck!*
            - *Final of 100uL of actual beads*
        - Spin at 2000rpm for 2min
        - Discard flow-through and replace bottom cap

        - Add 500uL of supernatant, cap the top of the column, and mix (DO NOT VORTEX)
        - Let gently incubate on rocker for 5min
        - Remove caps and place in 2mL centrifuge tube
        - Spin 2000rpm for 2min
        - Discard flow-through and replace bottom cap
        - Repeat until all supernatant has passed through (~3-4x)
    - Washing Protein (~1/2hr)
        - Cap the bottom, add 500uL wash buffer, cap the top, mix, agitate 5min gently, remove caps and place in 2mL tube, spin 2000rpm 2min, discard flow-through
        - Cap the bottom, add 500uL wash buffer, cap the top, mix, agitate 5min gently, remove caps and place in 2mL tube, spin 2000rpm 2min, discard flow-through
        - Cap the bottom, add 500uL wash buffer, cap the top, mix, agitate 5min gently, remove caps and place in 2mL tube, spin 2000rpm 2min, discard flow-through
    - Eluting Protein (~1/4hr)
        - Cap the bottom, add 200uL of elution buffer, cap the top, mix, agitate 5min gently, remove caps and place in FRESH Eppendorf tube, spin 2000rpm 2min
        - THE FLOW-THROUGH IS YOUR PURIFIED PROTEIN!

Buffer Examples; alter to fit your protein's ideal buffer

Wash Buffer (GENERAL STOCK BUFFER):

- 50mM pH 7.4 HEPES
- 500mM NaCl
- 25mM Imidazole

BUGBUSTER Lysis Buffer:

- 50mM pH 7.4 HEPES
- 500mM NaCl
- 25mM Imidazole
- 2x Bug Buster
- 2mg/ml lysozyme (small scoop)
- 0.2mg/ml DNAse (small scoop)

Elution Buffer:

- 50mM pH 7.4 HEPES
- 500mM NaCl

- 500mM Imidazole

Protein Expression/Purification of Recombinant Protein in pET29b+ - Washington – 2010

http://2010.igem.org/Team:Washington/Protocols/KunkelCapD

Expression

- 1.Transform pET29b+ DNA into BL21(DE3)* competent cells, plate on KAN.
  - a.*You can skip this step if there is already a glycerol stock in BL21(DE3)**
  - b.Combine 1uL of DNA + 20uL of cells in Falcon tube
    - i.Eppendorf is fine as well
  - c.Quickly immerse tube with cells in water batch at 42deg for 45sec
  - d.Remove and let rest on ice for 2min
  - e.Add 200uL of LB
  - f.Let recover, shaking at 37deg for 45min
  - g.Plate 200uL of cells onto a LB/Kan plate
- 2.Grow 50mL LB starter cultures in STERILE 250mL flask:
  - a.Thoroughly wash Flask with HOT WATER
    - i.Removes trace bleach/soap/etc which can inhibit cell growth
  - b.RINSE WITH diH2O (remove tap water particulates)
  - c.Autoclave for 20-40min
  - d.Cool down (1hr bench top should be enough)
  - e.50mL LB, 50uL 50mg/mL KAN
  - f.Innoculate with 1 colony or a scrape from glycerol stock
  - g.Grow O/N at 37C with shaking at 250 (let grow for >=18hrs).
  - h.Make glycerol stock using 500uL 50% glycerol + 500uL O/N culture.
    - i.This is only necessary if you do not already have a glycerol stock
- 3.Grow 0.5L cultures, in ZY Auto Induction media in 2L flask:
  - a.Thoroughly rinse out flask with HOT WATER
    - i.Removes trace bleach/soap/etc which can inhibit cell growth
  - b.RINSE WITH diH2O (remove tap water particulates)
  - c.5g Tryptone, 2.5g Yeast Extract + 465mL H2O.

        - i.Autoclave
        - ii.Let Cool down
        - iii.Make AUTOMIX (In separate 50ml falcon tube):
            - 1.500uL 1M $MgSO_4$
            - 2.500uL 1000X Metals Mix
            - 3.25mL 20X NPS
            - 4.10mL 50X 5052
            - 5.500uL 50mg/mL KAN
        - iv.Add contents of AUTMIX tube to Media
        - v.Add remaining 49.5mL of starter culture to media
        - vi.Grow at 18C for 24-30 hours.
- 4.Collect Cells
- 5.Spin at 4000rpm for 20 minutes.
- 6.Discard supernatant
- 7.Resuspend pellet in 5mL 1x PBS, pH 7.4
- 8.Transfer to 50mL falcon tubes.
    - a.Run gel to confirm expression. (optional)

Purification

- 1.Prepare buffers (recipes on next page)
    - a.Wash buffer: 50mM HEPES (pH 8), NaCl 500mM, 25mM Imidazole (pH 8)
    - b.Lysis Buffer: 50mM HEPES (pH 8), NaCl 500mM, 25mM Imidazole (pH 8), 2x Bug Buster, 2mg/mL lysozyme, 0.2mg/mL DNAse
    - c.Elution buffer: 50mM HEPES (pH 8), NaCl 500mM, 500mM Imidazole (pH 8)
    - d.Talon strip buffer: 100mM EDTA, pH 8.0
    - e.Talon recharge buffer: 100mM $CoCL_2$
- 2.Lyse Cells (Sonication)
    - a.Sonication
        - i.Make master mix of lysis buffer and add 10mL to each pellet and resuspend. Vortexing ok.
- 3.After each pellet is resuspended, pour resuspension into cleaned SS34 tube (holds 60mL roughly).
- 4.Add more lysis buffer so that its roughly 4/5 full
- 5.Remove Insoluble Matter
    - a.Balance samples in SS34 tubes (pairwise is sufficient)
    - b.Spin at >=18000rpm for >=30min at 4-15deg
        - i.If supernatent is still “goopy” add a little more DNAse and spin longer
- 6.Remove Particles

- a.Filter supernatant with 0.4uM Filter into new 50mL falcon tube.
  - i.If analyzing expression save Supernatant & Pellet Gel Samples (50uL)
- 7.Run Proteins over Column
  - a.Equilibrate 1mL NiNTA-Superflow Gravity columns with 10mL wash buffer.
  - b.Run supernatant over column 2X
    - i.If analyzing expression Save Load FT. Load FT Gel Sample
  - c.Wash column 1x with 20mL wash buffer with .5% Tween.
    - i.If analyzing expression Save Wash FT Gel Sample
  - d.Wash column 1x with 20mL wash buffer no Tween
  - e.Elute column with 15mL elution buffer
    - i.Collect in Vivaspin-20 Concentrator.
  - f.Save excess Elution buffer for blanking/diluting proteins later
- 8.Concentrate Proteins
  - a.Check A280 on using spec (e.g. nanodrop)
  - b.Spin at 8000 rpm for about 10-20min.
  - c.Target volume is 200uL
    - i.If this puts A280 above 30 stop at a larger volume, proteins can crash out when too concentrated.
- 7.Remove Excess Imidazole
  - a.Transfer 800uL to a dialysis tube (midi, 50-800uL), save the remaining protein
    - i.Novagen Cat No 71507-3
  - b.Let equilibrate in 1-4L of Dialysis buffer for at least 2 hours (overnight is fine)
  - c.Repeat so at least two rounds of dialysis have been done
- 8.Determine Protein Concentration
  - a.Check A280
  - b.Run Gel of Samples
    - i.Make 1:10 dilution of lysis, supernatent
    - ii.Dilute preconcentrated and final protein so A280 ~1.5
    - iii.Combine 5uL sample with 5uL 2x SDS Loading Buffer.
    - iv.Boil for 5min
    - v.Run 4-20% BioRad Gel for 35min at 200v
    - vi.Wash 3x with diH2O (rock 5min between each rinse)
    - vii.Stain using Thermo staining reagent
- 9.Store protein
  - a.For short term store in 4deg

- b.For long term flash freeze and store at -80deg
- 10.Clean columns
  - a.10mL 5M GuHCl + 500mM Imidizole + 1% nonionic detergent (Tween-20)
  - b.10mL of ddiH2O
  - c.10mL of 0.2M EDTA (pH ~7.0)
  - d.10mL of ddiH2O
  - e.10mL of 50mM CoCl2
  - f.10mL of ddiH2O
  - g.10mL of 300mM NaCl
  - h.10mL of ddiH2O
  - i.10mL of 20% EtOH
  - j.Cap bottom, add 5mL of 20% EtOH, cap top and store

Buffers

Wash Buffer (GENERAL STOCK BUFFER):

- 50mM pH 7.4 HEPES
- 500mM NaCl
- 25mM Imidizole

Lysis Buffer:

- 50mM pH 7.4 HEPES
- 500mM NaCl
- 25mM Imidizole
- 2mg/ml lysozyme (small scoop)
- 0.2mg/ml DNAse (small scoop)

Elution Buffer:

- 50mM pH 7.4 HEPES
- 500mM NaCl
- 200mM Imidizole

Kunkel Mutagenesis – Washington – 2010 http://2010.igem.org/Team:Washington/Protocols/KunkelCapD

Produce ssDNA (RECOMMENDED TO DO IN DUPLICATE! SEE NOTE AT END OF SECTION 2)

- Transform plasmid into chemically competent CJ236 cells
  - Plate onto a Chlor+YOUR ANTIBIOTIC plate and incubate overnight at 37C
- Inoculate 6 colonies into 3ml of LB + YOUR antibiotic (NO chlor here per NEB instructions)
- Grow for 4-6 hours at 37C, shaking at 200rpm (until cloudy)
- Add 3ul of M13K07 helper phage
- Continue growing for 1 hour at 37C, shaking at 200rpm
- Expand culture by diluting 1mL into 50ml of LB + antibiotic in 250ml flask
- Grow overnight at 37C, shaking at 200rpm

Harvesting ssDNA

- Spin down overnight culture in sterile 50ml Falcon tube at 7000rpm for 20 minutes at 4C
- Transfer supernatant (contains phage) to a new sterile 50ml Falcon tube
- Add 10ml 20% PEG/2.5M NaCl and mix thoroughly
- Incubate on ice for 45 minutes
- Spin down the phage at 7000rpm for 20 minutes at 4C
- Decant liquid and let tube stand upright to drain off the rest of the liquid
- Resuspend the pellet in 2mL 1xPBS (Vortexing is okay)
- Transfer the 2mL into two microfuge tubes (1mL in each)
- Spin at 14,000rpm for 5 minutes
- Transfer the supernatant to two new microfuge tubes, each with 300ul PEG/NaCl
- Vortex and incubate at room temperature for 10 minutes
- Spin down phage at 14,000rpm for 2 minutes
- Pipette off supernatant; do a second quick spin to collect residual liquid and pipette it off
- Resuspend the pellet (phage) in 1ml 1xPBS (Use that 1ml to resuspend the other pellet)
- Spin down at 14,000rpm for 5 minutes
- Transfer the supernatant (phage) to a new microfuge tube
- Harvesting ssDNA from Phage using Qiagen Qiaprep M13 kit (#27704)
  - DO NOT SPIN OVER 8000rpm when using this kit!
  - Final solution should be greater than 20ng/uL. If not, try again; ssDNA can randomly fail, so it is best to make 2 batches in parallel (works as a nice counter balance as well).

Kinasing Oligos

- Design mutagenic oligo using Stratagene's primer design and order the 5'->3' antisense oligo
  - This is specific for pET29b+, other vectors may require the sense oligo

    - QuikChange Primer Design Program
- Make Kinase Reaction Mix (make #rxn+2)
    - 3uL Kinase Buffer/rxn
    - 1uL of 10mM ATP/rxn
    - 1uL T4 Polynucleotide Kinase/rxn
    - 18uL ddiH2O/rxn
- Combine oligo and reaction mix in PCR strip tubes or 96well PCR Plate
    - Aliquot 23uL of Kinase Reaction Mix using repeater
    - Make sure all liquid at the bottom by tapping or briefly spinning
    - Add 7uL of 100uM oligo to the BOTTOM of each well and pipette up and down
- Seal, mix gently by tapping, and incubate at 37deg C (metal bath or PCR machine) for 1 hour
- Store on ice short term, or -20 long term
    - *These can be re-used in the future so no need to throw them away!*

Dilute Mutagenic Kinased Oligo (Optimal molar ratio is 1:4 dU-ssDNA:Oligo)

- Add 2uL of oligo into 200uL of diH2O
    - If doing a mutant with multiple oligos add 2uL of EACH oligo to the same tube
    - This dilution factor will achieve the desired 1:4 dU-ssDNA:Oligo molar ratio for most single stranded DNA preps.

Anneal Diluted Oligo and ssDNA

- Combine 0.2uL of T4 DNA Ligase Buffer with 2uL of ssDNA, (make #rxn+2)
- Aliquot 2.2uL of the mix into a in a fresh PCR plate or strip tubes
    - *Make sure all the liquid as the bottom by spinning down or tapping*
- Add 2uL of THE DILUTED kinased primer (single or mixed) to generate desired mutant to the bottom of the plate and PIPPETE UP AND DOWN TO MIX
    - **Always do a background as well where you have ssDNA with NO oligo (just add 2uL of water). This will allow to you to know if you mutations work and estimate your mutation efficiency so you can pick the appropriate number of colonies you need to screen in order to find the mutation desired.**
- Seal, mix (tapping or plate mixer)
- Run Siegel: Anneal in PCR machine **(USE heated lid)**
    - Starts at 95 degrees and ramps down to 25 over an hour
    - *I find the slow ramp decreases background and increases overall kunkel efficiency relative to the quick protocol, which does 95-2min; 50-2min; 25-forever*

Polymerize DNA

- Make polymerization reaction mixture (make #rxn+2)
    - 0.6uL 10x T7 Ligase Buffer/rxn
    - 0.4uL 25mM dNTPs/rxn
    - 0.4uL 10mM ATP/rxn
    - 0.4uL T7 Polymerase (unmodified from NEB)/rxn
    - 0.4uL T4 Ligase/rxn
- Add 2.2uL of polymerization reaction mixture to each annealing reaction
- Seal, mix (tapping or plate mixer)
- Incubate at room temperature for 1+ hrs.

Transform

- Dialyze DNA (ORDER IS IMPORTANT HERE!!!!)
    - Add ~40mL of diH2O to an empty Petri dish (till it is mostly full)
    - Mark up to 8 spots on the shiny side of a 0.025um membrane filter
    - Place the filter into the diH2O so shiny side is up and it is flat
        - **DO NOT SUBMERGE!**
    - CAREFULLY pipette the 5uL reaction onto the designated spots
    - Let dialyze for 20+ minutes
- Electroporate
    - CAREFULLY remove the reaction and pipette into the bottom of the crevice in the 0.1cm electroporation cuvettes and KEEP ON ICE
    - Add 30uL of electro competent BL21(DE3)* cells (Competent Cell Rack, Box labeled "Jasmine", tubes labeled E*)
    - Place into BioRad electroporater (only fit in 1 way) set to Bacteria and hit "Pulse"
    - IMMEDIATELY add 200uL of media (TB or LB, NO antibiotic) into the cuvette to extract cells, transfer to a fresh strip tube or PCR plate.
- Recover and Plate
    - Incubate 37C for ~1hr
    - On individual plates out ~200uL of each reaction
        - *I find it works best to do 12 plates at a time*
    - Add ~10 glass beads/plate
    - Add the 200uL of solution
    - Shake to spread
    - Turn plate and tap so beads are transferred to the lid, than pour out beads into 70% EtOH filled beaker.

    - Move to the next row.
- Dry plates at 37deg for ½ hr, lid-side up.
- Turn plates so agar side is up and incubate at 37deg overnight

Buffers -

20%PEG/2.5M NaCl

- 200g PEG (polyethylene glycol) 8000MW
- 141.6g NaCl
- Add dH2O to 1L
- Autoclave with a stir bar, stir immediately after autoclaving until cool, and store at 40C.

1xPhosphate Buffered Saline (PBS)

- 800ml dH2O
- 8g NaCl
- 0.2g KCl
- 1.44g Na2HPO4
- 0.24g KH2PO4
- Adjust pH to 7.4
- Add diH2O to 1L
- Autoclave

Ligation Protocol – UTDallas – 2010 http://2010.igem.org/Team:UTDallas/Protocols

- Determine insert to vector ratios
- Calculate the amount of insert needed if 50ng of vector is used (can use different amount of vector)
- In a PCR tube add the following:
    - 50ng of vector
    - Amount of insert based on ratios (calculated in second step)
    - 2uL of buffer
    - 2uL of DNA ligase
    - Amount of water to bring total volume to 20uL
- Incubate overnight at 14°C

Note: We used T4 DNA ligase and buffer from NEB

Gel Purification Protocol (from QIAquick Gel Extraction Kit) – UTDallas – 2010
http://2010.igem.org/Team:UTDallas/Protocols

- Excise DNA fragment from the agarose gel with a clean, sharp scalpel
- Weigh the gel slice in a microcentrifuge tube.
- Add 3 volumes of Buffer QG to 1 volume of gel (100mg~100uL)
- Incubate at 50°C for 10 min (until the gel slice has completely dissolved)

- After the gel slice has dissolved completely, check that the color of the mixture is yellow
- Apply the sample to a QIAquick column, and centrifuge for 1 min
    - Maximum volume of the column is 800uL. For samples larger than this, simply load and spin again.
- Discard flow-through and place QIAquick column back in the same collection tube
- To wash, add 750uL of Buffer PE to column and centrifuge for 1 min.
- Discard the flow-through and centrifuge for additional 1 min. at 13,000rpm
- Place QIAquick column into a clean 1.5 mL microcentrifuge tube
- To elute DNA, add 50uL of Buffer EB to the center of the QIAquick membrane, let the column stand for 1 min. and then centrifuge the column for 1 min.

Gel Electrophoresis Protocol – UTDallas – 2010 http://2010.igem.org/Team:UTDallas/Protocols

- Making a 1% agarose gel
    - 100mL 1X TBE buffer
    - 1g agarose
    - microwave until agarose dissolves
    - let mixture cool
    - when cool add 8-10uL ethidium bromide
    - stir gently, let cool
    - pour into plate with comb already in place
    - let harden
- Using the gel
    - Add loading buffer to DNA (for 100uL DNA, add 20uL loading buffer)
    - Load 2uL of DNA ladder into the gel
    - Load DNA into the gel
    - Run at 130V for 30min-1hr

Digestion Protocol – UTDallas – 2010 http://2010.igem.org/Team:UTDallas/Protocols

- Using a microcentrifuge tube add the following:
    - ~3000-5000 ng of DNA
    - 10uL Buffer 4
    - 10uL BSA
    - 5uL of appropriate enzyme (if doing a double digest, use 5 uL of both enzymes)
    - Amount of H_2O needed to make final volume 100uL
- Incubate at 37°C for 1hr and 30min

Note: We used the following enzymes from NEB: EcoRI-HF, PstI-HF, SpeI, and XbaI. All of which can be double digested with each other using Buffer 4.

Preparing LB+Appropriate Antibiotic Protocol – UTDallas – 2010
http://2010.igem.org/Team:UTDallas/Protocols

- 200 mL LB broth
- Autoclave
    - Put control thermometer in H_2O (from the sink)
    - Select vented container mode (Do Not Change Program)
- Let cool to 50°C
- Add antibiotic (50-100 ug/mL) (10 mg total)
    - Weigh on paper

    - Add to 0.5 mL DI H_2O
    - Add to LB mixture when cool enough
- Store at 4^oC

Preparing Agar Plates Protocol (Makes 12 (15mm) Plates) – UTDallas – 2010
http://2010.igem.org/Team:UTDallas/Protocols
- 300 mL DI H2O + 11 g LB agar
- Autoclave
    - Put control thermometer in H_2O (from the sink)
    - Select vented container mode (Do Not Change Program)
- Mix well after autoclaving; let cool to 50^oC
- Add antibiotic (50 to 100 µg/mL) (15 mg total)
    - Weigh on paper
    - Add to 0.5 mL DI H_2O
    - Add to LB mixture when cool enough
- Plate
    - Under flame open lids of all plates
    - Slowly pour agar into plate, avoiding bubbles, when it touches all edges stop pouring
    - Let sit under flames until gel solidifies
    - Replace lids on plates
- Store upside down at 4^oC

Preparing Competent Cells Protocol – UTDallas – 2010
http://2010.igem.org/Team:UTDallas/Protocols
- Place 1 colony in 5 mL of LB (with antibiotics if appropriate) Grow overnight at 37^oC and 200-300 rpm
- Inoculate 0.25 mL of the overnight strain into 25 mL of LB
- Shake at 37^oC until the OD650 is 0.6-0.7
- Harvest cells and resuspend in 12.5 mL ice cold 0.1M $MgCl_2$
- Harvest immediately and resuspend in 7.5 mL cold 0.1M $CaCl_2$
- Leave on ice for 30 minutes. Harvest and resuspend in 2.5 mL cold 0.1M $CaCl_2$
- Leave on ice for 30 minutes
- For long term storage, use 0.1M $CaCl_2$ in 15% glycerol at step 6 and store cells at -800^oC

Note: Harvest cells at 5000 rpm for 10 minutes at 4^oC

Miniprep Protocol (from QIAprep Spin Miniprep Kit) – UTDallas – 2010
http://2010.igem.org/Team:UTDallas/Protocols
- Harvest cells at 5400g 10 minutes 40^oC (possibly program 1)
- Resuspend pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube
- Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4-6 times
- Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times
- Centrifuge for 10 minutes at 13000 rpm (~17900g) in a table-top microcentrifuge
- Apply the supernatant (from step 4) to the QIA prep spin column by decanting or pipetting
- Centrifuge for 30-60 seconds. Discard the flow-through
- Wash QIA prep spin column by adding 0.75 mL Buffer PE and centrifuging for 30-60 seconds
- Discard the flow-through, and centrifuge for 1 minute to remove residual wash buffer

- To elute DNA, place the QIA prep column in a clean 1.5 mL microcentrifuge tube. Add 50 µL Buffer EB or water to the center of each QIA prep spin column, let stand for 1 minute and centrifuge for 1 minute.

Preparing Glycerol Stock Protocol – UTDallas – 2010
http://2010.igem.org/Team:UTDallas/Protocols

- Add 150 µL of 50% glycerol to 350 µL of cells
- Place in -80°C freezer

Transformation Protocol – UTDallas – 2010 http://2010.igem.org/Team:UTDallas/Protocols

- With a pipette tip, punch a hole through the foil cover of the DNA plate
- Add 10 µL of DI water
- Thaw competent cells on ice
- Add 1-2 µL of resuspend DNA and 50 µL of thawed competent cells to labeled tubes
- Incubate the cells on ice for 30 minutes
- Heat shock the cells at 42°C for 45 sec
- Incubate the cells on ice for 2 minutes
- Under flame, add 450 µL SOC broth
- Incubate at 37°C for 1 hour while rotating or shaking at 300rpm
- Spread cells on appropriate antibiotic LB plates (usually 100 µL)
- Incubate at 37°C for 18-24 hours
- Take a colony, put in 3 mL of LB + appropriate antibiotic
- Use resulting culture to miniprep DNA and make your own glycerol stock

Bacterial Transformation – Utah State University – 2010 **http://2010.igem.org/USU_protocol**

Once the target DNA has been successfully ligated into the plasmid vector, the plasmid must be transferred into the host cell for replication and cloning. In order to do this, the bacterial cells must first be made "competent." The term "competent" is to describe a cell state in which there exist gaps or openings in the cell wall which will allow the plasmid containing the target genes to enter into the cell. Several methods to make bacterial cells competent exist, such as the calcium chloride method and electroporation. The following is the method used by the USU team to insert the plasmids containing various biobricks into the cells.

Calcium chloride Method

- Ensure the necessary antibiotic agar plates have been prepared or begin their preparation now. Four plates per transformation will be necessary (two today, then two tomorrow for streaking). Also ensure that 10 ml liquid media is made up per transformation (also for tomorrow).

- If using Biobrick parts from iGEM distribution, use registry to identify appropriate will containing plasmid of interest and proceed to step 3, if using other DNA proceed to step 5.
- Add 10ul of sterile water to distribution well to dissolve DNA. Remove 10ul and place in 0.5ml bullet tube. Label tube with part number, use 2ul to transform and save the other 8ul in the BioBrick part box.
- Take competent cells (One Shot® TOP10 Chemically Competent E.coli, Invitrogen) from the -80˚C freezer and place on an ice bath.
- Add 2 µl of the DNA solution (or 4ul of ligation reaction) to the competent cells. Ensure the pipetting is done directly into the cell solution. Let cells incubate on ice for 30 minutes. Heat water bath to 42˚C.
- Heat shock cells in the 42˚C water bath for 30 seconds. Remove and place back in the ice bath for 2 minutes.
- In the hood, add 250 µl SOC media to each tube, bringing the total cell solution to 300 µl. Incubate at 37˚C for 1 hour.
- Add 200 µl of each transformed cell solution to the appropriate antibiotic plate. Use the Bunsen burner to create a "hockey stick" out of a glass pipette tip by holding over the flame until it bends. Allow to cool. Spread cell solution uniformly over the agar plate using the "hockey stick," then before discarding, spread residual solution on the "stick" over a second plate to get more a more sparse colony distribution.
- Parafilm all plates and place in 37˚C incubator 12-14 hours, or overnight if that is not possible.

Electroporation Method

Making competent cells for electroporation

- Streak out E.coli strain to get single colonies
- After ovenight incubation, pick a single colony. Inoculate 50ml of SOB Media. Incubate overnight at 37°C.
- Subculture to 1L of SOB Media with 5ml of the overnight culture
- Grow to O.D.550 =0.2 (3-5 hours)at 37°C.
- Pellet cells at 5,000 r.p.m. for 10 minutes in the Sorvcal, GSA rotor
- Resuspend the cells in 500ml of cold WB and recentrifuge
- Resuspend the cells again in 500ml of cold WB and centrifuge again.
- Resuspend the cells in the WB remaining in the tube after pouring off the supernatant. If necessary, adjust volume up to 4ml with cold WB

- Transfer 200ul aliquots into microfuge tubes and store at -70°C.

Electroporation

- Gently thaw the cells at room temperature, then put into ice
- In a pre-chilled microcentrifuge tube, mix 40µl of cells with 1µl (3-5µl) DNA. Mix the suspension well and place on ice for ½ to 1 minute.
- Set the machine to the following parameters: 25µF, 2.5kV, 200Ω.
- Transfer the cell solution to a pre-chilled 0.2 cm cuvette. Shake the suspension to the bottom of the cuvette.
- Pulse the cells (4-5 msec)
- Remove the cuvette and immediately add 1ml of cold SOC Media and resuspend the cells with a Pasteur pipet.
- Transfer the cells to a new tube and invubate them at 37°C for one hour.
- Dilute the cells in PBS or SS and plate them on selective media.

Streak Plates and Liquid Cultures from Transformed Colonies

After bacterial cells have been transformed, successfully transformed cells must be selected. Because 100% of the cells do not receive the desired plasmid and target gene, it is essential to select for cells that do have the target genes. The USU team uses antibiotic resistance to select for successful transformations. To do this, an antibiotic resistance gene is also added to the plasmid vector that contains the target genes. By doing so, it is possible to know that a cell was successfully transformed based on its ability to grow on an agar plate with antibiotics added. Because the cell is able to grow, the antibiotic resistance gene must be present as well as the target gene. From the agar plates containing the antibiotics, a colony is picked and transferred into a liquid culture for further analysis. The following is the method used by USU to clone the DNA and select for the successful transformation of various BioBricks in *E.coli*.

Method

- Prepare two 15 ml tubes per transformation, each with 5 ml media containing the appropriate antibiotic.
- Use a pipette tip to extract half of each colony and inoculate one agar plate per colony. Using a pipette with a tip, extract the other half of each colony and inoculate one liquid media tube per colony. Label all tubes and plates and place in the 37˚C incubator until the next morning.

Plasmid DNA Isolation – Utah State University – 2010 **http://2010.igem.org/USU_protocol**

Following successful bacterial cloning and isolation, it is important to verify that the target gene is in the cell and that the resultant plasmid is correct. To do this, it is a common practice to sequence the plasmid DNA. To obtain enough DNA for sequencing, the bacterial clones are grown in a liquid culture. The cells are harvested by centrifugation and then prepared for DNA plasmid extraction. DNA plasmid extraction can be done several ways, and the overall purpose is to lyse the cells and separate the plasmid DNA from all other cellular proteins, DNA, and debris. The following is the method used by the USU team to isolate plasmid DNA containing the various biobricks.

Method

- Prepare two water baths, one boiling and the other 68C.
- Centrifuge bactrerial cultures (3 to 5 ml) at 3K RPM for 20 min. Discard supernatant.
- Resuspend cell pellet in 200 µl of STET buffer. Transfer to 1.5 ml tubes.
- Add 10 µl of lysozyme (50 mg/ml) and incubate at room temperature for 5 min.
- Boil for 45 sec and centrifuge for 20 min at 13K RPM (or until pellet gets tight).
- Use a pipette tip or toothpick to remove the pellet.
- Add 5 µl RNase A (10 mg/ml) to supernatant and incubate at 68C for 10 minutes.
- Add 10 µl of 5% CTAB and incubate at room temperature for 3 min.
- Centrifuge for 5 min at 13K RPM, discard supernatant, and resuspend in 300 µl of 1.2 M NaCl by vortexing.
- Add 750 µl of ethanol and centrifuge for 5 min at 13K RPM.
- Discard supernatant, rinse pellet (which cannot be seen) in 80% ethanol, and let tubes dry upside down with caps open.
- Resuspend pellet in either sterile water or TE buffer.

Restriction Enzyme Digestion and Electrophoresis – Utah State University – 2010 **http://2010.igem.org/USU_protocol**

Restriction enzyme digestion is the process by which an insert DNA sequence is separated from the rest of the DNA molecule. Specific knowledge of the DNA insert is needed to determine which enzyme and conditions to use during the digestion reaction. Once the DNA sequence is known and the correct enzymes have been selected, the DNA may be digested. Listed below is the procedure used by USU to digest the plasmid DNA. After enzyme digestion, electrophoresis is used to separate the plasmid from the

insert. A gel is prepared and the respective reaction mixes are loaded into the gel. Using a DNA ladder, and knowing the size of the insert, the corresponding band can be seen and cut out of the gel. The insert may then be removed and isolated from the gel, thus yielding the desired DNA. The DNA from this may then be used in PCR reactions, sequencing, ligations for further experimentation, etc. Listed below are example protocols used by the USU team for a restriction enzyme digestion and subsequent agarose gel electrophoresis.

Method

- Resuspend DNA in 20 to 40 µl water, vortex, and do a brief centrifuge to get solution to the bottom of the tube.
- Add components to the digestion solution in the following order: DNA (23 µl), 10X restriction enzyme buffer (3 µl), Xba1 (2 µl), and Pst1 (2 µl). The volume and restriction enzymes can be varied, but it should be ensured that the total volume is 10X the amount of RE buffer. Tap tubes periodically and allow to digest at appropriate temperature while preparing electrophoresis gel.
- Prepare electrophoresis gel by adding 2 g agarose to 200 ml TAE (1% solution). This is best done in an Erlenmeyer flask of adequate volume as swirling will need to be done. Place in the microwave and microwave on high for 20 seconds at a time, pulling it out and swirling until solution is homogeneous again, then repeating (BE CAREFUL to watch the solution closely when swirling – it superheats and can boil over and cause severe burns). Continue until solution is seen boiling in the microwave then gently swirl again.
- Add 20 µl ethidium bromide to solution and swirl until dissolved evenly.
- Add 6 µl of 6X loading dye to each tube of digested DNA solution.
- Prepare the electrophoresis unit by orienting the basin sideways with rubber gaskets firmly against the side. Place desired well template in the basin.
- When the agarose solution is cool enough to comfortably touch the flask, pour into the basin until the solution is about ¾ of the way to the top of the well template.
- When the gel is solidified (should look somewhat cloudy), remove the well template and change basin orientation to have the wells closest to the negative pole (as the DNA will flow towards the positive pole). Pour 1X TAE buffer into both sides of the electrophoresis unit until it just covers the gel and fills the wells.
- By inserting the pipette tip below the TAE liquid and into the well, add 10 µl of DNA ladder solution to first (and last if desired) well, skip one well, then begin adding the digested DNA solutions to the wells by adding about 2 µl less than the total volume in the tubes to prevent air bubbles in the wells.
- Place the cover on the electrophoresis unit, plug into the power source, and turn on voltage to 70 V (this can be as high as 100 V if time is an issue), and press the start button. Separation should take

two to three hours. The yellow dye shows the location of the smaller nucleotide lengths and the blue dye shows the location of the larger nucleotide lengths. DNA separation can be observed as time goes on by turning off the power supply then gently removing the basin from the electrophoresis unit (be careful not to let the gel slip out of the basin) and placing on the UV transilluminator to see DNA bands. The basin can then be placed back in the electrophoresis unit for further separation if desired. Take care to not have the power supply on without the lid to the unit in place.

- When the desired level of separation is obtained, the basin can be placed on the transilluminator for picture taking. Place the cone-shaped cover over the transilluminator and place the digital camera in the top hole for pictures.

Media Preparation – Utah State University – 2010 **http://2010.igem.org/USU_protocol**

For all experimentation involving the need for bacterial biomass and experimentation, proper media is needed to grow the cells. We use Lysogeny broth media for *E. coli*. and BG-11 for cynobacteria. The following is the media composition.

BG-11 growth liquid media (1 L)

- 50x BG-11 Concentrated Media - 20 mL
- dH2O - 980 mL

Lysogeny Broth (LB) liquid media (1 L)

- dH2O - 1 L
- Bacto-Tryptone - 10 g
- NaCl - 10 g
- Yeast Extract - 5 g

Plate Preparation

- Before autoclaving, add 15 g Difco Agar to 1 L liquid media.

Autoclaving

- Add all composition into a 2L Erlenmeyer flask and bring the volume up to 1 L with ddH20. Mix by swirling. Cover top with foil.
- Autoclave for 45 minutes (liquid setting, 0 minutes drying time). For making plates, after the media cool enough, antibiotics are added. At last media are poured on plates and become solid.

Polymerase Chain Reaction (PCR) – Utah State University – 2010
http://2010.igem.org/USU_protocol

PCR is used to amplify a desired DNA sequence. The reaction is first set up by designing primers that will bind only to the desired regions of the DNA sequence. Once the primer and polymerase have been selected, the reaction parameters of time and temperature must be optimized. When the reaction works properly only the target DNA will be amplified into large quantities that may then be isolated and used for further experimentation. The following is the procedure used by USU for PCR reactions to amplify various biological parts. A useful set of primers are the universal BioBrick primers VF2 and VR that can be used to amplify almost any BioBrick part.

Method

- Obtain the following reagents from the freezer: DNA template (cells or DNA), 10X Taq buffer (+KCl, -Mg/Cl2), MgCl2, 10 mM dNTP Mix, Taq polymerase (take out of freezer only immediately when needed and put back), and sterile distilled H2O. Place all reagents on ice. Also obtain PCR (either 0.2 or 0.5 ml) tubes.
- Add the following reagents to a tube (50 µl reaction) in the following volumes and order:
    - sterile H2O - 32 µl
    - 10X buffer - 5 µl
    - dNTP Mix - 2 µl
    - MgCl2 - 3 µl
    - cells/DNA - 6 µl
    - Taq Polymerase - 0.25 µl
    - Primer 1 - 1 µl
    - Primer 2 - 1 µl
- MgCl2 volume can be varied (lower to increase specificity – just ensure total volume is 50 ul with H2O). If many reactions are to be constructed, a master mix can be made up to cut down on time and pipette tip usage (if this is done, ensure primers are added to the appropriate reaction, i.e. perhaps not to the master mix). Tap or vortex tubes and take to the thermocyler. Place all reagents back in the -20˚C freezer.
- Choose thermocycler temperatures. The Eppendorf Mastercycler will cycle between three temperatures: typical temperatures are 94˚C for denaturing, 50-60˚C for primer annealing, and 72˚C for polymerase extending. Lowering the annealing temperature decreases DNA specificity; 55˚C is a good temperature to begin if no trials have been made with the sample.

- Turn on thermocycler with the switch in the back of the unit and open the lid. The placement of the tubes depends on the size of the tube (0.2 or 0.5 ml) and whether or not a temperature gradient is to be used.
- If no temperature gradient will be used, tubes can be placed anywhere on the unit in the appropriately-sized hole. Select “Files” and press enter. Select “Load” and then “Standard.” If cells will be used in the reaction, include a 1-minute lysing step at the beginning (step 1); this will be followed by a 1-minute DNA denaturing step (step 2). If purified DNA will be used, set step 1 to 1 second. Set an annealing temperature for step 3. Ensure the lid temperature is 105˚C and the extending temperature is 72˚C. Press exit. If prompted to save, save by pressing enter three times. Press exit to return to the main menu. Choose “Start” on the main menu and select “Standard.” The program should begin.
- If a temperature gradient is to be used, temperature will vary according to column. A 20˚C range is the maximum range that can be used (+/- 10˚C). The range is made by setting a temperature for the middle column and then setting a +/- range. To see what the temperatures will be if a gradient is used, select “OPTIONS” on the main menu, then select “Gradient.” Select the size tube that is being used by pressing “Sel,” then press enter. Choose a temperature for the center column, press enter, then select a +/- range and press enter. The column number along with the corresponding temperature is shown. Decide tube placement based on this information. Press exit twice to return on the main menu. Select “Files” then “Load,” then “Gradient.” If cells are being used, set the cell lysing step (step 1) to 1 minute (1:00); if purified DNA is being used, set this time to 1 second (0:01). Step 2 should be 94C, Step 4 should be 72˚C, and the lid temperature should be 105˚C. Go to step 3 and set an annealing temperature for the center column. Leave the next two lines as they are, and change the gradient setting (“G”) to the +/- the center temperature amount. Press exit. If prompted to save, press enter three times; if not prompted to save, press enter once. Press exit to get back to the main menu. To begin cycle, select “Start,” then select “Gradient.” The program should begin.
- The thermocycler is set to store the completed reaction tubes at 4˚C when finished.

Ligation – Utah State University – 2010
http://2010.igem.org/USU_protocol

Ligation is the process by which the insert (target DNA gene) is inserted into a plasmid. Both the plasmid and insert have been digested and have the proper “sticky” or blunt ends which are compatible for joining the two DNA pieces together into one molecule. These two DNA pieces are placed in a reaction tube and the proper DNA ligase, buffer, and cofactors are added for the reaction to take place. When done properly, the ligation will result in a successful combination of the insert and plasmid into one plasmid. This newly formed plasmid may then be isolated using gel electrophoresis and then used for bacterial

transformation or other experimentation. The following is the procedure used by USU to ligate together various biobrick parts. **Method**

- Obtain the following reagents, some of which are in the -20˚C freezer: DNA vector, DNA insert, 10X ligation buffer, T4 DNA ligase (take out only when needed, then return immediately to freezer), and sterile distilled water.
- Ideally, it is desirable to have the concentration of insert ends (or moles of insert) be two to three times the concentration of vector ends (or moles of vector), with a total DNA concentration of 50-400 ng/µl in the reaction. If determining the DNA concentration is not possible, place two to three times the volume of vector as the volume of insert in the reaction. As this is often the case, place the following reagents in a thin-walled PCR tube in the following volumes:
  - Insert DNA - 10 µl
  - Vector DNA - 3µl
  - 10X ligation buffer - 2 µl
  - H2O - 34µl
  - T4 DNA ligase - 1µl
- This could also be done in different volumes depending on DNA concentration/total volume desired.
- Gently mix the tube, and place the tube in the PCR thermocyler, turn on the machine, select "Start," from the main menu, select "22" and press "Start." The thermocycler will keep the reaction at 22˚C.
- Incubate for 60 minutes. Heat-inactivate by placing tubes in 68C water bath for 10 minutes. Place in the freezer if storing for later use.

Site-Directed Mutagenesis – Utah State University – 2010 **http://2010.igem.org/USU_protocol**

QuikChange II Site-Directed Mutagenesis Kit (Stratagene) Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence.

- Prepare the control reaction as indicated below:
  - 5 µl of 10× reaction buffer (see Preparation of Media and Reagents)
  - 2 µl (10 ng) of pWhitescript 4.5-kb control plasmid (5 ng/µl)
  - 1.25 µl (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/µl)]
  - 1.25 µl (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/µl)]
  - 1 µl of dNTP mix
  - 39.5 µl of double-distilled water (ddH2O) to a final volume of 50 µl
  - Then add 1 µl of PfuTurbo DNA polymerase (2.5 U/µl)

- Prepare the sample reaction(s) as indicated below:

Note: Set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping the primer concentration constant.

  - 5 µl of 10× reaction buffer
  - X µl (5–50 ng) of dsDNA template
  - X µl (125 ng) of oligonucleotide primer #1
  - X µl (125 ng) of oligonucleotide primer #2
  - 1 µl of dNTP mix
  - ddH2O to a final volume of 50 µl
  - Then add 1 µl of PfuTurbo DNA polymerase (2.5 U/µl)
- If the thermal cycler to be used does not have a hot-top assembly, overlay each reaction with ~30 µl of mineral oil.
- Cycle each reaction using the cycling parameters as outlined in Table I of the Stratagene QuikChange II Site-Directed Mutagenesis Kit manual. We used an annealing temperature of 55C for 1 min and an extension temperature of 68C for 5 min and 18 cycles.
- Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to ≤37°C. If desired, amplification may be checked by electrophoresis of 10 µl of the product on a 1% agarose gel. A band may or may not be visualized at this stage. In either case proceed with Dpn I digestion and transformation.

Dpn I Digestion of the Amplification Products

- Add 1 µl of the Dpn I restriction enzyme (10 U/µl) directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip.
- Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.
- Transform into XL1-Blue Supercompetent Cells and proceed as previously described.

Fluorescence Testing in E. coli – Utah State University – 2010 **http://2010.igem.org/USU_protocol**

- Grow up cells containing GFP generator device overnight in 5 mL LB.
- In the morning, dilute cells 1:10 and measure O.D.600

- Innoculate 25 mL cultures of LB with enough volume from the overnight culture to have an initial O.D. 600 of 0.050
- Every 30 minutes, take sample from the culture and measure O.D. 600 (once O.D. becomes greater than 1.00, dilute sample taken from the culture 1:10 in LB, and multiply diluted O.D. by 10 to get the O.D. of the culture)
- Dilute the sample taken from the culture and dilute 1:100 in dH2O, measure the fluorescence of the GFP by using Spectropfluorophotometer. (blanking with 1:100 dilution of LB in dH2O; excitation/emission wavelengths of cycle-3 mutant GFP: 395/509).

Digestions – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols
Reaction Mix:

- 500 ng – Template
- 5 µl – 10x Buffer
- 1 µl – NEB Enzyme 1 (10 Units/µl) = 10 Units in 50 µl reaction
- 1 µl – NEB Enzyme 2 (10 Units/µl) = 10 Units in 50 µl reaction
- dH2O to 20 µl

TOTAL – 50 µl Procedure:

1. Calculate how much template is needed for digestion. Then calculate how much water needs to be added to make a 50 µl reaction.
2. Pipette appropriate amounts of dH20, template, and 10x Buffer in that order into a PCR tube (200 µl). Mix reagents together by vortexing and then tapping tube on desk to keep reagents on the bottom of the PCR tube.
3. Add appropriate amounts of Enzyme 1 and Enzyme 2. Once added gently swirl around reaction mix with pipette tip.
4. Incubate at 37o C for 1 – 2 hours
5. Run a gel of your digestions on a gel with low temperature melting agarose. Run at 100V for 1 hr. 30 min.
6. Look at gel under 320nm UV light. Conduct gel extraction purification.

NOTE: When digesting your vector it may be useful to use 2 µl of each enzyme in the 50 µl reaction and extending incubation time.

Electroporation (Transformation) – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols
Supplies:

- 100 µl – Electrocompetent Cells
- 2-5 µl – Ligation Reaction (10 – 100 ng DNA)
- 1 ml – SOC broth
- Electrocuvette

Procedure:

1. Gather all your supplies and keep them on ice (or in 4o C room).
2. Thaw frozen electrocompetent cells on ice and place in electrocuvette. Then add ligation reaction and tap the cuvette to mix well. DO NOT pipette up and down.
3. Keep on ice for 1 min.
4. Pulse once (On machine use Ec1 for 1mm cuvettes and Ec2 for 2mm cuvettes).
5. IMMEDIATELY after add 1ml of SOC broth and pipette up and down gently.

6. Incubate recovering cells in 37o C for 1 hr.

Plating

- After incubation, plate 200 µl of transformants on to appropriate selective plate and incubate at 37o C for 18+ hrs.
- If no colonies are formed plate out the rest of the transformants (~800 µl) and incubate at 37o C for 24 hrs.
- Pray it'll work.

Heat Shock Transformation Supplies:

- 50 µl – Chemically Competent Cells
- 2-5 µl – Ligation Reaction (10 – 100 ng DNA)
- 1 ml – SOC broth

Procedure:

1. Gather supplies, be sure to keep cells on ice
2. Thaw competent cells on ice for 5 minutes
3. Add 2-5 µl of ligation reaction to cells, swirl with pipette tip, and keep on ice for 2 minutes
4. Place transformation reaction in a 42*o* C water bath for 45 seconds
5. After heating put reaction on ice for 10 min
6. Add 1 mL of SOC broth and incubate at 37o C for 1 hr
7. Plate

Ligations – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols

Set-up:

1. Run a gel of your digestions and take a picture.
2. Estimate the intensities of your insert and vector where your insert (usually less intense) is considered 1 and your vector is x times as bright.
3. Once intensity is determined perform the following equation:
    - $$Li = 3Lv \frac{Si}{Sv} \frac{Iv}{Ii} \frac{Vi}{Vv}$$
    - L = Ligation volume S = Size of fragments I = Intensity of fragments V = Volume ran down gel i = Insert v = Vector
4. Ligation volume (Lv) for the vector should be approximately 2 µl.
5. The ratio for the ligation in the previous equation is 3:1 insert:vector but can be done 6:1 by replacing the 3 with a 6.

Reaction Mix:

- 2 µl – Vector
- Li µl – Insert
- 2 µl – 10x Buffer
- dH20 to 19 µl
- 1 µl – T4 DNA Ligase

TOTAL – 20 µl Procedure:

1. Add appropriate amounts of dH20, Vector, and Insert in a PCR tube (200 µl). Once added, heat mixture to 65o C for 5 min. This will remove all items that are already annealed to each other causing inefficient ligation.
2. Add then appropriate amounts of 10x Buffer and T4 DNA Ligase. Make sure not to pipette up and down or vortex to mix, this will shear the ligase. Gently swirl with the tip of your pipette to mix.
3. Incubate reaction at room temperature for 30 minutes.
4. After incubation heat inactivate the ligase by another incubation at 80o C for 20 min.

NOTE: The ligase buffer should smell like wet dog. If you smell nothing from the tube, then the buffer is old and useless.

Making Electrocompetent Cells – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols

SOB

2% tryptone
0.5% yeast extract
10 mM NaCl
2.5 mM KCl
10 mM MgCl2
10 mM MgSO4

SOC

SOB + 20 mM glucose
Sterile 10% glycerol (can be autoclaved) is needed for the washes. The volume of 10% glycerol needed is 2X the culture volume (for example, a 500 ml culture requires 1L of 10% glycerol).
Procedure (for 2, 250 ml cultures)

1. Inoculate 1 colony from a fresh plate of the strain to be made electrocompetent into 10 ml of SOB in a 125 ml flask and incubate for 16-18 hours at 37oC and 250 rpm.
2. Have ready 2, 1 L flasks containing 250 ml each of SOB pre-warmed to 37°C. Add two drops of the overnight culture to each of the flasks.
3. Shake at 37°C and 250 rpm until the cultures reach an OD600 of 0.5-0.7. Be sure to turn on centrifuge and cool rotor to 4°C well in advance of harvesting cells. Be sure to place 1 L of 10% glycerol on ice well in advance of harvesting cells
4. Place cultures on ice for 15 minutes. From this point on the cultures must be kept ice cold. Pour each 250 ml culture into chilled 500 ml (or 1000 ml) centrifuge bottles.
5. Centrifuge at 5000 rpm for 10 min. Pour off the supernatant and aspirate any residual broth.
6. Add 250 ml of glycerol to each of the centrifuge bottles and completely suspend the cells by pipetting up and down.
7. Centrifuge at 5000 rpm for 10 min. Pour off the supernatant, it is not necessary to aspirate. Completely suspend the cells as before.
8. Pour off the supernatant and suspend the cells in the residual glycerol by pipetting up and down.
9. At this point you can electroporate or freeze the cells away. To freeze, Add 100 microliters of the culture to microcentrifuge tubes on ice. Once you have used all of the culture transfer the tubes to dry ice for 10 minutes. Once the cultures are frozen transfer them to a -80°C freezer. The cultures should be good for >6 months.

Site Directed Mutagenesis – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols

Procedure modified from Stratagene QuikChange II Protocol

Materials

- PFU Ultra Polymerase (high fidelity)
- 10X Reaction Buffer
- DpnI (20U/μL)
- dNTPs
- Competent Cells

General Overview

1. Design primers
2. Mutant strand synthesis (PCR)
3. DpnI digestion of template
4. Transform and plate

Primer Design Considerations – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-

Illinois/Project/Protocols

- Both primers must contain desired mutation and anneal to same sequence on opposite strands of the plasmid.
- Keep the Primers aruond 25-45 bp long with 10-15 bp on either side of the mutated base.
- Keep the melting temperature greater than or equal to 78°C

Tm=81.5+,41(%GC)-675/N-%mismatch where N is the length of the primer

- Try to keep the primers to a minimum GC content of 40% and end the primer in GC for a clamp.
- Make sure primers are in excess to template

PCR – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols

Ingredient	Amount
10X Buffer	5 µL
Template	20 ng
Primer 1	125 ng
Primer 2	125 ng
10 mM dNTPs	1 µL
Polymerase	1 µL
H2O	Bring to 50 µL

Program:

95°C	3 min
95°C	30 sec
annealing temp	1 min
72°C	1 min/Kb
go to step 2	12X
72°C	1 min
hold at 4°C	

PCR Purification via Promega Kit

DpnI Digestion – UIUC-Illinois – 2010 http://2010.igem.org/Team:UIUC-Illinois/Project/Protocols

DNA	500 ng
Buffer 4	5 µL
BSA	.5 µL
DpnI	1 µL
H2O	Bring to 50 µL

Incubate at 37°C for 1 hour, inactivate at 80°C for 20 min.
Transform via electroporation

AarI Digest – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
1. Label PCR tubes.
2. Add the following reagents into the PCR tubes:
Aar1 Digest Reagents:

5 ug DNA

2.5ul Aar1 Enzyme

0.9ul Aarl oligo

6 ul 10x Aar1 Buffer

x ul dH2O

60 ul total reaction

Note: x ul dH2O may change in volume for different reactions in order to bring total volume up to 60 ul.
3. Briefly vortex and spin down the reaction.
4. Incubate reaction at 37C for 3 hours.
Note: PCR program can be set to 37C for 3 hours and cooled down to 4C indefinitely.

PCR with Phusion enzyme – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
1. Label PCR tubes.
2. Add the following reagents into the PCR tubes (in order):
PCR Reagents

23.7 ul dH2O

10 ul 5x HF Buffer

5 ul forward primer

5 ul reverse primer

5 ul dNTP

0.3 ul template

1 ul Phusion

50 ul total

3. Vortex and spin down the reaction.
4. Set up PCR program.

Cycle Step	Temperature	Time	# Cycles
Initial Denature	98C	3min	1
Denature Annealing Extension	98C 55C 72C	10s 30s 30s	30
Final Extension	72C	5min	1
Hold	4C		

Note: Times may differ for different reactions based on the size of the desired PCR product. Annealing temperature may also differ depending on primer properties.

Ligation – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Ligation Reagents:

50 ng vector

DNA insert(s)

Buffer

1 ul Ligase

Ligation Reaction:
1. Calculate amount of DNA insert and vector needed for reaction.
150 ng / (# bp in backbone/ # bp in insert) = ng needed
Concentration of insert or vector divided by ng needed = volume of DNA needed
2. Calculate amount of buffer needed depending on concentration for volume needed. A typical ligation reaction volume can be 20 ul.
3. Add reagents together, from smallest volumes to largest. Distilled water can be used to bring the volume up so that the reaction has the proper buffer concentration for the reaction. Ligase or enzymes in general should be added at the end.
4. Vortex or pipet up and down to mix. Spin down afterwards if vortexing.
5. Let reaction sit at room temperature for 5 to 20 minutes depending on ligase used. The ligase may start acting up if left too long.

Agarose Gel Electrophoresis – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Gel Electrophoresis Reagents/Materials:

Gel caster

Gel tray

Agar powder

TAE buffer

10, 000X Sybr Safe

50ml conical tube

Gel comb

Microwave

Large flask (microwave safe)

Preparing the agarose solution:
1. Pour in flask the amount of TAE buffer desired. This will be the volume of agarose solution made.
2. Measure out the amount of agar needed for desired concentration. For example to get a 1% agarose gel, you would add 1 gram of agarose powder to 100 ml of TAE buffer. Most concentrations commonly fall between 0.7- 2%. Higher concentrations of agarose are better suited for small fragment separation while lower concentrations are suitable for large fragments.
3. Microwave three minutes, may vary depending on volume in flask. Make sure cap is on loosely.
4. Using hot gloves, swirl bottle gently to ensure complete dissolving of agar. The solution should be clear when agar is completely dissolved. If it is not, microwave for another minute.
5. Place flask of agarose solution in 80C bath to store as to prevent solidifying of agarose solution.

Casting gel:
1. Secure gel tray in caster
2. Pour desired volume of premade agarose solution into 50 ml conical tube. Volume desired will vary depending on gel tray size and desired thickness of gel. Thin gels solidify quicker and can show faint bands clearer, but the wells of thicker gels can hold more DNA.
3. Add Sybr safe to agarose solution. For 10 ml of agarose solution, add 1ul Sybr Safe. The Sybr safe is used to stain and visualize the DNA when viewed under a blue light.
4. Invert tube several times until Sybr safe is equally distributed. Invert gently to avoid creating air bubbles.
5. Pour contents of conical tube into secured gel tray.
6. Place gel comb into notches of gel tray. Check that comb is evenly submerged in agarose. The comb will form wells in the gel to load DNA.
7. Dry at room temperature. The gel will appear opaque when done.

Colony PCR – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Colony PCR reagents:

12ul dH20

4 ul 5x gotaq green buffer

2 ul 10mM dntp

1 ul forward primer

1 ul reverse primer

0.1 ul GoTaq Polymerase

1. After adding reagents to PCR tube, pick a colony and touch the bottom of the PCR tube with the pipette.
2. Place PCR tubes into the PCR machine and set the cycling parameters to be optimal for the piece of DNA being copied.

Transformation of E. coli – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Transformation Reagents

LB Plates

Ice box

Water bath

37C Incubator

Competent cells & Plasmid

Spreading tool (beads)

1. Prewarm LB plates in the 37C incubator
2. Remove desired cells from -80C freezer (or other storage location) and melt over ice for at least 10 minutes. (Make sure to aliquot the correct amount of cells for transformation)
3. Pipette up 5ul of your plasmid DNA and gently pipette into the suspended cells. Do not mix by triterating the solution (pipetting up and down).
4. Let the cells sit on ice for 15-30 minutes.
5. Heat shock your cells for 75 seconds in a 42C water bath.
6. Quickly return your tubes to the ice bucket for 2-3 minutes.
7. When you are ready to plate, remove your prewarmed plate from the incubator. Using sterile technique, carefully pipette your entire tube of cells onto the LB plate.
8. Again using sterile technique, spread the cells across your plate using either sterile beads or another spreading tool.
9. Put the plate in the 37C incubator for 5-15 hours.

Gel Extraction/Purification of DNA – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Gel Extraction/Purification Materials & Reagents:

Sterile Blade

2ml microcentrifuge tube

1.5ml microcentrifuge tube

QIAGEN Gel Extraction Kit

Centrifuge

Heat Block/Bath

1. Using a sterile blade, carefully excise the desired band from your gel.
2. Transfer the agarose chunk into a new 2ml microcentrifuge tube.
3. Weigh the excised agarose chunk and add 3 times its weight in microliters of QG buffer.
4. Move the tube to a melting block for 10 minutes at 50C or until the agarose is completely melted.

5. Add isopropanol to the tube at a 1:3 ratio with QG, then vortex the solution briefly to help precipitate the DNA.
6. Transfer the solution to a Qiagen gel extraction/PCR purification spin column.
7. Spin for 1 minute at 13,000rpm and dump the supernatant.
8. Add 500µl of QG buffer. Centrifuge columns at 13,000 rpm for 1 min and dump or vacuum the flowthrough.
9. Add 750µl of PE Buffer. Centrifuge columns at 13,000 rpm for 1 min and dump or vacuum flowthrough.
NOTE: Wash the rim with the buffer.
10. Repeat Step 9
11. Centrifuge columns at 13,000 rpm for 1 min and dump supernatant. (Skip if you are using a vacuum)
12. Add 62µl of EB Buffer to spin column. Place spin column in a 1.5 ml tube. Centrifuge tune at 13,000 rpm for 1 min. Remove spin column.
13. Place tubes in a -20oC freezer box.

Miniprep – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Miniprep Materials

Qiagen Miniprep Kit

Centrifuge

1. Spin down bacterial culture at 3500 rpm for 5 minutes
2. Aspirate supernatant. Change tips for every sample.
3. Resuspend in 250µl of P1 Buffer. Vortex until cell pellet is not visible. Transfer into a 1.5 ml tube.
4. Add 250µl of P2 Buffer. Invert 5-6 times. Wait 3-5 minutes.
NOTE: When you open the cap, you should see a string of DNA.
5. Add 350µl of N3 Buffer. Invert 5-6 times or until colorless
6. Centrifuge at 13,000 rpm for 10 minutes. Label blue spin columns. Transfer supernatant into blur spin columns.
7. Centrifuge columns at 13,000 rpm for 1 min and dump supernatant or vacuum supernatant.
8. Add 500µl of PB buffer. Centrifuge columns at 13,000 rpm for 1 min and dump supernatant or vacuum supernatant.
9. Add 750µl of PE Buffer. Centrifuge columns at 13,000 rpm for 1 min and dump or vacuum supernatant.
NOTE: Wash the rim with the buffer.
10. Repeat Step 9
11. Centrifuge columns at 13,000 rpm for 1 min and dump supernatant. (Skip if you are using a vacuum)
12. Add 62µl of EB Buffer to spin column. Place spin column in a 1.5 ml tube. Centrifuge tune at 13,000 rpm for 1 min. Remove spin column.
13. Place tubes in a -20oC freezer box.

Maxiprep – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Maxiprep Materials & Reagents

250ml centrifuge tubes

Isopropanol

QIAGEN Maxiprep Stand

QIAGEN Maxiprep Kit

Large Centrifuge

50ml conical tubes

100% Ethanol

1. Pick a single colony and inoculate in 2-5 ml LB medium containing the appropriate antibiotic, Incubate for around 8 hrs at 37C with vigorous shaking.
2. Dilute 1/500 to 1/1000 into selective LB medium. For high copy plasmids, inoculate 100 ml medium with 100-200µl of starter culture. For low copy plasmids, inoculate 250 ml medium with 250-500µl of starter culture. Grow at 37C for 12-16 hrs with vigorous shaking.
3. Spin at 6000 x g for 15 minutes at 4C
4. Resuspend pellet in 10 ml P1 Buffer
5. Add 10 ml P2 Buffer, mix thoroughly, Incubate at room temperature for 5 mins.
6. Add 10 ml chilled P3 Buffer, mix immediately and thoroughly
7. Pour into the barrel of the QIAfilter cartridge. Incubate at room temperature for 10 mins.
8. Remove the cap from the QIAfilter cartridge outlet nozzle, insert the plunger into the QIAfilter Maxi cartridge and filter the cell lysate into a 50 ml tube.
9. Add 2.5 ml ER Buffer, mix by inverting 10 times, Incubate on ice for 30 mins.
10. Equilibrate a QIAgen-Tip 500 by applying 10 ml QBT Buffer and allow the column to empty by gravity flow
11. Apply the filtered lysate from step 9 to the QIAGEN-Tip and allow it to enter the resin by gravity flow.
12. Wash the QIAGEN-Tip with 30 ml QC Buffer, Repeat QC wash
13. Elute DNA with 15 ml QN Buffer
14. Precipitate DNA by adding 10.5 ml room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥ 15,000 x g for 10 mins. Carefully decant supernatant without disturbing the pellet
15. Air-dry the pellet for 5-10 min, redissolve the DNA in a suitable volume of endotoxin-free TE Buffer.

Antibody Staining – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Antibody Staining Reagents:

Transfected and Untransfected cells of the same cell type.

Cells with empty vector or put through electroporation without vector

Dylight 488-conjugated AffiniPure Goat anti-mouse IgG, F(ab')2,
Jackson Immuno Research cat# 115-485-072, Lot# 83241
[should be protected from light].

Pierce Human IgG, whole molecule, ThermoScientific, Cat# 31154,
Lot# LE1311903. (concentration=11mg/ml).

D-PBS CMF (Calcium, Magnesium free PBS).

Wash Buffer: 0.5% BSA, 0.1% Sodium Azide in D-PBS CMF.

FC Block: 1ul Human IgG in 1mL Wash Buffer, prepare fresh for each use.

Propidium Iodide(2mg/ml).

PI solution: 1ul Propidium Iodide in 1mL D-PBS CMF.

Work in an ice bucket (ethanol the bucket)
1. Pipette 1ml wash buffer into 1.5ml microcentrifuge tubes.
2. Add 1x10^5 cells into wash buffer in each tube.
3. Spin for 1 min for NKL and 2 minutes for CD8+ then aspirate. (Don't aspirate the pellet!)
4. Go to step 3, repeat one more time. (Meanwhile, prepare FC Block)
5. Resuspend in 100ul of FC Block. Icubate in ice for 15 min.
6. Add 1ul of Goat anti-F(ab')2 to each tube. Wrap the tube with foil. Incubate in dark for 30 minutes at 4 degrees. For the samples not being labeled, don't add the Goat antib-F(ab')2.
7. Add 1ml wash buffer to the Ab-labeled samples. Repeat step 3, two more times.
8. Resuspend in 250ul of wash buffer.
9. Take samples to the FACS machine.
10. Add 250ul PI solution to the samples the moment before you run the samples.

TOPO cloning – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Zero Blunt TOPO
- Follow manufacturer's protocol

Transfection – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Jurkat Cell Transfection Protocol
1. Cultivate the required number of cells for samples.
2. Prepare DNA for each sample.
3. Pre-warm the supplemented Cell Line Nucleofector® Solution V to room temperature. Either pre-warm an aliquot of culture medium at 37°C in a 50 ml tube (500 µl per sample) or add an additional 500 µl in the step below.
4. Prepare 12-well plates by filling appropriate number of wells with 1 ml of culture medium containing supplements and serum. Pre-incubate plates in a humidified37°C/5%CO2 incubator.
5. Take an aliquot of cell suspension and count the cells to determine the cell density.
6. Centrifuge the required number of cells (1 x 106 cells per nucleofection® sample) at 500xg at room temperature for 5 min. Discard supernatant completely so that noresidual medium covers the cell pellet.
7. Resuspend the pellet in room temperature Cell Line Nucleofector® Solution V to a final concentration of 1 x 106 cells/100 µl. Avoid storing the cell suspension longer than 15 min in Nucleofector® Solution as this reduces cell viability and gene transfer efficiency.
Important: Steps 8 - 13 should be performed for each sample separately.
8. Transfer the nucleofection® sample into an amaxa certified cuvette. Make sure that the sample covers the bottom of the cuvette, avoid air bubbles while pipetting. Close the cuvette with the blue cap.
9. Select the appropriate Nucleofector® program, X-01/X-001 or X-05/X-005 Insert the cuvette into the cuvette holder and press the "X" button to start the program.
10. After the program has finished (display showing "OK") take the cuvette out of the holder and incubate the sample in the cuvette for 10 min at room temperature.
11. If in step 3 you pre-warmed culture medium in a 50 ml tube, then after the 10 min incubation step, add 500 µl of this pre-warmed culture medium to the cuvette and transfer the sample into the prepared 12-well plates. If you added an additional 500µl to each well in step 4, then take 500 µl from the well, add to the cuvette, and transfer the sample back into the prepared 12-well plates.To transfer the cells from the

cuvettes, we strongly recommend using the plastic pipettes provided in the kit to prevent damage and loss of cells.
12. Press the “X” button to reset the Nucleofector®.
13. Repeat steps 8 - 13 for the remaining samples. Cultivation after 15.Incubate cells in a humidified 37°C/5% CO2 incubator. Following nucleofection®,nucleofection® gene expression should be analyzed at different times. Depending on the gene, expression is often detectable after 4 - 8 hours. If this is not the case, the incubation period may be prolonged up to 24 hours.

FACS – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
T-Cell Activation (NFAT-GFP readout by FACS)
Resuspend Jurkat and K562 cells at 1 million live cell/mL in RPMI supplemented with glutamine and 10% FBS (10G RPMI)
Add 100ul of each cell into a 96 well plate
Incubate overnight
Jurkat induction with anti-TCR (anti-CD3 (clone C305))
Resuspend Jurkat at 1 million live cell/mL in FBS supplemented with glutamine and 10% FBS
Add 100ul into a well in 96 well plate
Add 100ul of 2X induction media (2X C305 = 1:1000 dilution of C305 in 10G RPMI)
Incubate overnight
FACS
Imaging

IMAGING PROTOCOL – UCSF – 2010 http://2010.igem.org/Team:UCSF/Protocols
Open up granules and stain with anti-GFP (fixed cells)
Pre-warm all solutions/buffers that need to be warm (RPMI-1640, Myelocult, PBS)
DiI-labeling
1. Count NKLs and spin down 2e6 cells at 400g for 5 minutes
2. Resuspend in 2mL of pre-warmed RPMI-1640 (at 1e6/mL).
3. Add 10ul DiI stain and mix by swirling tube, etc
4. Incubate cells and dye for 10 minutes at 37 C
5. Centrifuge cells for 5 minutes at 400g
6. Aspirate off supernatant
7. Resuspend in 2ml Myelocult and incubate for ~5 minutes at 37C in TC incubator (“recovery”)
Lysotracker Blue labeling
LB1. Spin down cells at 400g for 5 minutes.
LB2. Resuspend at ~1e6/mL in 5uM LysoTracker Blue (diluted into RPMI-1640; note: 1:200 of 1mM stock). Incubate ~30 minutes at 37C in TC incubator.
LB3. Centrifuge cells for 5 minutes at 400g
LB4. Resuspend in (0.5 ml) Myelocult and incubate for ~5 minutes at 37C in TC incubator
Plating cells down on Fibronectin-coated Chambered Coverglass (8-well)
P1. Aspirate myelocult out of each well.
P2. Transfer 0.2mL cells to a fibronectin-coated well incubate at 37C for 15-30 minutes
P3. Check to make sure cells appear to be stuck (on scope)
P4. If not enough cells stuck, add more cells (repeat steps P2-P3).
Fix the cells / permeabilize / stain with anti-GFP Alexa647
F1. Fix in fixative solution (4% formaldehyde in PBS) for 15 minutes at room temperature with gentle agitation in the dark. Remove the solution.
F2. Wash cells twice in PBS for 1 minute each with gentle agitation. Remove PBS.
F3. Permeabilize the specimen with Permeabilization solution (0.25% Triton® X-100 in PBS) for 5 minutes at room temperature with gentle agitation in the dark. Remove the solution.

F4. Wash cells twice in PBS for 1 minute each with gentle agitation. Remove PBS.
F5. Add Blocking solution (5% FBS in PBS pH 7.4). Incubate for 15 min at room temperature with gentle agitation.
F6. ANTIBODY STAINING
-Add anti-GFP Alexa 647. (final conc: 10ug/mL, 1:20 overall dilution)
-Incubate for 0.5 hour at room temperature with gentle agitation.
F7. Decant antibody solution.
F8. Wash cells twice in PBS for 2 minutes each with gentle agitation. After the final wash, add PBS+BSA to the sample.
Prepare for microscopy
10/13 - We've tried 1:20 dilution and will stick with it.

Preparing Electrocompetent E. Coli Cells – **Penn State – 2010**

http://2010.igem.org/Team:Penn_State/Notebook

Day 1

1.Inoculate 25 mL of LB medium with the cell line you want and grow overnight with rotation at 37 degrees Celsius.

2.Autoclave the following:

a.Eight 125 mL flasks containing 50 mL LB or SOB
b.One liter flask containing 500 mL of millipore water
c.One 250 mL flask containing 100 mL 10% glycerol.

Day 2

3.Combine the eight flasks of LB until there are 4 flasks containing 100 mL each.

4.Inoculate each flask with 5 mL of the growing cell culture and grow for three hours with rotation at 37 degrees Celsius.

5.Cool the centrifuge with the correct rotor to 4 degrees

6.At noon pour the 400 ml of culture into eight 50 ml centrifuge tubes.

7.Place the tubes on ice for 30 mins.

8.Centrifuge tubes for 10 mins at 2000g (3500 RPM)

9.Remove supernatant and gently resuspend pellets in 10ml cold sterile water.

10.Centrifuge tubes for 10 mins at 2000g

11.Remove supernatant and gently resuspend pellets in 10 ml cold sterile water.

12.Hold on ice for 30 mins.

13.Centrifuge for 10 mins at 2000g

14.Remove supernatant and gently resuspend pellets in 10 ml cold 10% glycerol.

15.Transfer to 15 ml centrifuge tubes and hold on ice for 30 mins.

16.Centrifuge for 10 mins at 2000g at 4 degrees Celsius.

17.Remove the supernatant and add 500 ul of 10% glycerol.

18.Store tubes on ice while you label 45 microcentrifuge tubes.

19.Aliquot 100 ul per tube and douse the entire tube in liquid nitrogen.

20.Place tubes in the -80 degrees Celsius freezer ASAP!

Electrotransformation of E. Coli – **Penn State – 2010**

http://2010.igem.org/Team:Penn_State/Notebook

1.Chill electroporation cuvettes on ice

2.Prepare the SOC media by adding MgCl2 and Glucose solutions to prepare SOB media and pipette 900 ul into the desired amount of recovery vials

3.Remove # vials containing 100 ul electro-competent cells from the -80 degrees Celsius freezer.

a.Thaw vials on ice

4.Turn on electroporator and set voltage to 1.5 kV

5.Add 5 ul of ligated DNA sample to 100 ul thawed electrocompetent cells on ice.

a.Swirl tip around gently in cells to mix DNA and cells.

6.Place cells back on ice to ensure they remain cold.

7.Pipette 100 ul of cell-DNA mixture to cuvette.

8.Wipe off excess moisture from outside of cuvette.

9.Place cuvette in chamber of electroporator.

10.Pulse the cells by pressing button on electroporator twice.

11.Quickly use a pipette to remove the electroporated cell suspension from the cuvette and add it to the labeled recovery tube.

12.Let cells recover at room temperature for 1-2 hours.

13.Plate 100 ul of electroporated cells onto prewarmed LB-agar plate supplemented with appropriate antibiotic.

a.Incubate plate overnight at 37 degrees

14. Leave remaining SOB-cell mixture on the bench-top overnight.

Ligation – **Penn State – 2010** **http://2010.igem.org/Team:Penn_State/Notebook**

1. Mix together the following:

a. 5 ul deionized water
b. 2 ul of Reaction Buffer
c. 5 ul of digested downstream insert (or water if using only 1 insert)
d. 5 ul of digested downstream insert
e. 3 ul of digested vector
f. 1 ul of DNA ligase

2. Incubate at room-temp for 5 mins

3. Kill reaction for 20 mins at 80 degrees Celsius

DNA Digest – **Penn State – 2010** **http://2010.igem.org/Team:Penn_State/Notebook**

50 ul digest

1. Quickly vortex all ingredients (Buffer, BSA, DNA, Enzymes) before beginning.

2. Add the following in a micro-centrifuge tube:

a.5 ul of Buffer 2
b.1 ul of BSA
c.0.5 picomoles of DNA

d.Water to make 50 ul

3. Vortex Enzymes and add 20 units (1 ul) of each to the tube

4. Incubate reaction in a 37 degrees Celsius water bath for one hour

5. Heat kill the digest for 20 minutes at 80 degrees Celsius.

6. If digesting vector add 1 ul phosphotase and 5 ul of Phosphotase Buffer and incubate an additional 45 minutes.

7. Heat kill enzymes at 80 degrees for 20 minutes.

8. Store digested DNA in the freezer (-20 degrees Celsius).

Plasmid Mini Prep **– Penn State – 2010** **http://2010.igem.org/Team:Penn_State/Notebook**

1.Isolate single colony from a streaked plate and inoculate a culture.

a.Incubate for ~ 12-16 hrs

2.Pellet bacterial cells by centrifugation at 10,000 x g for 1 min

3.Resuspend bacterial pellet by adding 250 ul of Solution I/RNase A solution

4.Add 250 ul of Solution II and mix by inverting tube several times

5.Add 350 ul of Solution III and mix immediately by inverting several times until white precipitate forms.

6.Centrifuge at > 13,000 x g for 10 minutes at room temperature.

7.Prepare HiBind DNA miniprep Column (I) by adding 100 ul of Equilibration Buffer placed in a 2 mL collection tube.

a.Centrifuge for 60 seconds.
b.Discard flow-through liquid

8.Add the cleared supernatant by carefully aspirating it into Column (I) assembled in the 2 mL collection tube.

a.Centrifuge for 1 min

9.Discard flow-through liquid and add 500 ul of Buffer HB to wash the HiBind DNA Miniprep Column (I)

a. Centrifuge for 1 min
b. Discard flow through

10. Discard flow-through liquid and add 700 ul of DNA Wash Buffer diluted with absolute ethanol to wash the HiBind DNA Miniprep Column (I).

a. Centrifuge for 1 min
b. Discard flow through

11. Centrifuge the empty column for 2 min to dry the column matrix. DO NOT skip this step.

12. Place column into clean 1.5 mL microcentrifuge tube. Add 30 to 100 ul of Elution buffer directly onto column matrix.

a. Centrifuge for 1 min to elute DNA

Colony PCR – Penn State – 2010 http://2010.igem.org/Team:Penn_State/Notebook

1. Taq 10X buffer (each tube)

a. 36 ul H2O
b. 5 ul buffer thermopole
c. 1 ul DNTp
d. 1 ul primer 1 (Brk primer)
e. 1 ul primer 2 (Brk primer)

2. Add colonies (another set of tubes)

a. 6 ul H20
b. Colony (pipette up and down)

3. Add 5 ul of colony solution to buffer tube (save the 1 extra ul)

4. Add 1 ul of Taq polymerase to buffer tube

5. Thermocycler – login and use colony PCR protocol

a. Change 72 degrees extension time
  i. Shoot for 0:15 sec longer than necessary
  ii. Based on the # of base pairs, PCR does 1 kb per minute

Agarose gels – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

50 x TAE running buffer 1 l Tris-base 242 g Glacial acetic acid 57.1 ml 0.5 M EDTA 100 ml

Note: To prepare 0.5 M EDTA, dissolve 73 g EDTA in small volume of 1M NaOH, then ad H2O to 500 ml and adjust pH t0 8.0.

Usually make 1 % agarose – 4 g agarose (note: agarose is very expensive) in 400 ml 1x TAE buffer.

Heat in microwave to dissolve agarose (do not boil oer). Store at 60 C for continuous use.

To 30 ml agarose solution (30-40 ml full mini-gel and 15 – 20 ml half mini-gel) ad 1 ul ethidum bromide solution (1 % stock solution stored at 4C, small aliquot covered in aluminum foil stored on bench).

Pour gel and check for bubbles – let solidify for approx. 20 min.

Add approx. 2 ul Stop-mix to 10 ul sample and load 8 ul 1-kb ladder (400 ng --- each single band corresponds to approx. 20 ng DNA) as marker.

Run gel in 1 x TAE buffer at 120 V for 20 min (if current goes over 100 mA, the buffer is old or you have a different buffer in you gel than in the reservoir).

Note: You can pour several gels at once and store them in 1 x TAE buffer plus a drop of ethidium bromide at 4C (for approx. 1 week). For example, you get two gels out of a full mini tray by using two combs and cutting the gel in two pieces.

Attention !!!: Ethidium bromide is a carcinogen – wear gloves and dispose used gels in the corresponding waste.

Attention !!!: The UV-lamp of the photo documentation system is pretty strong – use the protective face shield if you don't want to end up with a bad sunburn.

Turn off monitor of photo documentation system after use.

Antibiotics – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

Stock-solutions (store at –20C)

Ampicillin 100 mg/ml, Chloramphenicol 50 mg/ml (in ethanol), Tetracycline HCl 30 mg/ml, Carbenicillin 100 mg/ml, Kanamycin 30 mg/ml, Spectinomycin 50 mg/ml.

Add 1 ul of antibiotic stock solution to for each 1 ml of medium. As antibiotics degrade quickly, add them to the liquid medium just before doing a cultivation. Allow media (plate) to cool before adding antibiotics (touch bottle without feeling burned). Store plates containing antibiotics at 4C.

Bicinchoninic acid (BCA) protein assay – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

(Pierce instructions: http://www.piercenet.com/Technical/default.cfm?tmpl=/Lib/ViewDoc.cfm&num=0731)

Sensitivity: 5 – 200 ug/ml protein concentration in the sample (not in reaction mix !!!)

Prepare working reagent: Mix 50 parts reagent A and 1 part reagent B

Reaction: 50 ul sample plus 1 ml working reagent Mix and incubate 30 min at 60C, stop by placing on ice (3 reactions per sample !)

Standard protein: BSA (prepare standard concentrations 10, 20, 30, 40, 50, 60, 70, 80, 100 ug/l)

Calibration curve: 50 ul BSA standard solution (10-100 ug/l, samples each concentration) plus 1 ml working reagent Mix and incubate 30 min at 60C, stop by placing on ice

Biological buffer systems – pH ranges

pH range

1.1 – 3.3 glycine/HCl, 1.1 – 4.9 Na-citrate/HCl, 3.6 – 5.6 acetic acid/NaOH (acetate buffer), 4.9 – 8.0 KH_2PO_4/Na_2HPO_4 (Soerensen buffer), 5.0 – 6.6 Na-citrate/NaOH, 5.8 – 8.0 KH_2PO_4/NaOH, 5.8 – 8.0 Na_2HPO_4/NaH_2PO_4 (isotonic), 7.2 – 9.0 Tris/HCl, 8.55 – 12.9 glycine/NaOH.

Dephosphorylation – CIP treatment – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

• To avoid religation of a vector backbone it is necessary to treat the cut plasmid with calf intestinal alkaline phoshpatase (CIP) to remove 5'-phosphate groups. As only the vector backbone lacks the 5'-phosphate groups, the 5'-phosphate groups provided by the insert are sufficient for the ligase to carry out the ligation of the two DNA strands (the remaining two nicks are later repaired in E. coli).

• Note: Never treat both insert and vector backbone with CIP.

• CIP treatment becomes necessary, if you want to ligate an insert into a single restriction site. For example, an EcoRI digested insert into a likewise EcoRI digested plasmid. Although you could theoretically discriminate on an agarose gel between cut and non-cut vector and isolate the band corresponding to the cut vector from the gel, in most cases you get some cross-contamination from uncut plasmid (which can run in different supercoiled forms and apparent sizes) in your gel-extract. Since the transformation efficiency of uncut plasmid is much higher than your ligation products, you will get a huge background of plasmids without inserts.

• For CIP treatment:

Add 1-2 ul CIP to the digestion mixture and incubate together with the restriction enzymes at 37C.

See NEB catalog and web-page: http://www.neb.com/neb/frame_cat.html http://www.neb.com/neb/frame_tech.html

DNA-Sequencing – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

ABI dye terminator cycle sequencing – preparation of sequencing reaction for BMGC DNA-Sequencing Facility (U of M):

3-4 ul mini-prep plasmid DNA (1-2 ug DNA required) plus 4 ul sequencing primer (1 pmol/ul) (3.2 pmol required). Add ddH_2O to total volume of 12 ul

Extraction of DNA from agarose gels – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

A preparative agarose gel to separate DNA fragements (e.g. digestion products, PCR products) from other DNA fragments is often required. Load entire DNA containing solution (containing 20% Stop-mix) on a preparative of a gel (use preparative combs) and run gel. Carefully cut out the desired DNA band (do not contaminate with other DNA bands) using a clean scalpel and transfer into a 2 ml tube.

For extraction use one of the following Qiagen Kits:

Qiagen Qiaquick Gel Extraction Kit: (recommended for most applications)

Note: Fragments < 100 bps can not be isolated. Note: Use Qiagen EB buffer pH 8.5 for elution.

>>>> Refer to the Qiaquick Spin Handbook for instructions.

Qiagen Qiaex II Gel Extraction Kit (use for DNA-shuffling)

Note: Fragments <100 bps can be isolated – this kit must be used for the extraction of 50 bps during DNA-shuffling. Note: Use Qiagen EB buffer pH 8.5 for elution.

Additional information can be found in the "Qiagen DNA cleanup and gel extraction handbook" at http://www.qiagen.com/literature/cleanlit.asp#qiaexii)

Flow Cytometry – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

• Inoculate single colony of freshly transformed DH5αPro or TOP10 cells in 4 ml LB medium containing 50μg/ml chloramphenicol (or appropriate antibiotic).

• Grow the culture overnight at 37 C with shaking (250 rpm).

• Next day re-inoculate the cultures into 4 ml fresh LB medium having antibiotics and varying inducer concentrations. Inducer concentrations can be varied from 0-1mM of IPTG or 0-200 ng/ml aTc.

• Collect the samples at different time intervals of 3, 6 and 9 hours.

• Monitor the growth rate by measuring optical density at 600 nm.

• Measure the fluorescence in a Becton Dickinson FACS Calibur flow cytometer equipped with a 488 nm argon laser and a 515-545 nm emission filter (FL-1) and a 585-610 nm emission filter (FL-2).

• Make sure that machine has settings for E. coli.

• To measure the fluorescence, add 3-5 µl of the growing culture in ~1 ml PBS (phosphate buffer saline, pH-7.5). Measurement should be done at low flow rate (~1000 events/second).

• For each sample, collect 50,000 events.

• Analyze the fluorescence in both FL-1 and FL-2 channel using FlowJo software (BD Biosciences).

• Determine the background fluorescence by using controls (cells having empty plasmid vector).

Glycerol cultures – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

Add to 2 ml of a mid-log or 1 ml of a fresh stationary phase culture 1 ml sterile glycerol solution (65 % (v/v) glycerol, 0.1 M $MgSO_4$, 25 mM Tris/HCl pH 8.0). Mix and store at –80C. To inoculate from a glycerol culture – scrape some ice from the top of the culture – never allow the culture to thaw (freezing and thawing destroys cells).

Ligase Free Cloning – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

1. Plasmid preparation: Cut the plasmid/vector with appropriate enzymes (for cloning into pUCBB-GFP, digest it with BglII and NotI). Gel purify the vector fragment. Measure the vector concentration by nano-drop (you need 100ng vector for 1 cloning reaction)

2. Insert preparation: Each primer should have the following: • 15-20 bp overlap with the gene of interest • 15-20 bp overlap with the linearized plasmid including restriction site • Primers for CSD lab biobrick vectors should add the following to the gene specific sequence: - BB-BglII-F 5'-AGAAGGAGGAGATCT---Start - BB-NotI-R 5'-GATGCTCGAGGCGGCCGC---End (reverse complement) i.e., …TAAGCGGCCGCCTCGAGCATC-3' is added to the end of the gene sequence,. PCR (2 or 4 x 50µl) for the insert using Vent following the usual protocol. Gel purify the insert. Measure the insert concentration by nano-drop (you need 100ng insert for 1 cloning reaction).

3. Transformation of competent E. coli If necessary, use speed-vac to concentrate your vector/insert, so that you can have 100ng vector + 100ng DNA in about 5-25 µl total volume. 1 tube of chemically competent E.coli + 100ng vector + 100ng insert. Incubate on ice, 20 minutes. Heat shock 35-40s. Add 800µl SOC, incubate at 37°C for 1 hour with shaking (recovery). Spin down the cells: 4000rpm, 5 minutes. Remove approximately 900µl SOC, so the volume of the cell pellet + remaining SOC is less than 120µl. Resuspend the cells in the remaining SOC, plate the cells on an appropriate antibiotic plate. Invert the plate & incubate at 37°C overnight.

Ligation – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

(with NEB-ligase) Typical reaction volume either 10 or 20 ul:

For example: 20 – 50 ng cut vector (also called backbone) preparation 50 – 200 ng cut insert preparation 1 ul 10 x ligase buffer (note: Gibco buffer is 5 x fold) 0.5 ul ligase ad. H2O to 10 ul total volume

Incubate 1 h at RT (or overnight at 4 C, although there will rarely better results than at RT) and transform complete mixture.

Note: Thaw ligation buffer completely – no precipitate visible – and mix thoroughly before using. Ligase buffer must be aliquoted in 10-20 ul portions and these frozen aliquots are then used for ligation. Repeatedly thawing and freezing of ligation buffer kills the ATP in the buffer.

Pipette ligase carefully – no excess drops on the tip to ensure accurate volume. Never vortex reactions containing DNA modifying enzymes – always mix by aspirating with the pippetor.

Sticky-end ligation: Use a 1:2-3 ratio of vector:insert. Blunt-end ligation: Use a 1:1 ratio of vector:insert.

Always check DNA-concentrations of vector on insert on the same gel prior to ligation. (You can compare the intensity of your DNA bands with that of the bands of the 1-kb ladder: if you load 8 ul kb-ladder (400 ng) then each band corresponds to approx. 20 ng)

Too high vector and insert concentrations do not result in good ligation efficiencies – it is better to try different concentrations of vector and insert.

Be sure that both vector and insert preparation are pure – e.g. by purifying with the Promega Wizzard Kit or Qiagen gel extraction kit.

Additional tips and protocols are found on NEB's web-page: http://www.neb.com/neb/frame_cat.html http://www.neb.com/neb/frame_tech.html

Loading buffer for agarose gels (Stop-mix) – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

For 100 ml loading buffer:

4 M Urea 24.02 g, 10 mM EDTA 372 mg, 50% glycerol 50 ml, 0.1 % bromphenol blue 0.1 g. Add H2O to 100ml

Note: For separation of fragments < 100 bp use Stop-Mix without dye.

Media – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

LB (low-salt Luria Bertani) (standard E. coli medium)

tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l (low-salt medium).

Pouring plates

For 1L LB agar, add 15g agar to the above. Let the media cool until you can touch the bottle (flask) without feeling burned. Don't cool too much or the agar will solidify. Add the appropriate antibiotic. Pour plates so that bottom of the plate is covered (25-35ml). Flame media briefly to get rid of bubbles, but not too long otherwise antibiotic gets killed and plastic melts. Let the plates cool before refrigerating to avoid condensation inside the plate. Store plates upside down so that no liquid drops on the media – write any information on the bottom of the plate so that losing the top of the plate will not mess up your experiment.

Microscopy – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

Preparations were viewed using a Nikon Eclipse E800 photomicroscope equipped with brightfield, DIC, phase and fluorescence optics including a 120 W X-Cite epi-fluorescence illuminator with UV (excitation filter 330-380, barrier 420 nm), blue (excitation filter 470-490 nm, barrier 520-580 nm) and green (excitation filter 510- 560 nm, barrier 570- 620 nm) filter sets. The samples were viewed using either a 4X, 0.13 n.a. plan fluor; 10X, 0.30 plan fluor; 20X, 0.50 n.a. plan fluor; 40X, 0.75 n.a. plan fluor; 60X, 1.40 n.a. plan apo; or100X, 1.3 n.a. plan apo objectives.16-bit digital images were collected using a Roper CoolSnap HQ monochrome camera and captured to a Pentium IV 2.6 GHz personal computer using Image Pro Plus AMS version 6.3 software (Media Cybernetics, Silver Springs, MD 20910).X, Y and Z-motor movements, filters and shutters were managed using a Ludl MAC 3000 controller (Ludl Electronic Products Ltd., Hawthorne, NY 10532) interfaced to the ImagePro software.The instrument is housed and maintained by the University of Minnesota - College of Biological Sciences, Imaging Center, http://www.cbs.umn.edu/ic/.

PCR reaction – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

1. Typical reaction mixture (100ul) :

10 x PCR buffer (w/o added MgCl2) 10 ul, 25 mM MgCl2 a) 8 ul (final concentration 2 mM), dNTP Mix (2.5 mM each dNTP) 8 ul (final concentration 200uM of each dNTP), Primer 1 (100 pmol/ul) 1 ul (100 pmol), Primer 2 (100 pmol/ul) 1 ul (100 pmol), Template DNA b) 1-10 ul (10 –200 ng),. Taq 0.5 ul (2.5 U)

2. Sometimes different MgCl2 concentration (1 – 5 mM) can increase PCR yield. I recommend, however, to optimize other parameters first, e.g. annealing temperature, as suboptimal MgCl2 concentrations increase the error-rate of Taq

polymerase. 3. Don´t use too little template, as early errors introduced by the Taq polymerase quickly amplify to significant error-rates.

Cycle conditions:

95 C 2 min (initial denaturation step) 1x Followed by 25-32 cycles of

95 C 1 min (denaturation), 55 C 1 min (annealing), 72 C 1 min per 1.5 kb template (extension).

72 C 7 min (final extension step)

Depending on the PCR reaction, denaturation and annealing temperature and times are different. Denaturation times can be shorter in case of low-melting temperature, but maybe longer for PCR with genomic DNA templates. The annealing temperature depends on the melting temperature of the primer (note: calculate melting temperature only from the annealing part of the primer, not extensions that provide e.g. restriction enzyme recognition sequences). Usually, a annealing temperature 5C below the calculated melting temperature works well.

Note: Use 200 ul thin-wall tubes, place the tubes into the corresponding smaller spaces of the heating block of the Eppendorf Mastercycler.

PCR reaction can also be carried out in smaller volumes (e.g. 50 ul), this can sometimes be beneficial for the reaction performance.

Refer to the instruction manual of the Eppendorf Mastercycler for setting up a gradient, cycle extension or modified ramp times.

For difficult PCR reactions, addition of 2 – 20 % (v/v) DMSO (molecular biology grade) to the reaction mixture can help to obtain better PCR yields (try 2, 5 and 10% first). If this does not help, performing the PCR reactions with the Q-solution of the Qiagen PCR Kit might help (or using all components of the kit – although addition of Q-solution to a standard reaction works just as well).

dNTP-Mix: 2.5 mM each nucleotide

10 ul dATP (100 mM stock solution) 10 ul dCTP (100 mM stock solution) 10 ul dGTP (100 mM stock solution) 10 ul dTTP (100 mM stock solution) 360 ul H_2O

aliquot 100 ul portions and store at –20C

For use: thaw carefully and keep on ice, freeze immediately after use.

Oligonucleotides

Always a 100 pmol/ul stock solution is prepared by dissolving the freeze dried pellet in H_2O. Oligo-concentrations are found on the manufacture reference data sheet for each oligo.

Remember: dNTP's and Taq polymerase are very expensive – use them with consideration.

A comprehensive list of tips and hints for PCR are found in the Qiagen handbook "Critical factors for successful PCR" available athttp://www.qiagen.com/literature/brochures/pcr/index.html.

Primer design:

- 15 bps minimum length of annealing primer part – Primer needs to be 5-8 bps longer if annealing part is very AT rich in order to increase melting temperature - try to design primer with a 50% GC content - primer should contain at least one G or C at its 3'prime end to ensure optimal annealing where the polymerase starts DNA-extension.

Plasmid preparation – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

Purified plasmid preparations are prepared from overnight cultures of E. coli cells harboring the plasmid of interest (do not forget to add the appropriate antibiotic) using Qiagen Kits.

Small-scale plasmid preparations (up to 20 ug of high-copy plasmid DNA) are done with the Qiaprep Miniprep Kit from 1-5 ml overnight cultures. • This scale is sufficient for most applications.

>>>> Refer to the Qiaprep Miniprep Handbook for instructions.

Medium-scale plasmid preparations (up to 100 ug of high-copy plasmid DNA) are done with the Qiaprep Miniprep Kit from 50 ml overnight cultures. • Note, that the obtained plasmid DNA may contain more salt impurities than the Minipreparation and my need additional purification with the Promega Wizard Kit for efficient digestion with some enzymes.

>>>> Refer to the Qiagen Plasmid Purification Handbook for instructions.

Qiagen handbook plasmid purification and other information can be downloaded at: http://www.qiagen.com/literature/plklit.asp

Purification of PCR products and DNA – TSS method – Minnesota – 2010 **http://2010.igem.org/Team:Minnesota/Protocols**

DNA (plasmids, digestion reaction mixtures, PCR reactions etc.) can be purified and concentrated with the:

Promega Wizard PCR and DNA prep purification Kit:

Note: Use 3 ccc luer lock syringes to apply resin and wash buffer to the columns.

Important: Use 10 mM Tris/HCl buffer pH 7.5 for elution (do not use the Qiagen EB buffer which has a higher pH of 8.5)

Note: PCR products need to be purified with this kit prior to DNase digestion.

>>>> Refer to the manual for instructions. Additional information can be found at: http://www.promega.com/tbs/tb118/tb118.html

Restriction enzyme digestion – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

Most of the enzymes used in the lab are purchased from NEB. The NEB-catalog and the NEB homepage provide you with reaction buffer charts and lots of other information on restriction enzymes, that will give you some ideas as to why your digestion might not work--- see http://www.neb.com/neb/frame_tech.html andhttp://www.neb.com/neb/frame_cat.html.

Single enzyme digestion:

For example:

10 ul plasmid DNA (mini-prep, typically 500 ng/ul plasmid DNA) 3 ul 10 x reaction buffer (check for enzyme specific buffer) 1 ul restriction enzyme (10-20 Units/ul) ad. H2O to 30 ul

incubate for 1-2 hrs at 37C (!!!! some enzymes require different reaction temperatures, e.g. SmaI 25C etc.) and check result on agarose gel.

Double enzyme digestion:

For example:

10 ul plasmid DNA (mini-prep, typically 500 ng/ul plasmid DNA) 3 ul 10 x reaction buffer * 1 ul restriction enzyme I (10-20 Units/ul) 1 ul restriction enzyme II (10-20 Units/ul) ad. H2O to 30 ul

incubate for 1-2 hrs at 37C (!!!! some enzymes require different reaction temperatures, e.g. SmaI 25C etc.) and check result on agarose gel.

• Chose a 10 x reaction buffer in which both enzymes work – simultaneous digestions may not be possible for all enzyme combinations (Then you have to purify reactions (e.g. using Promega Wizzard) before submitting to a second digestion)

• Tip: Some enzymes do not cut well if less than 10 bps extend beyond their recognition sequence. This can happen when simultaneously digesting with a second enzyme (especially, if this enzyme is more active) cutting near that recognitions sequence (for example in a multiple cloning site). You can look up the cleavage reactivity of enzymes close to the end of DNA-fragments in the NEB-catalog or at (http://www.neb.com/neb/frame_tech.html).

o To increase your digestion efficiency you should first add the less efficient enzyme to the digestion mixture and incubate for 1 hr and check a small aliquot of the digestion mixture on a agarose gel. If the plasmid is efficiently cut, add the less critical enzyme to the mixture and incubate for another hr. ---- By doing so (if cutting a plasmid), you can at least be sure that the less efficient enzyme has efficiently cut (you won’t be able to see whether or not the second enzyme did cut).

When designing oligo nucleotides for cloning experiments keep in mind that many enzymes need their recognition sequence at least 4 bps from the DNA-end for efficient cutting (see appendix of NEB catalog or http://www.neb.com/neb/frame_tech.html)

SDS gel electrophoresis – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

• Refer to Mini-Protean III Cell Instruction Manual for assembly of electrophoresis cell and running SDS-PAGE using the Laemmli Buffer System (preferably 12% gel).

• Stock solutions for running gel buffer, stacking gel buffer, 30% acrylamide/bis, TEMED solution are kept at 4C, 10% APS and 10% SDS solutions are kept at –20C.

• SDS-Sample Loading buffer:

10 ml glycerol 5 ml mercaptoethanol 3 g SDS 2.5 ml 0.05 % bromphenol solution (filtered) 12.5 ml stacking gel buffer

(store in aliquots at –20C)

Sample preparation:

For example, 10 ul protein sample + 90 ul sample buffer denature 5 min at 95C and centrifuge load 5-20ul on gel

For protein expression rate analysis of complete cells, • centrifuge 1 ml E. coli culture and wash cells with 50 mM Tris/HCl buffer pH 7.5 • dissolve cells in 200 ul sample buffer and denature 10 min 95C • centrifuge cells and load 5-20 ul cleared supernatant onto gel • if high DNA concentrations make sample to viscose, dissolve cells of 1 ml culture in 100 ul 50 mM Tris/HCl buffer pH 7.5 and disrupt cells by repeatedly thawing and freezing, centrifuge and add 2 ul (1:10 diluted DNAse in5 0 mM Tris/HCl buffer pH 7.5) to cleared supernatant and incubate 20 min 37C to degrade DNA, add 200 ul sample buffer and proceed as described above.

Staining: Use ready to use Coomassie Stain according to instructions – destain in water.

Dry gels according to BioRad's instructions using the gel drying frame and drying solution.

Transformation of E. coli – TSS method – Minnesota – 2010
http://2010.igem.org/Team:Minnesota/Protocols

TSS solution:

100 ml PEG 4000 15 g DMSO 5 ml (add after autoclaving) 1 M MgCl2-solution 5 ml LB add to 95 ml

Adjust pH to 6.5 prior to autoclaving.

After addition of DMSO aliquot TSS solution in 10 – 15 ml portions and store at –20C (TSS can get contaminated very quick).

Preparation of competent E. coli cells:

• Cultivate overnight E. coli culture* (*LB, add appropriate antibiotics if competent cells containing a plasmid for co-transformation are required) to inoculate main culture* 1:100 with overnight culture. Note: 50 ml culture will give 10 aliquots of competent cells Use larger culture volumes (e.g. 100 ml) to prepare more aliquots.

• Grow main culture at 37C and 260 rpm to ensure rapid growth to OD 0.4 – 0.6 (typically 2 – 3 hrs, fast growing cells to OD 0.4 reach highest transformation efficiencies) • Centrifuge cells for 10 min at 4000 rpm (4C) • Carefully resuspend cell pellet in cold (4C) TSS solution (2 ml TSS for each 50 ml culture volume). • Incubate resuspended cells for 5 min on ice and aliquot 200 ul competent cells in 15 ml sterile tubes. o Note: Handle cells carefully and keep them always on ice as they get very fragile during the TSS treatment. • Shock-freeze aliquoted cells in liquid nitrogen and store cells at –80C.

Transformation of TSS competent cells:

• Quickly thaw frozen competent cells at RT and place on ice. • Add 1 ul – 20 ul DNA solution (0.5 ul plasmid prep, 10 or 20 ul ligation mixture) to a 200 ul aliquote competent cells and mix carefully. • Incubate 20 min on ice. • Heat shock cells for 35 s in a water bath at 45C – Immediately add 800 ul LB (without antibiotics) and place on ice. • Incubate transformation mixture for 1 h at 37C to allow recovery. • Plate 10 ul (plasmid transformation) of mixture on LB plate. In case of ligation mixture, briefly spin down cells and decant most of the supernatant. Carefully resuspend cells in remaining liquid and plate on 4-6 plates.

Test-transformation:

• For checking transformation efficiency of competent cells:

Transform 10 ng of plasmid DNA (1 ul of pUC19 reference plasmid prepared by diluting 1:50 500 ug/ml pUC19 from Pharmacia) and plate 50 ul of 1 ml transformation mixture on LB plate:

Obtained colony number x 2 x 104 = transformation efficiency/ug DNA

• Transformation efficiency for E. coli cells should be > 106 to ensure good cloning results.

Preparation of Electrocompetent cells – Georgia State – 2010 http://2010.igem.org/Team:Georgia_State/Protocols

1. Inoculate 500ml of L-broth with 1/100 volume of a fresh overnight E.coli culture

2. Grow the cells at 37°C on shaker to an OD600.(dilute culture if desired OD is exceeded.

a. Best results are obtained with cells harvested at early to mid log phase so the desired OD may vary based on specific strain growth conditions

3. Chill cells on Ice for approximately 20 minutes. Keep cells on Ice for all subsequent steps in procedure and pre-chill all tubes before adding cells. If possible, centrifuge at 4°C.

4. Transfer cells to chilled 50 ml Falcon tubes and centrifuge at 4000x g for 15 minutes

5. Pour off and discard supernatant. Resuspend pellet in 50ml of ice-cold 10% glycerol. Centrifuge at 4000 x g for 15 min. Pour off and discard supernatant.

6. Resuspend pellet in 25 ml of ice-cold 10% glycerol. Centrifuge at 4000 x g for 15 min. Pour off and discard supernatant.

7. Resuspend Pellet in 20 ml of ice-cold 10% glycerol. Centrifuge at 4000 x g for 15 min. Pour off and discard supernatant.

8. Resuspend cell pellet in a final volume of 2ml of ice-cold 10% glycerol. Cell concentration should be about 1-3 x 1010 cells/ml

9. Make aliquots of 100µl and store at -80°C.

Preparation of Heat Shock Competent Cells – Georgia State – 2010 http://2010.igem.org/Team:Georgia_State/Protocols

Preparation of Seed Stock 1. Streak TOP 10 cells on an SOB plate and grow for single colonies at room temperature 2. Pick single colonies into 2mL of SOB medium and shake overnight at room temperature 3. Add 15% glycerol 4. Aliquot into 1mL samples 5. Place in -80°C Preparing competent cells 1. Prechill 2mL centrifuge tubes 2. Inoculate 250mL of SOB medium with 1mL vial of seed stock and grow at 20°C to an OD600nm of 0.3 3. Aim for a lower OD not higher if possible for 16 hours at room temperature should work 4. Centrifuge at 3000g at 4°C for 10 minutes 5. Pellets should be resuspended in 80mL of ice cold ccMB80 buffer 6. Incubate on ice for 20 minutes 7. Centrifuge again at 4°C and resuspend in 10mL of ice cold ccMB80 buffer 8. Test the OD of a mixture of 200uL of SOC and 50uL of the resuspended cells 9. Add chilled ccMB80 to yield a final OD of 1.0 – 1.5 10. Incubate on ice for twenty minutes 11. Aliquot into chilled 2mL chilled centrifuge tubes 12. Store at – 80°C

Transformation of DNA using Electroporation – Georgia State – 2010 http://2010.igem.org/Team:Georgia_State/Protocols

1. Thaw DNA and cells 2. Add 40uL of cells in a fresh microcentrifuge tube along with 1uL of DNA 3. After gently mixing transfer the contents of the microcentrifuge tube into an electroporation cuvette 4. Have the micropulsor set to Eco1 5. Hit the pulse button and hold it until you hear it beep 6. Immediately add 1mL of SOC broth to the electroporated cells 7. Transfer the contents of the cuvette to a micro centrifuge tube and inoculate the tubes at 37°C for an hour 8. Plate 20uL and 100uL on selective media plates 9. Also plate 100uL on regular media plates as a control to test the viability of the shocked cells 10. Inoculate these plates overnight for 14-16hrs at 37°C

Transformation Using Heat Shock – Georgia State – 2010 http://2010.igem.org/Team:Georgia_State/Protocols

1. Thaw the cells and DNA on ice 2. In a microcentrifuge tube at 50uL of competent cells with 2uL of DNA 3. Let it sit on ice for 30minutes 4. Heat shock at 42°C for 60seconds 5. Let it sit on ice for 5minutes 6. Add 200uL of SOC broth 7. Incubate at 37°C for 2hours 8. Plate 20uL and 100uL on Selective media 9. Plate 100uL on normal media as a control to test viability of the heat shock cells

Preparing 1% Agarose Gels: – Georgia Tech – 2010
http://2010.igem.org/Team:GeorgiaTech/Protocols

1. Added 180 mL 1x TBE and 1.8g agarose
2. Heated up until agarose was no longer visible
3. Let cool (approx. 10 min)
4. Added 180 uL Ethidium bromide (1000X)

PCR Purification – Georgia Tech – 2010 http://2010.igem.org/Team:GeorgiaTech/Protocols

1. Added 5 volumes of Buffer PBI to 1 volume of the PCR sample and mix (in a clean 1.5 mL eppendorf)
2. Checked that the color of the mixture was yellow (similar to Buffer PBI without the PCR sample).
3. To bind DNA, transferred the sample to the column and centrifuge at 17,900g for 30 – 60 secs.
4. Discarded flow-through. Placed the column back in the same tube.
5. To wash, added 0.75 mL Buffer PE to the column and centrifuged for 30 – 60 secs.
6. Discarded flow-through and place the column back in the same tube. Centrifuged the column for an additional 1 min.
7. Placed the column in a clean 1.5 mL microcentrifuge tube.
8. To elute DNA, 50 µL autoclaved milliQ water to the center of the membrane, let the column sit for 1 min, and then centrifuged.

Heat shock transformation of the plasmids into our bacteria – Georgia Tech – 2010 http://2010.igem.org/Team:GeorgiaTech/Protocols

1. Left cells and ligation reaction products on ice.
2. Added plasmid (5uL) to cells. Mix gently by swirling pipette tip in mixture (DO NOT ASPIRATE).
3. Left cells on ice for 30 min.
4. Applied heat shock of 45 seconds in 42C bath.
5. Put tubes on ice for 2 min.
6. Added 250 uL of LB (room temp.)
7. Incubated 1 hour at 37 C
8. Plated 100uL and left plate in the 37 degrees incubator
9. Incubated overnight at 37C.

Gel Extraction – Georgia Tech – 2010 http://2010.igem.org/Team:GeorgiaTech/Protocols

1. Excise DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg or approximately 100 µL).
3. Incubate at 50ºC for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2 – 3 min during the incubation.
4. After the gel slice has completely dissolved, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 mL collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Recommended: Add 0.5 mL of Buffer GQ to QIAquick column and centrifuge for 1 min.
10. To wash, add 0.75 mL of Buffer PE to QIAquick column and centrifuge for 1 min.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).
12. Place QIAQuick column into a clean 1.5 mL microcentrifuge tube.
13. To elute DNA, add 50 µL of Buffer EB (1 mM Tris-Cl, pH 8.5) or water (pH 7.0 – 8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.
14. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Plasmid DNA Purification with Mini Prep Kit – Georgia Tech – 2010
http://2010.igem.org/Team:GeorgiaTech/Protocols

1. Resuspend pelleted bacterial cells in 250 µL Buffer P1 and transfer to a microcentrifuge tube.
2. Add 250 µL Buffer P2 and mix thoroughly by inverting the tube 4 - 6 times.
3. Add 350 µL Buffer N3 and mix immediately and thoroughly by inverting the tube 4 – 6 times.
4. Centrifuge for 10 mins at 13,000 rpm (~17,900 xg) in a table-top microcentrifuge.
5. Apply the supernatants from step 4 to the spin column by decanting or pipetting.
6. Centrifuge for 30 – 60 secs. Discard the flow-through.
7. Recommended: Wash the spin column by adding 0.5 mL Buffer PB and centrifuging for 30 – 60 secs. Discard the flow-through.
8. Wash spin column by adding 0.75 mL Buffer PE and centrifuging for 30 – 60 secs.
9. Discard the flow-through, and centrifuge for an additional 2 min to remove residual wash buffer.
10. Place the column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 30 µL autoclaved water to the center of each spin column, let stand for 1 min, and centrifuge for 1 min.

Plasmid Prep - Gaston Day – 2010

http://2010.igem.org/Team:Gaston_Day/Notebook

SOLUTIONS: TENS (make fresh each time) To 4.5 ml TE add: 250 ul 10% SDS 250 ul 2N NaOH Sodium Acetate 3.0M; pH 5.5 TE with 10 ug/ml of RNAse A

PROCEDURE: 1. Spin 1.5 ml of overnight culture for 30 sec in microfuge

2. Aspirate off all but 100 ul of the supernatant and resuspend the pellet by vortexting

3. Add 300 ul of TENS and mix by inversion. The solution should become viscous.

4. Add 150 ul of sodium acetate and votex. A fine white precipitate should form.

5. Centrifuge for 2.5 minutes at 10K.

6. TRANSFER the supernatant to a clean tube and add 2 volumes (1 ml) of room temperature EtOH.

7. Vortex and pellet DNA by centrifugation for 2-5 minutes at 10K.

8. Wash pellet with 70% ethanol and allow the pellet to dry.

9. Resuspend the pellet in TE with RNAseA.

10. Digest 5-10 ul as usual.

Digest Ligation - Gaston Day – 2010

http://2010.igem.org/Team:Gaston_Day/Notebook

1. Remove the 10X T4 DNA Ligase Reaction Buffer* from the freezer to thaw. You can remove the T4 DNA Ligase enzyme from the freezer at this point but leave the ligase in a cold box to keep it close to -20○ C. Thawing is fast if the buffer tube is immersed in room temperature water. Once thawed, agitate the 10X T4 DNA Ligase Reaction Buffer until all precipitate goes into solution.

2. Add 11ul of H2O to a 200ul PCR tube.

3. Add 2ul from each of the digest to the tube.**

4. Add 2ul of 10X T4 DNA Ligase Reaction Buffer to the tube.

5. Add 1ul of the T4 DNA Ligase to the tube.

6. The total volume in each tube should now be 20ul. Ensue the ligation is well mixed by flicking the tube. You can spin the tube in a microcentrifuge for a few seconds to collect the liquid in the bottom of the tube again.

***Repeated freeze-thaw cycles of the buffer can degrade the ATP in the buffer thereby making the ligation reaction less efficient. It is wise to aliquot the buffer into 10ul aliquots prior to freezing**

****There is no need to purify the restriction digests via gel electrophoresis in any other method.**

Transformation - Gaston Day – 2010 http://2010.igem.org/Team:Gaston_Day/Notebook

1. Thaw tube of NEB 10-beta Competent E. coli cells on ice for 10 minutes.

2. Add 1-5ul containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. **DO NOT VORTEX.**

3. Place the mixture on ice for 30 minutes. Do not mix.

4. Heat shock at exactly 42○ C for exactly 30 seconds. Do not mix.

5. Place on ice for 5 minutes. Do not mix.

6. Pipette 950ul of room temperature SOC into the mixture.

7. Place at 37○ C for 60 minutes. Shave vigorously (250 rpm) or rotate.

8. Warm selection plates to 37○ C.

9. Mix the cells thoroughly by flicking the tube and inverting then perform several 10-fol serial dilutions in SOC.

10. Spread 50-100ul of each dilution onto a selection plate and incubate overnight at 37○ C for 24-36 hours of 25○ C for 48 hours.

CPEC Cloning – Duke – 2010 http://2010.igem.org/Team:Duke/Project/Protocols

Materials

- Phusion™ High-Fidelity PCR Kit (FINNZYMES, Cat. No. F-553)
- Thermocycler

Preparation

```
5x Phusion HF Buffer    4 ul
10 mM dNTPs             0.4 ul
Vector                  50 ng/1kb
Insert                  x ng*
Phusion DNA Polymerase  0.2 ul
-------------------------------
H2O                     to 20 ul
```

*The amount of insert is determined so that the molar ratio for vector and insert is 1 to 2.

Procedures

```
98°C             30sec
  10X
    98°C         10 sec
    Annealing**      30 sec
    72°C         x sec***
72°C             5min
4°C             hold
```

** Anneal at Tm + 3°C. The Tm should be calculated with the nearest-neighbor method.
***The extension time is usually calculated according to the shortest piece with 15 sec /kb if the cloning is not complicated. For example, if there is only one insert and is shorter than the vector, say, 600 bp, then I will use 15 sec for extension. Refer to the published paper for detailed information.

Transformation – Duke – 2010

http://2010.igem.org/Team:Duke/Project/Protocols

1: Thaw 1 tube of competent cells on ice;

2: Add 1-50ng of plasmid into competent cells while stirring gently;

3: Keep the tube covered by ice for 30min;

4: Heat-shock the competent cells in water bath for 45s at 42C;

5: Put the tube on ice for 2 min;

6: Add 450uL of SOC medium and put it in a 37C shaker for 1 hour;

7: Dilute and spread an appropriate amount on an LB agar plate with the appropriate antibiotcs;

8: Place the plate upside-down in a 37C incubator for 16-18 hours.

Plasmid Extraction (Miniprep) – Duke – 2010

http://2010.igem.org/Team:Duke/Project/Protocols

1:Pick a single colony from a freshly streaked selective plate and inoculate a culture of 1–5 ML LB MEDIUM CONTAINING THE APPROPRIATE SELECTIVE ANTIBIOTIC. INCUBATE FOR 12–16 H AT 37 ‹C WITH VIGOROUS SHAKING;

2: THE BACTERIAL CELLS BY CENTRIFUGATION AT > 8000 RPM (6800 X G) IN A CONVENTIONAL, TABLE-TOP MICROCENTRIFUGE FOR 3 MIN AT ROOM TEMPERATURE (15–25 ‹);

3: RESUSPEND PELLETED BACTERIA IN 250 UL OF RESUSPENSION BUFFER AND TRANSFER TO A MICROCENTRIFUGE TUBE;

4: ADD 250 UL OF LYSIS BUFFER AND THOROUGHLY MIX BY INVERSION. IMPORTANT: DO NOT ALLOW LYSIS TO CONTINUE FOR MORE THAN 5 MIN;

5: ADD 350 UL OF NEUTRALIZATION BUFFER AND THOROUGHLY MIX BY INVERSION;

6: CENTRIFUGE FOR 10 MIN AT 13,000 RPM (ALL CENTRIFUGATION MAY BE PERFORMED AT 13,000 RPM);

7: DECANT SUPERNATANTS FROM STEP 6 INTO A SPIN COLUMN;

8: CENTRIFUGE FOR 30-60S AND DISCARD THE FLOW THROUGH;

9: ADD 750 UL OF WASH BUFFER AND CENTRIFUGE FOR 30-60S;

10: DISCARD FLOW-THROUGH, CENTRIFUGE FOR AN ADDITIONAL MINUTE TO REMOVE RESIDUAL WASH BUFFER;

11: PLACE THE SPIN COLUMN IN A CLEAN MICROCENTRIFUGE TUBE, ADD 20-50UL OF ELUTION BUFFER OR DDH2O TO THE CENTER OF THE SPIN COLUMN, LET IT STAND FOR 1 MIN AND CENTRIFUGE FOR 1MIN.

12: A SECOND ELUTION STEP MAY BE PERFORMED TO INCREASE DNA YIELDS.

Restriction Digest – Duke – 2010

http://2010.igem.org/Team:Duke/Project/Protocols

Reaction Mix: •100 µg/mL BSA •1X NEB2 buffer •1 µL BioBrick enzyme 1 •1 µL BioBrick enzyme 2

DNA samples should be .2-1ng, and deionized, sterile H2O should be used to bring the solution to H2O to 50 µL

1.Add restriction enzyme buffer. Add BSA. Add DNA. Add each enzyme. Also, the enzyme is in some percentage of glycerol which tends to stick to the sides of your tip. To ensure you add only 1 µL, just touch your tip to the surface of the liquid when pipetting. Incubate for 2 hours at 37°C. Incubate for 20 mins at 80°C to heat inactivate enzyme. This step is sufficient to inactivate even Pst I.

2.Incubate 4°C until you pull the reaction out of the thermal cycler. Protocol from Openwetware:

http://openwetware.org/wiki/Engineering_BioBrick_vectors_from_BioBrick_parts/Restriction_digest

PCR – Duke – 2010

http://2010.igem.org/Team:Duke/Project/Protocols

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Materials

- Phusion™ High-Fidelity PCR Kit (FINNZYMES, Cat. No. F-553)
- Thermocycler

Preperation

5x Phusion HF Buffer	10 ul
10 mM dNTPs	1 ul
DNA template	1 pg – 10 ng
Forward primer (10 uM)	2.5 ul
Reverse primer (10 uM)	2.5 ul
Phusion DNA Polymerase	0.5 ul
--------------------	-----
H2O	to 50 ul

Procedure

```
98°C         30sec
 30X
    98°C      10 sec
    Annealing* 30 sec
    72°C      15 sec per 1 kb
72°C         5min
4°C          hold
```

* Anneal at Tm + 3°C. The Tm should be calculated with the nearest-neighbor method.

Ligation – Duke – 2010
http://2010.igem.org/Team:Duke/Project/Protocols

1.Add appropriate amount of deionized H2O to sterile 0.6 mL tube

Add 1 µL ligation buffer to the tube.

Vortex buffer before pipetting to ensure that it is well-mixed.

Remember that the buffer contains ATP so repeated freeze, thaw cycles can degrade the ATP thereby decreasing the efficiency of ligation.

Add appropriate amount of insert to the tube.

Add appropriate amount of vector to the tube.

Add 0.5 µL ligase.

Vortex ligase before pipetting to ensure that it is well-mixed.

Also, the ligase, like most enzymes, is in some percentage of glycerol which tends to stick to the sides of your tip. To ensure you add only 0.5 µL, just touch your tip to the surface of the liquid when pipetting.

Let the 10 µL solution sit at 22.5°C for 30 mins Denature the ligase at 65°C for 10min

Dialyze for 20 minutes if electroporating

Use disks shiny side up

2.Store at -20°C

Two-Day Cloning System – Caltech – 2010

http://2010.igem.org/Team:Caltech/Cloning

We perfected an efficient 2-day cloning method for BioBricks to accomplish the large number of ligations we wanted. Constructs should be designed to accomplish as many ligations steps in parallel as possible.

Procedure

1. Day 1
    1. Miniprep cultures to extract DNA to be digested (if applicable).
    2. Digest (2.5hr) and ligate (1hr) appropriate DNA using Standard Assembly and (a) linearized backbone, (b) digested vector containing RFP or some other pigment gene, or (c) vector digested with Alkaline Phosphatase (CIP).
    3. Transform 2µL ligation product into desired cells via electroporation. Plate on LB-agar plates with appropriate antibiotic and grow overnight.
2. Day 2
    1. Perform CPCR on 2-10 colonies from each ligation plate to find clones with correct inserts. Depending on the backbone used, various fractions of the colonies may contain the incorrect insert, and will determine the number of colonies that must be tested. Make sure to make the parallel LB liquid cultures.
    2. CPCR product should be analyzed using standard agarose gel electrophoresis. Clones with the correct-sized inserts can be miniprepped the following morning for further cloning.
3. Day 3 (optional)
    1. Miniprepped samples can be sent out for sequencing to verify. This is only necessary when the construct is complete or to check progress on a very large construct.

Colony PCR – Caltech – 2010

http://2010.igem.org/Team:Caltech/Cloning

Materials

- Autoclaved water
- PCR tubes
- LB broth & plates
- Qiagen PCR Core Kit: dNTPs, Taq, Buffers
- Forward & reverse primers

Procedure

1. 85.5µL autoclaved water were added to PCR tubes - one for each colony to be amplified.
2. Inoculate the tube with cells from the desired colony. With the same toothpick, simultaneously inoculate a 5mL LB liquid culture (with antibiotic) and streak an LB-agar plate.
3. Incubate PCR tubes in a thermal cycler for 10 minutes at 100°C. Remove and cool.
4. To each tube, add: 10µL 10x PCR buffer, 2µL 10mM dNTP mix, 1µL 10µM forward primer, 1µL 10µM reverse primer, 0.5µL Taq DNA polymerase. Note: it is generally much more efficient to combine all these in a single tube and scale up for multiple reactions.
5. Replace tubes in thermal cycler and initiate CPCR program (below).
6. Analyze reactions via 1% agarose gel electrophoresis:
    1. Add 2µL NEB 6x Gel Loading Buffer to 10µL CPCR product.
    2. Load into gel. Run for 30-40 minutes at 90-100V.
    3. Stain/destain in EtBr and image using UV illumination.

Program

1. Initial denaturation: 94°C, 30s
2. 30 cycles:
    1. Denaturation: 94°C, 3 min
    2. Annealing: 54°C, 30s
    3. Elongation: 72°C, 2 min
3. Final extension: 72°C, 2 min
4. Finish: 5°C hold

Primers

- Forward: 5'-GAA TTC GCG GCC GCT TCT AGA G-3'
- Reverse: 5'-CTG CAG CGG CCG CTA CTA GTA-3'

Sequencing Primers – Caltech – 2010

http://2010.igem.org/Team:Caltech/Cloning

Materials

- Forward Primer:
  - 5'-GAATTCGCGGCCGCTTCTAGAG-3'
- Reverse Primer:
  - 5'-CTGCAGCGGCCGCTACTAGTA-3'

Procedure

1. All sequencing was performed by Laragen.

Adding Antibiotics to LB-agar – Caltech – 2010
http://2010.igem.org/Team:Caltech/Cloning

This method allows you to make LB-agar plates with no antibiotic, and add the antibiotic when needed.

Materials

- 1000x stock antibiotic solution
- LB-agar plates, clean
- Glass beads, sterile

Procedure

1. Warm plate to 37°C.
2. Add 50µL of 1000x antibiotic to the surface of the agar.
3. Distribute antibiotic evenly using glass beads.
4. Dispose of beads and allow plates to dry in biohood or near flame.

Glycerol (Freezer) Stocks – Caltech – 2010
http://2010.igem.org/Team:Caltech/Cloning

Materials

- 0.5mL overnight LB culture
- 0.5mL 50% glycerol (sterile)
- Cryotube or 1.5mL Eppendorff
- Dry ice/ethanol or LN2

Procedure

1. Mix overnight cell culture with equal parts 50% glycerol solution in cryotube.
2. Label and add to dry ice/ethanol bath.
3. Freeze for 10 minutes. Store at -80°C.

Transformation by Electroporation – Caltech – 2010

http://2010.igem.org/Team:Caltech/Cloning

Materials

- N samples of DNA to be transformed, 1 positive control sample
- N+1 electroporation cuvettes
- N+1 50µL aliquots of EC cells (we used DH5α)
- N+1 mL SOC (Super-optimal broth + 10mM glucose)
- N+1 LB-agar plates of appropriate resistances

Procedure

1. Chill electroporation cuvettes and thawed EC cells on ice. Do not let EC cells warm to above ice-cold.
2. Add 1µL of ligation product to the 50µL aliquot. Don't forget an aliquot for positive control.
3. Transfer DNA/cell mixture to a cuvette and pulse at 2.5V. Rescue the cells immediately by adding 0.75mL warm SOC to the cuvette.
    1. Ensure that the time constant is above 3.0 for each.
4. Incubate for 1 hour at 37°C.
5. Plate the entire mixture on an LB-agar plate with the appropriate antibiotic resistance, grow overnight.

Making Competent Cells – Caltech – 2010

http://2010.igem.org/Team:Caltech/Cloning

Materials

- Desired cells
- 1-2L ice cold autoclaved water
- 1L 2YT media
- 2x Erlenmeyer flasks, autoclaved
- Sterile centrifuge tubes large enough to hold 0.2-1L of culture

Procedure

NOTE: The cells must be kept as close to 0°C as possible after they are chilled. All utensils must be completely sterile, as no antibiotics are added to the competent cells, and contamination is a serious danger.

1. Incubate two 5mL overnight cultures of the desired cells in 2YT media at 37°C.
2. Use the cultures to inoculate two 100-500mL cultures of 2YT in autoclaved Erlenmeyer flasks. Incubate for 2-4 hours.
3. Transfer to centrifuge tubes (balanced), chill on ice for 20-30 minutes, and centrifuge at 4000xG for 10 minutes.
4. Decant supernatant. Resuspend pellet in an equal volume of ice-cold autoclaved water. Centrifuge again.

5. Decant, resuspend pellet in a half-volume of ice-cold water. Centrifuge.
6. Decant, resuspend pellet in 1-5mL ice-cold 10% glycerol. Centrifuge.
7. Decant, resuspend in 0.5-1mL 10% glycerol.
8. Flash-freeze 50µL aliquots in dry ice/ethanol and store at -80°C.
9. To be sure that the process was successful, perform positive and negative transformation controls using your new cells.

Plasmid DNA Extraction – TEC-Monterrey – 2010 http://2010.igem.org/Team:Tec-Monterrey/Protocols

Wizard® Plus SV Minipreps DNA Purification System was used for DNA extraction. Slight changes were made to the Centrifugation protocol:

Production of Cleared Lysate

1. Pellet 20 mL of overnight culture for 15 minutes at 8,000 x g.

Washing

12. Centrifuge for 2 minutes at 14,000 x rpm with the microcentrifuge lid open to allow evaporation of any residual ethanol.

Elution

14. Add 50 µL of Nuclease-Free Water to the Spin Column. Centrifuge at 14,000 rpm for 1 minute at room temperature.

Restriction Digestion – TEC-Monterrey – 2010 http://2010.igem.org/Team:Tec-Monterrey/Protocols

1. Review the Digestion Calculator, available through here, to make the proper calculations. Adjust the final volume of the reaction and be sure to add the DNA concentration and the proper enzymes to be used (with their corresponding quantities).
2. Add all of the components but the enzymes to a 0.2 ml PCR tube. Make sure that the buffer used is the one recommended for the combination of enzymes to be used.
3. Carefully, add the enzymes, preferably without taking them out of the freezer.
4. Incubate the tubes at 37˚C for two hours.

Agarose Gel Electrophoresis – TEC-Monterrey – 2010 http://2010.igem.org/Team:Tec-Monterrey/Protocols

The following procedure is for the preparation of a 1% agarose gel.

Materials:

- Agarose (Electrophoresis grade)
- 1X TAE
- Electrophoresis chamber
- Electrophoresis tray
- Transilluminator
- SYBR Safe
- Microwave
- Beaker

Procedure:

1. Take a beaker and place 30 mL of 1X TAE.
2. Weigh 0.3 gr of agarose and add it to 1X TAE solution.
3. Place beaker inside microwave and heat it until agarose dissolves completely.
4. Allow agarose to cool down to 60°C and add 3 µL of SYBR Safe 10 000X.
5. Mix solution and pour it in a taped gel tray.
6. Add a comb and let the gel harden for about 15 minutes.
7. Remove the comb and the tape and put the gel tray in the electrophoresis chamber.
8. Add enough 1X TAE to completely cover the gel.
9. Add DNA loading buffer to the samples and load them.
10. Run the gel at a voltage between 60V and 120V, depending on the desired resolution/time.
11. Visualize or cut the DNA by putting it in the transilluminator.

Purification of DNA Fragments from an Agarose Gel – TEC-Monterrey – 2010
http://2010.igem.org/Team:Tec-Monterrey/Protocols

Wizard® SV Gel and PCR Clean-Up System was used for purification of DNA fragments obtained from an agarose gel or ligation reaction. Slight changes were made to the DNA Purification by Centrifugation protocol:

Washing

6. Empty the Collection Tube, incubate at room temperature for 5 minutes and recentrifuge the column assembly for 2 minutes at 8,000 x rpm to allow evaporation of any residual ethanol.

Elution

8. Add 30 µL of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 x g for 3 minutes.

DNA Ligation – TEC-Monterrey – 2010 http://2010.igem.org/Team:Tec-Monterrey/Protocols

T4 DNA Ligase protocol by Invitrogen was used to ligate DNA, aided by a Ligation Calculator available through here.

Preparation of Electrocompetent Cells – TEC-Monterrey – 2010 http://2010.igem.org/Team:Tec-Monterrey/Protocols

The following protocol is for preparing electrocompetent *E.coli* cells.

Materials:

- LB-agar plate
- LB medium
- 500 mL chilled autoclaved dH2O, stored at 4°C
- Chilled 10% glycerol/dH2O solution, stored at 4°C
- 1 L flask
- 50 ml tubes
- 0.2 mL tubes
- Ice bucket
- Ice
- Refrigerated centrifuge

Procedure:

1. Streak *Escherichia coli* DH5α cells onto LB-agar plate with no antibiotics and incubate at 37°C overnight.
2. Pick one colony and place it in a 50 mL tube with 20 mL LB medium. Incubate overnight on a shaker at 37°C and 350 rpm.

3. Add 250 ml of LB medium to a flask and add the overnight culture until an OD600 of 0.1 is reached.
4. Place the flask on a shaker at 37°C, 350 rpm until an OD600 between 0.4-0.6 is reached.
5. Transfer the diluted culture to 50 mL tubes.
6. After this step, the cells must be kept at 4°C at all times. Place the cells on ice for 15 minutes.
7. Cool the centrifuge to 4°C.
8. Centrifuge the tubes for 10 min at 8000g at 4°C.
9. Remove supernatant and gently resuspend pellets with 10 mL cold sterile water by pipetting. Add the rest of the water to a total volume of 50 mL.
10. Centrifuge a second time for 10 min at 8000g at 4°C.
11. Remove supernatant and gently resuspend pellets with 10 mL cold sterile water by pipetting. Add the rest of the water to a total volume of 50 mL.
12. Centrifuge a third time for 10 min at 8000g rpm at 4°C.
13. Remove supernatant and gently resuspend pellets with the remaining water (if it's too little, add some more).
14. Calculate and add glycerol so that the final concentration is 10-15 %.
15. Resuspend the cells and aliquot 50 µL per 0.2 mL tube (tubes on ice) and store at -80°C.

Transformation via Electroporation – TEC-Monterrey – 2010 http://2010.igem.org/Team:Tec-Monterrey/Protocols

The following protocol is for transforming plasmid DNA into *Escherichia coli* DH5α cells.

Materials:

- Electrocompetent cells
- Plasmid DNA (from a ligation reaction)
- Ice
- Ice bucket
- Electroporation cuvette (2mm gap width)
- Electroporator (Biorad Gene Pulser Xcell)
- LB-agar plate with appropriate antibiotic
- 1mL SOC at room-temperature

Procedure:

1. Chill electroporation cuvettes, DNA samples and tubes on ice.
2. Once cuvettes are cold, remove electrocompetent cells from -80°C freezer and thaw on ice.
3. Turn on electroporator and set voltage to 2.5 kV.
4. Dial a micropipette to 1 or 2µL of DNA sample.
5. Dial a micropipette to 50µL of electrocompetent cells.
6. Dial a micropipette to 1000µL and pipet in SOC. Place micropipette on counter such that tip doesn't touch anything.
7. Pipet 1-2µL of DNA sample and place inside the cuvette.
8. Pipet 50µL of electrocompetent cells inside the cuvette ensuring they mix with the DNA sample. Do not pipet up and down.
9. Place cuvette back on ice to ensure it remains cold.
10. Tap the cuvette on the counter gently so that cells are at the bottom and to remove any air bubbles.
11. Wipe off excess moisture from outside of cuvette.
12. Place in chamber of electroporator so that the cuvette sits between electrodes.
13. Pulse the cells with a shock by pressing button on electroporator.
14. Remove cuvette from the chamber and immediately add SOC.
15. Transfer cuvette to 37°C incubator and shake at 350 rpm to promote aeration. Incubate for 1 hr.
16. Plate 100 µL transformation onto LB-agar plate supplemented with appropriate antibiotic.
17. Incubate plate overnight at 37°C until colonies appear.

Characterization
The following protocol is for characterizing the PoPS amplifier by measuring absorbance and fluorescence.
Materials:

- LB medium
- 50 ml tubes
- Antibiotic
- Incubator
- Spectrophotometer
- Microplate reader
- Clear 96-well microplate
- 0.1, 0.5, 1, 10, 50, 100 and 500 µM L-Arabinose solutions

Procedure:

1. Pick three different colonies that contain the desired part to be characterized and place each of them in a different 50 mL tube with 5 mL LB medium.
2. Add 1 µL of the corresponding antibiotic per each mL of LB medium.
3. Pick one colony that does not contain the plasmid with the part that will be characterized (control) and place it in a 50 mL tube with 5 mL LB medium.
4. Incubate all the 4 tubes for 16 hours on a shaker at 37°C and 350 rpm.
5. Check the OD600 of the cultures and dilute with fresh LB medium until an OD600 of 0.1 is reached. Make sure that you have at least 7 mL of each culture.
6. Add 1 µL of the corresponding antibiotic per each mL of LB medium.
7. Incubate all the 4 tubes on a shaker at 37°C and 350 rpm until an OD600 of 0.6 is reached.
8. Fill each well with 198 µl of inoculum and 2 µl of L-Arabinose at different concentrations. Make 3 repetitions of each colony with the different concentrations of L-Arabinose.
9. The microplate is then read by the microplate reader by using the following protocol:
   i. Set temperature to 37°C
   ii. Kinetic reading lasting 4 hours with measurements every 5 minutes.
   iii. Absorbance (600 nm filter) and Fluorescence (Excitation: 485 nm, Emission: 528 nm).
   iv. Shaking in intensity 2 for 5 seconds before every reading.
10. Export the results of the well data to an Excel sheet for further interpretation.

Alkaline Lysis Mini Plasmid Preps – UNAM CINVESTAV – 2010 http://2010.igem.org/Team:Mexico-UNAM-CINVESTAV/Protocols

1. Grow O/N in 1.5 ml LMM or Terrific broth (see Reagents) with 75µg/ml Amp
2. Pour into ependorf tube and spin down cells at 7-8K for 2 min
3. Aspirate s/n and resuspend in 50µl 25mM Tris pH 8, 10 mM EDTA; leave lids open
4. Add 100µl of freshly prepared 1% SDS, 0.2M NaOH (5ml = 100ul 10M NaOH added to 4.4ml DDW then 500µl 10% SDS). Add it forcefully and you don't need to vortex
5. Add 75µl KoAc solution and vortex
6. Add 100µl of phenol/CHI3, close lids, vortex
7. Spin 13K for 2 mins
8. Remove supernatant, add to 500µl ethanol. Vortex and spin at 13 K for 5 min
9. Aspirate s/n, removing all ethanol
10. Resuspend in 50µl TE

11. Digest 2-5ul, adding 1µl preboiled 10mg/ml RNAse A (see Reagents for preparation).

KOAc solution:

- 60 ml 5M potassium acetate
- 11.5 ml glacial acetic acid
- 28.5 ml DDW

DNA Isolation for Low-Melting Point Agarose (using elu-tip method) **– UNAM CINVESTAV – 2010**
http://2010.igem.org/Team:Mexico-UNAM-CINVESTAV/Protocols

1. Excise fragment from gel and estimate volume.
2. Add 1/100 volume of 1 M Tris pH 7.5, 1/50 volume of 0.5 M EDTA, and 1/100 volume of 5M NaCl.
3. Incubate at 68°C for 10 minutes.
4. Vortex and remove to a small tube.
5. Incubate at 37°C for 5 minutes.
6. Phenol extract 2-3 times (phenol at 42°C, spin at 13 K for 2 minutes.)
7. Ether extract 1 time. Place tubes at 65°C, then speed-vac 2 to 5 minutes. Repeat procedure about 4 times to get rid of residual ether.
8. Ethanol precipitate DNA and suspend pellet in 1 ml of low salt buffer (from elu-tip column protocol)
9. Following the elu-tip protocol booklet, wash the column by pushing 5 ml of low salt buffer through the matrix at a rate of 0.5-1.0 ml/minute. The column may be incubated in the low salt buffer ³ 2 hours to improve recovery.
10. Load DNA sample onto the column slowly (1-2 drops/second). NOTE: When recovering DNA from low-melt temperature agarose, use of the pre-filter is not recommended. Consult the protocols booklet for specific parameters of different types of nucleic acid purification (i.e. DNA purification when LMP agarose isn't used).
11. Wash the column with 2-3 mls of pre-warmed (42°C) low salt buffer.
12. Elude DNA with 0.4 ml of high salt buffer. Do a total of 2-3 washes as desired to be certain of good recovery.
13. Ethanol precipitate DNA and resuspend in TE or dH2O.

Transformation Protocol Using Heat Shock – UNAM CINVESTAV – 2010
http://2010.igem.org/Team:Mexico-UNAM-CINVESTAV/Protocols

1. Take competent E.coli cells from –80oC freezer.
    a. Use DH5α cells in most cases.
    b. If want to cut at XbaI or other DAM- enzyme site, use SCS110 cells which are deficient in Dam and Dcm methylases.

1. Turn on water bath to 42oC.
2. Put competent cells in a 1.5 ml tube (Eppendorf or similar). For transforming a DNA construct, use 50 ul of competent cells. For transforming a ligation, use 100ul of competent cells. You may need more or less cells, depending how competent they are.

3. Keep tubes on ice.
4. Add 50 ng of circular DNA into E.coli cells. Incubate on ice for 10 min. to thaw competent cells.
5. Put tube(s) with DNA and E.coli into water bath at 42oC for 45 seconds.
6. Put tubes back on ice for 2 minutes to reduce damage to the E.coli cells.
7. Add 1 ml of LB (with no antibiotic added). Incubate tubes for 1 hour at 37 oC.(Can incubate tubes for 30 minutes, unless trying to grow DNA for ligation which)is more sensitive. For ligation, leave tubes for 1 hour.)
8. Spread about 100 ul of the resulting culture on LB plates (with appropriate antibiotic added – usually Ampicillin or Kanamycin.) Grow overnight.
9. Pick colonies about 12-16 hours later.

Double ligation (Richard Lab) Procedure – UNAM CINVESTAV – 2010
http://2010.igem.org/Team:Mexico-UNAM-CINVESTAV/Protocols

1. Mix together the following:

   a. 5µl digested front part (EcoRI and SpeI).

   b. 5µl digested rear part (XbaI and PstI).

   c. 4µl desionized water.

   d. 3µl digested vector (EcoRI and PstI)

   e. 2µl of Reaction Buffer for T4 Ligase.

   f. 1µl of DNA Ligase.

1. Incubate at room-temp for 10 mins (or at 4ºC overnight)
2. Stop Reaction for 15 min at 80ºC

- PCR **– TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab

Read more

Taq Polymerase Hot Start
PCR Pippeting plan:
1 µl template
1 µl dNTP 10 µM
1 µl G1004 (Primer) 10 µM
1 µl G1005 (Primer) 10 µM
5 µl 10x Taq-buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$)
0,2 µl Taq-Polymerase (add last) 5,000 U/ml

40.8 µl Water
Final volume 50µl

Processing: (program saved as **IGEMPCR**)

- preheating of PCR chamber to 94 °C

--> insert sample

- 2 min at 94 °C

- loop 35x:

- 30 sat 94°C (according to IGEM protocols)
- 30 s at 56 °C
- 45s at 72°C

- 7 min at 72°C
- stay at 4°C

colony PCR – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab

- Colony PCR
  - pick colonies and resuspend them in 20 µl LB+Antibiotic (each)
  - PCR of 2 µl of each sample, 2 µl as negative control (Program: ColonyPCR, modified), store remaining 18 µl for overnight cultures
  - afterwards, mix 15 µl of each PCR product with 3 µl GLPn and load to Gel
  - make overnight cultures of positive clones by adding the remaining 18 µl to 5 ml LB+AB

program:colonypcr

- preheating of PCR chamber to 94 °C

--> insert sample

- 5 min 30 sec at 94 °C
- loop 35x:
  - 30 sat 94°C (according to IGEM protocols)
  - 30 s at 58 °C
  - 60s at 72°C
- 7 min at 72°C
- stay at 4°C

Close

- DNA Purification

Read more

PCR samples

ZYMO RESEARCH DNA Clean&Concentration Kit – TU Munich – 2010

http://2010.igem.org/Team:TU_Munich/Lab

Protocol and Information

1. In a 1.5 ml microcentrifuge tube, add 2-7 volumes of DNA Binding Buffer to each volume of DNA sample (see table below). Mix briefly by vortexing.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR, cDNA, DNA fragment	5 : 1	500 µl : 100 µl
ssDNA (e.g., M13 phage)	7 : 1	700 µl : 100 µl

1. Transfer mixture to a provided Zymo-Spin™ Column1 in a Collection Tube.
2. Centrifuge at ≥10,000 x g for 30 seconds. Discard the flow-through.
3. Add 200 µl Wash Buffer to the column. Centrifuge at ≥10,000 x g for 30 seconds. Repeat wash step.
4. Add ≥6 µl water2,3 directly to the column matrix. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge at ≥10,000 x g for 30 seconds to elute the DNA. Ultra-pure DNA in water is now ready for use.

QIAquick purification Kit – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab

Handbook

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove

mineral oil or kerosene. For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. **We changed it to 3 min @ 6000rpm !**
5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**repeat!**
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Gel samples

ZYMO RESEARCH Gel DNA Recovery Kit Product informartion **– TU Munich – 2010**
http://2010.igem.org/Team:TU_Munich/Lab

Protocol

1. Excise the DNA fragment1 from the agarose gel using a razor blade or scalpel and transfer it to a 1.5 ml microcentrifuge tube.
2. Add 3 volumes of ADB to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB).
3. Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved2. For DNA fragments >8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g. 100 µl agarose, 300 µl ADB and 100 µl water).
4. Transfer the melted agarose solution to a Zymo-SpinTM I Column in a Collection Tube.
5. Centrifuge at ≥10,000 x g for 30-60 seconds. Discard the flow-through.
6. Add 200 µl of Wash Buffer to the column and centrifuge at ≥10,000 x g for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add ≥6 µl of water3,4 directly to the column matrix. Place column into a 1.5 ml tube and centrifuge ≥10,000 x g for 30-60 seconds to elute DNA.
   Ultra-pure DNA in water is now ready for use.

Miniprep

Protocol:

1. Add 600 µl of bacterial culture grown in LB medium to a 1.5 ml microcentrifuge tube.

2. Add 100 µl of 7X Lysis Buffer (Blue)1 and mix by inverting the tube 4-6 times. Proceed to step 3 within 2 minutes. After addition of 7X Lysis Buffer the solution should change from opaque to clear blue, indicating complete lysis.
3. Add 350 µl of cold Neutralization Buffer (Yellow)2 and mix thoroughly. The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form. Invert the sample an additional 2-3 times to ensure complete neutralization.
4. Centrifuge at 11,000 – 16,000 x g for 2-4 minutes.
5. Transfer the supernatant (~900 µl) into the provided Zymo-Spin™ IIN column. Avoid disturbing the cell debris pellet.
6. Place the column into a Collection Tube and centrifuge for 15 seconds.
7. Discard the flow-through and place the column back into the same Collection Tube.
8. Add 200 µl of Endo-Wash Buffer to the column. Centrifuge for 15 seconds. It is not necessary to empty the collection tube.
9. Add 400 µl of Zyppy™ Wash Buffer2 to the column. Centrifuge for 30 seconds.
10. Transfer the column into a clean 1.5 ml microcentrifuge tube then add 30 µl of Zyppy™ Elution Buffer3 directly to the column matrix and let stand for one minute at room temperature.
11. Centrifuge for 15 seconds to elute the plasmid DNA.

Close

- Digestion

Read more

Restriction Digest – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab

Enzyme	10 units is sufficient, generally 1µl is used
DNA	1 µg
10X NEBuffer	5 µl (1X)
BSA	Add to a final concentration of 100 µg/ml (1X) if necessary
Total Reaction Volume	50 µl
Incubation Time	1 - 1.5 hour
Incubation Temperature Enzyme dependent	XbaI, SpeI, PstI, SpeI : 37 °C

activity of restriction enzymes in NEB buffers

Biobrick standard – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab

Protocols for IGEM standard digestion

Dephosphorylation – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab

using Antarctic Phosphatase

1. Add 1/10 volume of 10X Antarctic Phosphatase Reaction Buffer to 1-5 µg of DNA cut with any restriction endonuclease in any buffer.
2. Add 1 µl of Antarctic Phosphatase (5 units) and mix.
3. Incubate for 15 minutes at 37°C for 5´ extensions or blunt-ends, 60 minutes for 3´ extensions.
4. Heat inactivate (or as required to inactivate the restriction enzyme) for 5 minutes at 65°C.
5. Proceed with ligation.

from NEB

Close

- Ligation **– TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab

Read more

Using T4 Ligase, New England Labs

- 1 µl T4 Ligase (10.000 U)
- 50 ng plasmid
- 3x mol(plasmid) insert
- 2 µl T4 Ligase 10x buffer
- add H_2O to reach final volume of 20 µl
- incubation at 22°C for 1 h
- storing at 16 °C for 40 min

Biobrick Standard

Standard BioBrick assembly

Close

- Transformation **– TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab

Read more

At Woehlke's S1-Lab !!!

1. Thaw competent cells on Ice
2. Add DNA, pipette gently to mix
3. Let sit for 30 minutes on ice
4. Incubate cells for 45 seconds at 42°C
5. Incubate cells on ice for 2 min
6. Add 1 ml LB0
7. Incubate for 1 hour at 37oC on shaker
8. Spread 100-300 µl onto a plate made with appropriate antibiotic.
9. Grow overnight at 37 °C.
10. Save the rest of the transformants in liquid culture at 4 °C

modified from open wetware

Close

- Gel electrophoresis **– TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab

Read more

Agarose Gels

usual volume needed: 80 ml Optimum resolution according to NEB

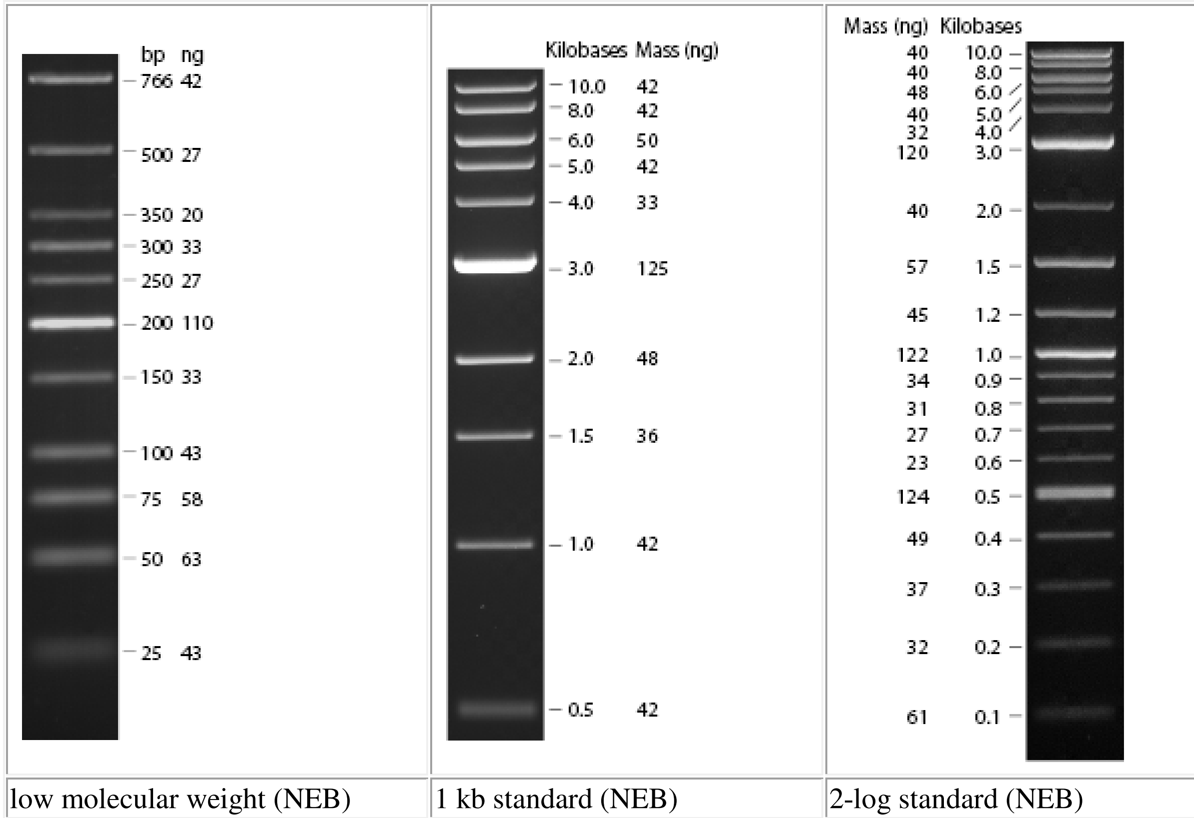
further information on optimizing gel electrophoresis, e.g. recommanded voltage per cm2 gel

stain

- SybrGold (invitrogen)
  - Cover Gel with 1x TAE
  - Add SybrGold to a 1:10000 dilution
  - cover with aluminium foil (light sensitive)
  - shake&incubate 20 min (for 2% Agarose Gels at least 45 min!)

- SybrSafe
  - used just like SybrGold

standards



low molecular weight (NEB)	1 kb standard (NEB)	2-log standard (NEB)

Polyacrylamide Gels – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab
Preparation of Gels
Recipe for denaturing gels:

Gel type	1 big gel	2 big gels	1 small gel	2 small gels
Urea	28.8 g	57.6 g	x	x
Acrylamide 40%	22.5 ml	45 ml	x	x
Buffer 10x	6 ml	12 ml	x	x
End volume (reach by adding water)	60 ml	120 ml	x	x
APS	600 µl	1200 µl	x	x
TEMED	60 µl	120 µl	x	x

- Dissolve Urea in Acrylamide-buffer mixture (use Ultrasound bath), this may take more than an hour!
- Tighten the Gel chamber
- add water to desired end volume
- Add APS, then TEMED, mix
- Pipette mixture into gel chamber
- Add desired comb
- let gel polymerize overnight; add buffer in the evening

Running of Gels
mix samples 1:1 with formamide loading dye (stored @ -20°C) carefully remove comb blow air into pockets with a 50 µl syringe fill samples into pockets run the gel (usually about 200 V)
Close

Close

***In vivo* Measurement – TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab
Read more
Bacterial Cell Growth
Bacteria from over night cultures were diluted 1:50 into 20 ml culture in LBamp and incubated at 37 °C. Upon $OD_{600}$of 0.7-0.8 the cultures were induced with 0.4% Arabinose and 0.4% Arabinose + 1mM IPTG, respectively. Subsequently Cultures were incubated at 25°C for at least 12 h.
Fluorescence Measurement
Cell samples for the fluorescence measurement were diluted to OD_{600}=0.03 and analyzed in a JASCO fluorimeter. eGFP excitation wavelength was set to 501 nm and mCherry fluorescence was measured with an excitation at 587 nm. Standard parameters for the fluorimeter included scanning speed of 100 nm/ min and data points every 0.2 nm as well as medium detector sensivity. The cuvette holder was temperated to 25 °C. The resulting spectra were corrected for instrumental wavelength dependencies and quantum yield of the fluorescent proteins. A pure LBamp spectrum was subtracted and the corrected spectra were normalized using eGFP fluorescence as reference.
Close

***In vitro* Translation – TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab
Read more
The Promega Kit is used according to the provided protocols. Further Information about this Kit can be found in theParts Registry.
Fluorescence kinetics are recorded for at least 3 hours, settings are applied as in the in vitro measurement.
Close

***In vitro* Transcription – TU Munich – 2010** http://2010.igem.org/Team:TU_Munich/Lab
Read more
Buffers
Three different buffers were used for *in vitro* transcription experiments:

- For Epicentre E. coli RNA Polymerase the recommended buffer was used
- For T7 RNA Polymerase experiments two buffers were tested:
    - T7 RPO buffer as recommended and used by members of the Simmel group
    - T7 RPO "paper buffer", as used in the paper of XXX

Of all buffers 2x concentrated stocks were prepared. Malachite green was usually added to the buffer stocks.

	E. coli RPO buffer	T7 RPO buffer	T7 RPO buffer "paper"
Tris	40 mM	40 mM	40 mM
pH	7.5	7.1	7.9
MgCl2	10 mM	40 mM	6 mM
KCl	150 mM	/	100 mM
Triton X-100	0.01%	/	/

Sample Preparation
Different concentrations were tested for malachite green and DNA templates. Components of a standard experiment are listed in the table below.

	E. coli RPO buffer	T7 RPO buffer	T7 RPO buffer "paper"
buffer	1x	1x	1x
DTT	10 mM	10 mM	10 mM
Malachite green	5-10 µM	5-10 µM	5-10 µM
NTP's	1 mM	4 mM	0.8 mM
RPO	2 U	125 U	125 U

In each run up to 4 samples are measured simultaneously. Components that are the same in each of the 4 samples (buffer, DTT, NTPs, RPO, Water) are prepared as a 4.1x MasterMix in a loBind tube and split to the 4 cuvettes. Final Volume of each sample is 100 µl.

Cary Eclipse – TU Munich – 2010 http://2010.igem.org/Team:TU_Munich/Lab
For fluorescence measurements, a Cary Eclipse Spectrofluorimeter with a Multicellholder (4 cells) is used. Kinetics are recorded at 37° C. Excitation wavelength is 630 nm, emission is followed at 650 nm and 655 nm. After each kinetics measurement, spectra are to be recorded.
Close

Colony PCR – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
CP1.1

How to amplify DNA from bacterial colonies and solutions

Important remarks

All solutions should be kept at ice until run of PCR

Materials
Premix Pfu-PCR

For 1 PCR reaction:

• 5 µL Pfu-buffer + $MgSO_4$

• 1.5 µL 10mM dNTP mix

• 1.5 µL 10pmol/µL forward primer

• 1.5 µL 10pmol/µL reverse primer

• 0.5 µL Pfu polymerase enzyme (add just before PCR run)

• 40 µL H_2O

Total vol.: 5O µL

Premix TAQ-PCR (no proofreading):
For 1 PCR reaction

- 2.5 µL 10x TAQ-buffer + $MgCl_2$
- 0.5 µL 10mM dNTP mix
- 1.25 µL 10pmol/µL forward primer
- 1.25 µL 10pmol/µL reverse primer
- 19.25 µL Water
- 0.25 µL TAQ polymerase enzyme **(add just before PCR run)**

Total vol.: 25 µL

The TAQ polymerase has no proofreading, and should therefore only be used for size determination of DNA fragments. When PCR-product is to be purified and used for further experiments always use Pfu polymerase!!! Make enough premix for your number of colony +1.

Protocol
Colony PCR:

1. Select and transfer a single colony to a PCR tube with a pipette tip (afterwards use the same for plating out on plates)

2. Add all of the H_2O used in the premix to the PCR tubes and place them in the microwave at full power for 2 min. with an open lid.

3. Make the premix (without water). Do not add enzyme until just before premix is added to the PCR tubes. Mix the premix by pipetting up and down (do not vortex!)

4. Add premix to each PCR tube.

5. Run PCR

PCR of DNA in solutions:

1. Transfer 1-2 µL of DNA to each PCR tube (to obtain the correct total volume adjust the volume of the H_2O)

2. Make the premix(do not add enzyme until just before premix is added to the PCR tubes). Mix the premix by pipetting up and down (do not vortex!)

3. Add premix to each PCR tube.

4. Run PCR.

PCR program:

Other programs have been used as well

Pfu-PCR

1	Start	95°C	2 min
2	Denaturing	95°C	30 sec
3	Annealing	54°C	30 sec
4	Elongation	72°C	1 min
5	GOTO 2		rep. 29x
6	End	72°C	5 min
7	Hold	4°C	

TAQ-PCR

1	Start	94°C	2 min
2	Denaturing	94°C	1 min
3	Annealing	55°C	1 min
4	Elongation	72°C	2min
5	GOTO 2		rep. 29x
6	End	72°C	5 min
7	Hold	4°C	

CP1.2

Updated Taq protocol for determination of product size. Due to Taq's lack of proofreading, only use this protocol for determining sizes.

Protocol:
1. A single colony is transfered to each eppendorf tube with a pipette tip. (The same tip is used to plate out on a LA+antibiotic plate afterwards)
2. Add 30 ul H2O to each tube.
3. Microwave with open lid at full power for 2 minutes.
4. Prepare Pre-Mix (number of colonies+1) Distribute 19ul to each tube.
5. shortly spin down PCR tubes
6. Load and set PCR machine
7. Add TAQ polymerase at last moment. Make sure to get it under the surface of the solution.
8. Run PCR reaction.

Pre-mix:
5ul 10x TAQ buffer
2ul MgCl2 (Increase in 0.25ul incriments if the DNA you want to extract is longer than 3kb.)
2ul 10pmol/ul forward primer
2ul 10pmol/ul reverse primer
1ul 10mM dNTP mix
7ul H2O

1ul TAQ polymerase -> NB! Pre-Mix is made without TAQ polymerase!
PCR program

1	Start	94°C	2min
2	Denaturing	94°C	1min
3	Annealing	55°C	1min
4	Elongation	72°C	2min
5	GOTO 2		rep. 29x
6	End	72°C	3min
7	Hold	4°C	-

Elongation time is adjusted according to the length of the template. (1 min for every 1Kb)
CP1.3

Updated Taq protocol for size determination. Due to Taq's lack of proofreading, only use this protocol for size determination.

Protocol:
1. A single colony is transfered to each eppendorf tube with a pipette tip. (The same tip is used to plate out on a LA+antibiotic plate afterwards)
2. Add 15 ul H2O to each tube.
3. Microwave with open lid at full power for 2 minutes.
4. Prepare Pre-Mix (number of colonies+1) Distribute 9.5ul to each tube.
5. shortly spin down PCR tubes
6. Load and set PCR machine
7. Add TAQ polymerase at last moment. Make sure to get it under the surface of the solution.
8. Run PCR reaction.

Pre-mix:
2.5ul 10x TAQ buffer
1ul MgCl2 (Increase in 0.25ul increments if the DNA you want to extract is longer than 3kb.)
1ul 10pmol/ul forward primer
1ul 10pmol/ul reverse primer
0.5ul 10mM dNTP mix
3.5ul H2O

0.5ul TAQ polymerase -> NB! Pre-Mix is made without TAQ polymerase!
PCR program

1	Start	94°C	2min
2	Denaturing	94°C	1min
3	Annealing	55°C	1min
4	Elongation	72°C	2min
5	GOTO 2		rep. 29x
6	End	72°C	3min
7	Hold	4°C	-

Elongation time is adjusted according to the length of the template. (1 min for every 1Kb)
--Tipi 07:40, 22 July 2010 (UTC)

Making competent cells of *E. coli* for transformation – SDU-Denmark – 2010
http://2010.igem.org/Team:SDU-Denmark/protocols
CC1.1

How to make competent cells for transformation

Compotent cells enough for 12 transformations

Important remarks
Use 2 ml eppendorf tubes

Cool eppendorf tubes at 4°C prior to use

Cool 50 ml 50 mM CaCl2 at 4°C prior to use

Materials
• Overnight culture of TOP10 cells in LB media

• Ice cold 50mM CaCl2

• LB media (pre-heated to 37°C)

Protocol
1. Dilute the culture to OD550 = 0,02 in 110 ml of LB. Incubate at 37°C with shaking until OD550 reaches 0.5

2. Divide the cells in 2x55 ml and transfer to Falcon tubes (the can hold only 55 ml). *From now on proceed with the 2 tubes in parallel*

3. move the CaCl2 to -20°C

4. Harvest cells by centrifugation at 4100 rpm (2160 G) at 4°C for 10 min.

5. Discard the supernatant (keep the cells on ice!)

6. Resuspend cells gently in 5 ml ice cold CaCl2 (50 mM) taken from -20°C and kept on ice.

7. Repeat the centrifugation step.

8. Discard the supernatant and resuspend cells in 1.2 ml of icecold CaCl2 (keep the cells on ice!)

9. Leave the cells on ice for 30 min => now the cells are ready for transformation.

Transformation – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
TR1.1

Important remarks
Pre-heat LB media to 37°C

Pre-dry LA plates with the appropriate antibiotics.

Pre-cool 2 mL eppendorf tubes.

Keep cells on ice at all times!!

Remember controls:
Positive control with your uncut vector

Negative control with no inserted DNA

Materials
• Freshly made compotent *E. coli* cells.

• LA plates with appropriate antibiotics

• LB media

Protocol
1. Transfer 5 µl DNA (plasmid or ligation mix) to precooled eppendorf tubes. (Use only 1ul if DNA is taken from distribution plates.)

2. Transfer 200 µl of competent *E. coli* cells to the tube and mix by pipetting up and down **(keep the cells on ice at all times)**

3. Leave on ice for 30 min.

4. Heat shock for 90 sec. at 42°C in a water bath, do not shake tubes.

5. Place on ice for 2 min.

6. Add 1.5 mL of preheated LB media (37°C)

7. Incubate at 37°C for 1 hour with gentle shaking.

8. Plate 2 plates with 150 µl mixture on LA plates with the appropriate antibiotics.

9. Pellet the remaining cells 5 min at 3500 rpm and discard approximately 900 µl of the supernatant.

10. Resuspend cells and plate out on LA plates with appropriate antibiotics.

11. Incubate all plates ON at 37°C
Restriction digest – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
RD1.1

How to digest DNA using Fermentas fast digest restriction enzymes.

Important remarks
Remember to load 2-5µL uncut product next to the marker and take a picture of this for later documentation.

Materials
Restriction mixture:

For 1 digest reaction.

- 12 μL H_2O (or 13 μL if only one restriction enzyme is used)

- 1 μL enzyme A

- 1 μL enzyme B

- 2 μL Fast Digest green buffer

- 5 μL PCR product

Multiply restriction mixtures if more digested PCR product is needed

Protocols
1. Cast an agarose gel of suitable percentage for purification of the cut product

2. Mix the restriction mixture in en eppendorf tube by pipetting up and down

3. Leave for 5 min. at 37°C (no shaking!)

4. Immidiately load the restriction mixture in the gel

5. Run the gel and cut out and purify correct sized bands.

Ligation – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
LG1.2

How to assemble DNA biobricks

Materials
Ligation mixture:

For 1 ligation reaction

- 2 μL 10x T4 DNA ligase buffer

- 1 μL T4 DNA ligase (add last!)

- 5 μL PCR product (cut) of each brick which is to be ligated – or 1 part plasmid and 5 part bricks

- Add H_2O to reach a total volume of 20μL

Protocol
1. Prepare the ligation mixture and mix by pipetting up and down

2. Leave the mixture overnight at 17°C

2a. If there is no time leave the ligation solution at 22.5°C for 30mins. Then denature the ligase at 65°C

for 10min.

3. Use ligation solution for transformations
--Tipi 06:48, 20 July 2010 (UTC)
DNA extraction from gel – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
DE1.1
Gel extractions were done according to the protocol of Fermentas with the exception that we introduced an additional centrifugation step after washing to remove surplus ethanol.
DE1.3
Gel extractions were done according to the protocol of GE Healthcare with the exception that we introduced an additional centrifugation step after washing to remove surplus ethanol.

Genomic DNA purification – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
GP1.1

How to extract and purify genomic DNA

Important remarks
All steps should be carried out at room temperature.

Be sure to mix thoroughly when adding the solutions.

Addition and removal of chloroform should be carried out in a fume hood.

Materials

- Lysis solution
- Chloroform
- Precipitation solution (80 µL is diluted in 720 µL of H2O just prior to use)
- 1.2M NaCl solution
- Ice cold ethanol (70%)
- H2O

Protocol
1. Mix 200 µL of sample (overnight culture) with 400 µL Lysis solution and incubate at 65°C for 5 min.*If a frozen sample is used lysis solution should be added before thawing and incubated at 65°Cfor 10 min. with occasional inverting the tube.*

2. Immediately add 600 µL of chloroform, gently emulsify by inversion (3-5 times) and centrifuge the sample at 10.000 rpm for 2 min.

3. Prepare precipitation solution.

4. Transfer the upper aqueous phase containing DNA to a new tube and add 800 µL of freshly prepared precipitation solution, mix gently by several inversions at room temperature for 1-2 min. and centrifuge at 10.000 rpm for 2 min.

5. Remove supernatant completely (do not dry) and dissolve DNA pellet in 100 µL of 1.2M NaCl solution by gentle vortexing (make sure that the pellet is completely dissolved) *To avoid loosening the pellet, keep the tube in the same angle as when placed in the centrifuge!*

6. Add 300 µL of cold ethanol, let the DNA precipitate (10 min. at -20°C) and spin down (10.00 rpm, 3-4 min.).*Pour off the ethanol and dissolve DNA in 15 µL of sterile dH2O by gentle vortexing.*

7. Measure DNA concentration on nanodrop.

8. Store DNA at -20°C

Plasmid miniprep kit

MP1.1

Plasmids are isolated from cultures by transferring 5 mL overnight culture to a 15 mL falcon tube and spinning down at 4000g for 15 min and removing supernatant.

Then the GeneJet Plasmid miniprep kit from Fermantas was used according to manufacturers recommendations.

MP1.2

Plasmids are isolated from cultures by transferring 10 mL overnight culture to a 15 mL falcon tube and spinning down at 4000g for 15 min and removing supernatant.

Then the GeneJet Plasmid miniprep kit from Fermantas was used according to manufacturers recommendations.

MP1.3

Plasmids are isolated from exponential growing cultures by:

1. Transferring 2.5mL overnight culture to 15mL preheated LB medium and growing cells for an additionally 2 hours.

2. Transfer all 17.5mL new culture to a 50mL falcon tube and spin down at 4000g for 15 min.
Proceed with the the GeneJet Plasmid miniprep kit from Fermantas was used according to manufacturersrecommendations.

Preparation of Agarose gelmix for gel electrophoresis – SDU-Denmark – 2010

http://2010.igem.org/Team:SDU-Denmark/protocols

AG1.1

Important remarks

Agarose concentration is dependent on the size of the DNA fragment that needs to be seperated (see the door of the incubator in the gel room)

Addition of EtBr is carried out in a fume hood.

Materials

• Seachem Agarose

• TAE buffer

• EtBr

Protocol

1. For a 1% agarose gel mix 3 g agarose and 300 mL TAE buffer in a 500 mL flask.

2. The mixture is heated for 5 min. at max temperature in micro-wave. *Remember to note name, date and –EtBr on the flask.*

3. Place flask in the incubator for 20 min or at room temperature until cooled to 60°C.

4. Add 5 droplets of EtBr and mix.

5. Cast gel and leave for 20 min until the gel is set. *Remaining agarose solution is placed in incubator for later use.*

6. Load and run gel. *Load 5 μL of DNA marker*

Extraction af Carotenoids and polyene chromophores – SDU-Denmark – 2010

http://2010.igem.org/Team:SDU-Denmark/protocols

EX1.1

1. Incubate *E. Coli* in 110 ml LB media with appropriate antibiotics at 37 degrees celcius for 20 hours

2. Harvest cells by centrifugation at 4000g for 15 min

3. Resuspend cells in 8 mL acetone and sonicate the sample for 2x 30 sek

4. Centrifuge the samples at 16000g for 2 min and collect 2 mL of the supernatant

5. Measure absorbance using UV-Vis spectrophotometer at 450 nm

EX1.2

1. Incubate *E. Coli* in 110 ml LB media with appropriate antibiotics at 37 degrees celcius for 20 hours

2. Harvest cells by centrifugation at 4000g for 15 min

3. Re-suspend cells in 8 mL acetone and sonicate the sample for 2x 30 sek

4. Centrifuge the samples at 16000g for 2 min and collect 2 mL of the supernatant

5. Measure absorbance using an HPLC at 450 nm for bata-carotene and 382 nm for retinal analysis, For this particular purpose, we use a Poroshell 120 EC-C18 (4,6 x 150 mm 2,7 micron)column, and the eluents used are as follows:
A-buffer: 100% methanol with 0,1% trifluoroacetic acid.
B-buffer: A mixture consisting of 60% methanol and 40% acetone with 0,1% trifluoroacetic acid.
Due to the chemical properties of beta-carotene and retinal, respectively, retinal will come through the column before beta-carotene when a gradient is run from 100% A-buffer to 100% B-buffer.
Afterwards, the solutions of purified retinal or beta-carotene are studied using UV-vis photospectrometry and the values and spectra are compared to those of the same compounds of known concentrations.
Again, this gives both qualitative and quantitative indications of whether the compound in question is present and, if it is, in what concentration. Usage of the HPLC and instruction on how to use it was kindly provided by FLINT

Photosensor characterisation – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocolsPS1.1

Materials

- Diluted LB media
- Difco Agar
- 1μM retinal (final concentration)

Swimming motility plates

1. LB media is mixed with 0.3% difco agar and autoclavated

2. The appropriate antibiotic and 1µM retinal (final concentration) is added to the autoclaved media (NB: for the media used for the control plates, no antibiotic or retinal is added)

3. Plates are cast and incubated overnight at room temperature

4. 15 minutes prior to the experiment the plates are dried at 37°C.

Sample preparation
1. 5mL LB media containing the appropriate antibiotic is inoculated with a colony. The culture is grown overnight at 37°C and 180rpm.
2. The overnight culture is diluted in 5mL LB media containing the appropriate antibiotic to reach an OD550 of 0.02 and are incubated at 37°C and 180rpm until it reaches an OD550 of 0.5.
3. 1µM retinal (final cnocentration) is added to the culture (NB: no retinal is added to the cultures containing the control cells)
4. The tubes containing the cultures are wrapped in tin foil (creates darkness) and are subsequently grown for 2 hours at 37°C and 180rpm.

Motility assay
1. 2x2.5µL of culture is placed on each plate, and the plates are placed in a specially engineered lightbox, so that ½ of each plate is illuminated with blue light and the other ½ is kept in dark.
2. The plates are incubated at 37°C for 24 hours.
3. Pictures are taken

Microscopy
1. 5µL of bacterial culture is placed in the center of a microscopy slide dimensions 7.5cm x 1.5cm and a cover slide is used to cover the culture.
2. To eliminate any flow in the system, which can be mistaken for bacterial motility, the cover slide is sealed with ordinary mail polish.
3. Samples are examined under the microscope.
PS1.2
A protocol for optimizing the motility of E.coli MG1655 to use for microscopy
This protocol is designed based on preceeding pilot studies
Materials:
• LB media
• Motility buffer (20mM potassium phosphate and 0.1mM EDTA dissolved in ddH2O [1]
• 1mM retinal
Protocol: 1. A colony is inoculated in 5mL LB media with appropriate antibiotic.
2. The culture is incubated for 12h. at 22° and 160rpm
3. The cultures containing the unmodified bacteria (controls) is then diluted 1:10 in motility buffer, at which point these are ready for microscopy
4. The culture containing the modified bacteria is added 1uM retinal (final concentration), and is incubated for an additional 2h in darkness at 22°C and 160rpm.
5. The culture is then diluted 1:10 in motility buffer and are ready for microscopy.
Microscopy
1. 5µL of bacterial culture is placed in the center of a microscopy slide dimensions 7.5cm x 1.5cm and a cover slide is used to cover the culture.
2. To eliminate any flow in the system, which can be mistaken for bacterial motility, the cover slide is sealed with ordinary mail polish.
3. Samples are examined under the microscope.

Growth rate assay – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
GA1.1
Materials
• LB media
• Spectrophotometer

Protocol
1. 5mL LB media containing the appropriate antibiotic is inoculated with a colony .
2. The culture is incubated over night at 37°C and 180rpm.
3. The optical density at 550nm (OD550) of the overnight culture is measured and the culture is diluted in 15mL fresh LB media containing the appropriate antibiotic to reach an OD550 of 0.02. The culture is then incubated at 37°C and 180rpm.
4. OD550 of the colony is measured every hour for the first 12 hours, and after 24 hours.
Flagella staining – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols

The following protocols are based on knowledge recived from [2]
FS1.1
Day 1: The bacteria were grown in 5 ml-LB media overnight. The solutions used for staining were prepared.

Solution I: The following components were added in the listed order:
• 5 g of tannic acid dissolved in 9.65 ml distilled water.
• 150 µl 9% $FeCl_3$
• 100 µl 1% NaOH
• 200 µl formalin

Solution II:
• 2 g silver nitrate was dissolved in 10 ml distilled water
• 10% aqueous ammonia solution was added until the silver nitrate was dissolved. Approximately 2 ml.

Day 2: The bacteria were boosted in 5 ml LB-media to ensure that they were in the exponential growth phase when used for staining. They were diluted to approximately an OD550 of 1.

Preliminary bacteria work:
• The bacteria were centrifuged 15 min at 4000rpm
• The pellet was resuspended in LB-media to an OD550 of 3.

Staining protocol:
• A clean glass slide was used and Poly-L-Lysin was added onto a small area.
• 20 µl of the bacteria solution was plated on the slide and allowed to air dry.
• The slide was flooded with solution I and allowed to stand for 30 min before it was washed with distilled water.
• Solution II was added and was incubated at room temperature for 10 min and was washed with distilled water.
• The slide was flooded with a carbol-fuchsin solution and air-dried before washed with distilled water.

PBS were added to the area containing the bacteria and they were covered with a cover slide. The slides are now ready for examination under the microscope.
FS1.2

Day 1: Bacteria were platede on agar plates and incubated at 37 degrees celcius overnight. The staining solutions were prepared.

Solution I:
The following components were added in the listed order:
• 5 g of tannic acid was dissolved in 9.65 ml distilled water.
• 150 µl 9% $FeCl_3$
• 100 µl 1% NaOH
• 200 µl formalin

Solution II:
• 2 g silver nitrate was dissolved in 10 ml distilled water.
• 10% aqueous ammonia solution was added until the silver nitrate was dissolved. Approximately 2 ml.

Day 2: A bacterial colony was dissolved in LB-media.

Staining protocol:
• A clean glass slide was used and Poly-L-Lysin was added onto a small area.
• 20 µl of the bacteria solution was plated on the slide and allowed to air dry.
• The slide was flooded with solution I and allowed to stand for 30 min before it was washed with distilled water.
• Solution II was added and was incubated at room temperature for 10 min and was washed with distilled water.
• The slide was flooded with a carbol-fuchsin solution and air-dried before washed with distilled water.

PBS were added to the area containing the bacteria and they were covered with a cover slide. The slides are now ready for examination under the microscope.
Stability assay – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols
SA 1.1
Stability assay

Materials

• LB media
• LA plates
• LA plates with appropriate antibiotic
• 0.9% NaCl

Protocol

1. 5mL LB media with appropriate antibiotic is inoculated with a bacterial colony.
2. The culture is incubated over night at 30°C and 180rpm
3. 100µL of the culture is serially diluted in 900µL 0.9% NaCl until a dilution of 10^7 is reached.
4. 100µL of the 10^5, 10^6 and 10^7 dilutions are spread onto LA plates, and LA plates with the appropriate antibiotic, respectively.
5. Plates are incubated at 37°C for 16-24 hours. The following day the colonies formed on the plates are counted and cfu are determined.
6. 500µL of the 10^5 dilution is added to 4.5mL of fresh LB media without any antibiotics.
7. The new culture is incubated over night at 30°C and 180rpm
8. The experiment is carried out for 5 days.

(NB: antibiotic is only added to the first culture. The remaining days the bacteria are grown in cultures without any antibiotics)

Scanning Electron Microscope – SDU-Denmark – 2010 http://2010.igem.org/Team:SDU-Denmark/protocols

SEM 1.1

Day 1: The bacteria was cultivated overnight in 5 ml LB-media at 37 degrees celcius.

Day 2: The overnight culture was centrifuged 15 min at 4000prm. Afterwards the pellet was resuspended in distilled water. We aimed to get approximately 10^6 bacteria in 10 µl solution which was plated on double adhesive tape at the top of the grid. The solution was allowed to air dry and the remaining fluid disappeared as samples were exposed to the vacuum in the electron microscope.

The sample was examined with different electron intensity and magnification. We found that the best picture was taken with a electron intensity of 10 kV and a magnitude on 10kx.

Charactarization of K389016 (VirA/G reporter device mRFP)

CK1.1

Materials:

• 20mM acetosyringone (39.3 acetosyringone dissolved in 1mL DMSO and 9mL ddH2O)
• LB media
• 35µg/mL chloramphenicol

Protocol:

1. A colony is inoculated in 5mL LB media with 35µg/mL chloramphenicol and incubated over night at 37°C and 180rpm.
2. Parallel cultivations of 25mL LB media, 35µg/mL chloramphenicol and acetosyringone concentrations of 0uM (control), 100uM, 200uM and 400uM respectively.
3. The cultures were incubated in a waterbath of 37°C and 180rpm
4. The optical density at 550nm (OD550) was measured every two hours and samples were freezed at -80°C and used for fluorescence measurements (excitation at 584nm, emmitation at 607nm)

Scanning EM – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list

This is a standard method for preparing samples for Scanning Electron Microscopy. Specimens vary greatly and the final protocol should be decided by referring to text books and publications.

Fixation

Fix in 2% Gluteraldehyde in Sorensons Phosphate Buffer overnight (minimum).
Rinse in Sorensons Phosphate Buffer 2x 15 mins.

Dehydration

25% ethanol: 30 mins.
50% ethanol: 30 mins.
75% ethanol: 30 mins.
100% ethanol: 1 hr.
100% ethanol: 1 hr.
Once in 100% ethanol, samples are dried using a Baltec Critical Point Dryer.

Mounting

Mount on an aluminum stub with Achesons Silver ElectroDag.

Gold coating

Coat with 15 nm of gold using a Polaron SEM Coating Unit.

IPTG Induction – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Aim
The aim of this protocol is to prepare slides for the *Bacillus subtilis* 168 cells which have *yneA* integrated into the chromosome and are induced by IPTG at various concentrations.
Materials
Flasks
Colonies of *Bacillus subtilis* 168 cells with different plasmid insertions.
Inoculation Loop
Shaking incubator
1M IPTG stock solution
Appropriate antibiotic solutions
1.2% agarose
12 well glass slide
LB broth
Spectrophotometer
Protocol
Take 20 ml of LB broth with appropriate concentration of antibiotics in a flask and inoculate it with a particular colony of *Bacillus subtilis* 168 cells with a particular plasmid insertion.
Put it onto a shaking incubator at 37°C overnight.
Next morning, check the OD_{600} by using spectrophotometer and record OD for future references and to check whether all the cells are in the same growth phase or not.
Now, in a flask add 10ml of LB broth with an appropriate concentration of antibiotics and add 100µl of the appropriate overnight culture in it.
Measure OD_{600} after every 30 minutes and record it every time.
In the meantime, prepare flasks with proper labels and also dilute 1M stock solution of IPTG to a range from 0.02mM, 0.2mM, 1mM and 2mM IPTG solutions.
When the OD of all the cell population comes to 0.1 then take 5ml of the sample from the flask and transfer it to a universal tube and add appropriate amount of the IPTG solution in it. In this way we will have 4 universal tubes for a particular sample having different concentrations of IPTG.
Incubate the cells at 37°C on a shaking incubator for 120 minutes and in-between time; check the OD of the cell culture after every 30 minutes.
After 120 minutes put the cell cultures out on the bench at room temperature and start preparing the slide.
For slide preparation, first clean the slide with 100% ethanol.
Now make 10 ml of 1.2% agarose solution (agarose and water solution).
Now on the slide add 500µl of the agarose solution and but a long coverslip over it and be careful that all the wells are been filled up by the agarose solution.
After 5 minutes, remove the coverslip by sliding it horizontally so that we don't disturb the agarose layer.
Now we take 0.5µl of each sample and load it in each well separately and put the coverslip back on the slide.
Observe the slide using normal phase contrast or fluorescent microscope.

Extreme Base Resistance Test – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Aim
The aim of this experiment is to test that the chassis is able to acclimatize to the increase in the environmental pH.
Materials Required
Bacillus subtilis 168 colony plates

Potassium modified Luria broth (LBK)
HEPES (a dibasic compound which would act as a buffer)
Conical flasks
Procedure
Day 1
Grow *B. subtilis* 168 cultures in LBK buffered with 100mM HEPES at pH 7 and incubate them overnight at 37 °C for 16 hours.
Day 2
Make 2 different LBK solutions buffered with 50mM HEPES at either pH 7 or pH 9.
Dilute the overnight cultures by 100 fold in the LBK solution mentioned above.
Let the bacteria grow until it reaches at the OD600 of 0.3.
Prepare a LBK solution buffered with 50mM HEPES at pH 10.
Dilute the cultures by 200 fold in the LBK broth mentioned in the above step and incubate them for 2 hours at 37 °C.
Plate serial dilutions of the culture mentioned in the above step on a LBK plate and also plate the original culture in the medium at pH 7.
Let the cells to grow and see the growth of the colony next morning.

Slide Preparation – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Grow overnight culture in 5 ml of LB broth, with appropriate antibiotics if required, at 37°C.
Dilute the overnight culture to a ratio of 1:100 (overnight culture: LB with appropriate antibiotic).
Incubate at 37°C for 1 hour.
Set up the following broths with different concentrations of IPTG in 5 ml of LB broth:
2mM IPTG
1mM IPTG
0.2mM IPTG
0.02mM IPTG
Broth only
Inoculate the broth containing the different concentrations of IPTG with the appropriate cultures and incubate at 37°C for two hours.
Prepare the slides by pipetting 500 µl of 1.2% agarose. Gently place the coverslip over the slide.
Wait for about 5 minutes for the agarose to harden. Remove the coverslip by gently sliding the coverslip horizontally from the slide. This is to ensure that agarose layer remains undisturbed.
Transfer 200 µl of each sample to microfuge tubes and label accordingly. Add 0.5 µl of membrane dye to each sample.
Place 0.5 µl of each sample in each well of the slide and label accordingly. Place a coverslip on top of the slide.
The slide is now ready for microscopy.

Making glycerol stocks – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials
1.5ml microcentrifuge tubes
Glycerol
Protocol
Inoculate 5ml THY Broth with a single colony and incubate, stationary at 37°C for 8 hours.
0.5ml culture mixed with 0.5ml of sterile 50% glycerol (filter sterile).
Store at -80°C in microcentrifuge tubes.

LB agar plates – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
The materials mentioned below are used to make up 1 liter of the standard LB agar plates:
10 g of tryptophan
5 g of yeast
5 g of NaCl
15 g of agar
The materials mentioned are used to make up 1 liter of LB starch agar plates:
10 g of tryptophan
5 g of yeast
5 g of NaCl
15 g of agar
10 g of starch
Protocol
Dissolve the materials in 800 ml of distilled water. (Note: The agar will not dissolve in distilled water.)
After most of the materials are dissolved, top up the final volume to 1 liter with distilled water.
Autoclave the mixture.
After autoclaving, allow the mixture to cool down before adding the appropriate antibiotic.
Pour appropriately 25 ml of the mixture into petri dishes.
Note: Aseptic technique must be employed after the autoclave part.

Urease test – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
Pipettes
Universal tubes
Streaking loop
Christensen's Urea Agar
Make up 1 liter of the agar mixture containing:
Gelatin peptone 1.0 g
Potassium dihydrogen phosphate 2.0 g
Sodium chloride 5.0 g
Agar 20 g
Top up to 1 liter and apply gentle heating to dissolve. Sterilize at 115°C for at least 20 min.
Add the following to the molten base:
D(+)-Glucose 1.0 g
Phenol red, 0.2% 6 ml
Note: Ensure that the work was done using aseptic technique.
Transfer 10 ml of the molten base to universal tube and allow the agar to set in a slanted position.
Store the harden agar in the fridge.
Procedures
Perform the experiment using aseptic technique.
Pick up single colony of *B. subtilis* 168 and streaking it onto the slanted agar tube.
Loosen up the cap to allow air exchange between the interior of the tube and the external environment.
Incubate the tubes overnight at 37°C.
Set up the tubes as indicated:
Negative control - Without *B. subtilis* 168

Test 1 (Duplicate) - Inoculated with *B. subtilis* 168
Test 2 (Duplicate) - Inoculated with *B. subtilis* 168

Arginine Test – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials Required
Plate containing *Bacillus subtilis* 168 colonies.
Flame (streaking) loop
LB media consisting of arginine
Auto pipette
Bunsen Burner
Universal Tube
pH indicator paper
Procedures
Perform the experiment using aseptic technique.
Transfer *B. subtilis* 168 colonies into universal tubes containing 5 ml of LB media and allowed to grow overnight at 37° C.
Transfer 1 ml of the overnight culture to another universal tube containing 4 ml of the following media:
Control (1) - LB media
Control (2) - LB media with 10 mM of arginine
Control (3) - LB media plus *B. subtilis* 168
Test (1) - LB media with 10 mM of arginine plus *B. subtilis* 168
Test (2) - LB media with 10 mM of arginine plus *B. subtilis* 168
Incubate the culture at 37° C with shaking.
Record the pH at every 30 min interval. Use 20 ul of the culture and measure the pH using the pH indicator paper.

DNA extraction – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials Required
Cells grown from yesterday
Centrifuge
Pipette
Lysozyme
Cell lysis solution
RNase solution
Protein precipitation solution
DNA hydration solution
Isopropanol
70% ethanol
Procedures
Cell lysis
Pellet cells by centrifugation at 3600 rpm for 10 minutes.
Pour off supernatant.
Add 0.5 ml of cell suspension solution, gently pipet up and down to Resuspend and transfer to 1.5 ml eppendorf tube.
Add 25 µl of lysozyme and invert tube 25 times.
Incubate for 30 minutes at 37°C while inverting the tube occasionally.
Centrifuge at 13000 rpm for 10 minutes to pellet the cells, and then remove the supernatant.

Add 0.5 ml of cell lysis solution to the cell pellet and gently pipet up and down to lyse the cells.
Heat sample for 30 minutes and mix every 5-10 minutes.
RNase treatment
Add 3 µl of RNase A solution to the cell lysate
Mix by inverting 25 times and incubate at 37°C for 60 minutes
Protein precipitation
Cool samples on ice.
Add 0.5 ml of protein precipitation solution to each tube.
Invert the tubes to mix the protein precipitation solution uniformly with the cell lysate.
Place samples on ice for 5 minutes.
Centrifuge at 13000 rpm for 30 seconds or until the precipitated proteins form a tight pellet.
DNA precipitation
Pour the supernatant containing the DNA into a clean eppendorf tube. (The samples may be kept at -20°C overnight at this stage.)
Add 0.5 ml isopropanol to each tube.
Mix by inverting gently for 50 times.
Centrifuge at 13000 rpm for 1 minute. The DNA should be visible as a small white pellet.
Pour off the supernatant and drain the tube on a clean absorbent paper.
Add 0.5 ml of 70% ethanol and invert the tube several times to wash the DNA.
Centrifuge at 13000 rpm for 1 minute. Carefully pour off the ethanol.
Drain the tubes on clean absorbent paper. Allow to air dry for 10-15 minutes.
DNA hydration
Add 100 µl DNA hydration solutions to each tube.
Rehydrate DNA by incubating the sample for 1 hour at 65°C, followed by overnight incubation at room temperature. Tap the tube periodically to aid in dispersing the DNA.
For storage, centrifuge briefly and store at -20°C.

Transformation of *Bacillus subtilis* – Newcastle – 2010
http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
Bacillus subtilis 168
10 µl of transformation DNA
Pipettes
2X 15 ml falcon tubes
2X 50 ml falcon tubes
Eppendorf tubes
Agar plates containing the appropriate antibiotic
Water bath
Centrifuge
SMM medium (1 liter)
2.0 g of ammonium sulphate
14.0 g of dipotassium hydrogen phosphate
6.0 g of potassium dihydrogen phosphate
1.0 g of sodium citrate dehydrate
0.2 g of magnesium sulphate
Top up the rest of the medium with water
MM competence medium
10 ml of SMM medium
125 µl of Solution E (40% glucose)

100 µl of Tryptophan solution (at a concentration of 2 mg/ml) –
60 µl of Solution F (1M $MgSO_4$)
10 µl of 20% Casamino acids
5 µl of 0.22% Fe-NH_4-citrate
Starvation medium
10 ml of SMM medium
125 µl of Solution E (40% glucose)
60 µl of Solution F (1M $MgSO_4$)
Protocol
This protocol will stretch for 2 days and aseptic technique has to be applied for all steps.
Protocol for Day 1
Inoculate a single colony of *Bacillus subtilis* 168 into a 15 ml falcon tube containing 10 ml of MM competence media.
For control, transfer 10 ml of MM competence media without the bacteria.
Incubate overnight in a shaking incubator at 37ºC.
Protocol for Day 2
Transfer 0.6 ml of the overnight culture into 50 ml falcon tube containing 10 ml of MM competence medium.
Incubate the tubes for 3 hours at 37ºC.
Warm up the starvation medium to 37ºC in a water bath.
Add 10 ml of starvation medium (prewarmed) into each tube and incubate for a further 1-2 hours at 37ºC.
Transfer 0.4 ml of the medium not containing *B. subtilis* into one Eppendorf tube.
Add 10 µl of water into the tube.
Transfer 0.4 ml of the medium containing *B. subtilis* into two Eppendorf tubes.
Add 10 µl of DNA into one tube.
Add 10 µl of water into one tube.
Incubate the samples for 1 hour at 37ºC in the shaking incubator.
The Eppendorf tubes have to be aerated, therefore incubate the tubes on their side.
Centrifuge the Eppendorf tubes at 13,000 rpm for 2 minutes.
Discard 0.3 ml of supernatant from each Eppendorf tubes.
Resuspend the pellet in the remaining supernatant and plate it onto agar plates containing the appropriate antibiotic.
Incubate the plates overnight at 37ºC.

Nano Drop Spectrophotometer – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Nanodrop uses absorbance to measure the concentration and purity of DNA, RNA and protein. The ideal concentration of DNA is 150 ng/ml.
Materials required
Pipettes
Nanodrop machine
Appropriate blanking solution
Appropriate samples
Procedures
Log onto computer and select Nanodrop program from the desktop (ND 1000)

Wipe the pedestal and top of the Nanodrop machine with a tissue. Place 3 µl of water to nib of pedestal and press blank to clean.

After blanking, wipe the water off and equalize the Nanodrop using 3 µl of the appropriate buffer used to resuspend the sample. (Example: Miniprep samples should be equalized with EB buffer).
Use DNA-50 for DNA samples.
Wipe to remove buffer and apply 3 µl of sample to nib. Press measure.
If dealing with multiple samples, clean the equipment with water at regular intervals (about every 10 samples)
After measurements, clean the equipment with 3 µl of water on the spectrometer and press blank. Wipe and log off.

QIAquick Gel Extraction Microcentrifuge Protocol – Newcastle – 2010
http://2010.igem.org/Team:Newcastle/Protocol_list
Materials
Scalpel
Eppendorf tubes
Pipettes
QIAquick column
UV transluminator
Buffer QG
Buffer PE
Buffer EB
Isopropanol
70% ethanol
Protocol
Before extraction, clean the UV transilluminator and scalpel with 70% ethanol.
Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. (Minimize the exposure of the gel to UV as much as possible.)
Weight the gel slice and add 3 volumes of buffer QG to 1 volume of gel (100 mg ~ 100 µl).
Incubate at 50°C and invert the tube gently at regular interval until the gel has completely dissolved.
After the gel has dissolved completely, check that the color of the mixture is yellow.
Add 1 gel volume of isopropanol to the sample and mix.
Place a QIAquick spin column in a 2 ml collection tube.
To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min.
Discard the flow through and place the QIAquick column back into the same tube (max volume: 750 µl).
Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min. Discard the flow through and place the QIAquick column back into the same tube.
To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min. Place the QIAquick column back into the same tube.
Centrifuge the column for a further 1 min.
Transfer the column into a clean 1.5 ml microcentrifuge tube.
To elute DNA, add 30 µl of Buffer EB to the center of the QIAquick membrane and allow to stand for 1 min.
Centrifuge the column for 1 min and transfer the eluate to a clean 1.5 ml micriocentrifuge tube.

To measure the purity of the sample, use a Nanodrop Spectrophotometer.

Gel electrophoresis – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list

Materials

50X TAE buffer

Make up 1 liter of 50X TAE buffer with the following:

242 g of TRIS base

57.1 ml of acetic acid

100 ml of 0.5 M of EDTA (pH 8.0)

Top up to 1 liter with water

SafeView

Agarose

DNA ladder

Eppendorf tube

Gel making tank

Gel running tank

Procedure

Make up 1% agarose gel (1 g of agarose per 100 ml of TAE buffer) and using the microwave to dissolve the agarose.

Allow the molten agarose to cool down before adding 5 µl of Safeview dye.

Set up the gel tray containing the gel comb and transfer 60 ml of molten agarose gel into the gel tray (take care not to cover the gel comb).

Wait for 30 min to allow the gel to harden.

Transfer harden gel into the gel tank and add TAE buffer until the gel is completely submerged.

Depending on the nature of the sample, 3 µl of GeneRuler™ 1 kb Plus DNA Ladder is used for analysing DNA.

Loading buffer is then added together with the sample before loading onto the gel matrix.

Run gel at 90V until the desired separation is achieved and visualize using the gelDoc.

Restriction digestion – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
Eppendorf tubes
Pipettes
Appropriate DNA/plasmid
Appropriate restriction enzymes
10X buffer
Water
Procedures
Add the following as mentioned below to make up to a final volume of 20 µl of reaction mix:
15 µl of DNA/plasmid
1 µl of restriction enzyme 1
1 µl of restriction enzyme 2
2 µl of 10X buffer
1 µl of water
Incubate the digestion mixture at 37°C for 3 hours
Notes
No more than 10% of enzyme should be used for a single reaction - glycerol inhibits reaction
10x buffer must be diluted to 1x i.e. 10% final volume.

Ligation – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Procedures
Set up the ligation mix according:

Reagents	**1:3(µl)**	**1:5(µl)**	**Vector(µl)**
Vector	1	1	1
Insert	3	5	N/A
10X BUFFER	1	1	1
T4 Ligase	1	1	1
H2O	4	2	7
Total Volume	10.0	10.0	10.0

For sticky end ligation, incubate the tube containing above mentioned chemicals on the bench for a period of 3-4 hours or overnight.
Important point to note is that 1:3 dilution means that during ligation, for 1 unit of vector, there are 3 units of insert and in 1:5 ligation, for 1 unit of vector there is 5 units of insert.
Notes
For standard ligation, 100-200 ng of vector DNA is required and this can be checked using nanodrop protocol (refer to Nanodrop Spectrophotometer protocol).
Ligation calculation can be used to calculate the amount of vector and insert required for the ligation but 1:3 or 1:5 ligation ratios are used more frequently.

Minipreps using the Qiagen kit – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list

Plasmid extraction
Materials required

Eppendorf tubes
Pipettes
Appropriate overnight cultures
Buffer P1 (In the fridge)
Buffer P2
Buffer N3
Buffer PB
Buffer EB
QIAprep spin column

Procedures
Overnight culture should have been done the day before. Refer to growing an overnight culture.
Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
Add 250 µl Buffer P2 and mix throughly by inverting the tube 4-6 times.
Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
Centrifuge for 10 minutes at 13,000 rpm in a table-top microcentrifuge.
Apply the supernatant to the QIAprep spin column by decanting or pipetting.
Centrifuge for 30-60 seconds. Discard the flow-through.
Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30-60 seconds. Discard the flow-through.
Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30-60 seconds.
Discard the flow-through, and centrifuge for an additional 1 minute to remove residual wash buffer.
To elute DNA, place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 50 µl Buffer EB or water to the center of each QIAprep spin column, let stand for 1 minute, and centrifuge for 1 minute.

Growing an Overnight Culture – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials Required
LB Broth
Colonies of bacteria on plate
Innoculation loop

Universal tube
Pipette
Procedures
Transfer 5 ml of LB or appropriate media into a universal tube.
Heat the innoculation loop till red hot to sterilize the loop.
Allow the innoculation loop to cool down for approximately 30 seconds.
The innoculation loop is then used to pick a single colony of bacteria on the agar plate.
Innoculate the LB or appropriate media with the bacteria.
Resterilize the loop.
The culture is then left overnight at 37°C in a shaking incubator at 200 rpm.

Transformation of *E. coli* – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
E. coli DH5α (200 µl)
Appropriate vector DNA
Heat block
Bucket of ice
Pipettes
Eppendorf tubes
1.5% agar plate containing appropriate antibiotics
Protocol
Thaw a 200 µl aliquot of *E. coli* DH4α and add the transforming DNA (10 ng of vector DNA in 10 µl).
Incubate on ice for 30 minutes.
Heat-shock the cells for 120 seconds at 42°C, and place on ice again for 3-4 minutes.
Add 1 ml of LB broth and incubate the cells at 37°C for 1-1.5 hr in a water bath with gentle shaking.
Plate 200 µl of transformed *E. coli* onto 1.5% agar plate containing the appropriate selection markers.
Incubate the plates overnight at 37°C.

Preparing competent *E. coli* – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
Conical flask
300ml of LB broth
1/20 volume of an overnight culture of the desired strain
Ice and ice bucket
eppendorf tubes
TFB1
TFB2
1.5 ml Microfuge tubes
Ethanol dry ice
-80°C freezer
Preparation of 100 ml of TFB1

Number	Chemicals required	Volume
1	KAc	30 mM
2	CaCl2	10 mM
3	KCl	100 mM

4	Glycerol	15% (v/v)
5	Distill water	900 ml
6	500 mM MnCl2.4H2O	100 ml

Preparation of 100 ml of TFB2

Number	Chemicals required	Volume
1	CaCl2	75 mM
2	KCl	10 mM
3	Glycerol	15% (v/v)
4	Distill water	900 ml
5	Na-MOPS pH 7.0	100 mM

Procedures

Inoculate 300 ml of LB broth in a conical flask and inoculate with 1/20 volume of an overnight culture of the desired strain.

Grow the cell at 37°C, 200 rpm in an orbital incubator.

Chill cells by placing the flask on ice and harvest by centrifugation at 4°C for 10 minutes.

Resuspend the pellet in 100 ml of ice cold TFB1 and gently shake the tubes whilst placed on ice.

Repeat the above mentioned step and carefully resuspend pellet in 20 ml ice cold TFB2.

Aliquot 200 µl volumes of the cell suspension into cold sterile microfuge (1.5 ml) tubes and flash freeze in dry-ice .

Store at -80°C.

Gibson Cloning – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list

Background

This is a one-step isothermal reaction method for assembling overlapping DNA fragments. Please see *Enzymatic assembly of DNA molecules up to several hundred kilobases*, Gibson et al. (2009).

Reaction buffer recipes

5X isothermal reaction buffer

5X final	**Amount**	**Stock concentration**
25% PEG-8000	0.75 g	powder
500 mM Tris-HCl pH 7.5	1.5 ml	1M
50 mM $MgCl_2$	75 ul	2 M
50 mM DTT	150 ul	1 M
1 mM dATP	30 ul	100 mM
1 mM dTTP	30 ul	100 mM
1 mM dCTP	30 ul	100 mM
1 mM dGTP	30 ul	100 mM
5 mM NAD	300 ul	50 mM
H_2O		Make up to 3 ml final volume

1.33X Master Mix

1.33X	Amount
5X isothermal buffer	100 ul
T5 exonuclease 1.0 U/ul	2 ul
Phusion DNA pol 2 U/ul	6.25 ul
Taq DNA ligase 40 U/ul	50 ul
H_2O	216.75 ul (375 ul final volume)

This makes 25 aliquots of 15 ul each. Store at -20°C. Although the mixture can be freeze-thawed and is stable for a year, we generally plan experiments to use an entire aliquot so they never go through the freeze-thaw cycle. The 1.33X Master Mix is in this case based on the volume of the Taq ligase from the manufacturer. We make the mix straight into the Taq ligase tube because it is important to work quickly with everything on ice. The T5 exonuclease may be difficult to accurately measure out and the water volume is great enough to allow for a 10X dilution so 20 ul of the dilution can be added to the mix.
PCR product purification
PCR products that come from a plasmid template need to be purified on a 1% agarose TAE gel (adjust the gel and well size to accommodate the entire PCR (Phusion) reaction. This procedure is generally a good idea to reduce unwanted assemblies from minor aberrant PCR products. The Qiagen MiniElute gel extraction kits have been successful for this purpose as the final volume/concentration is approximately right for the assembly protocol.
One-step isothermal assembly
For ~6 kb fragments, use 10-100 ng DNA of each DNA in 5 ul final volume. Scale accordingly for fragment sizes (molar ratios).
On ice, add 5 ul DNA to 15 ul thawed 1.33X Master Mix
Incubate at 50°C for 60 minutes
N.B.: The amount of T5 exonuclease varies with the size of the overlap. Current amount is for ~40bp.

Transformation of *E. coli* may be done with 5-20 ul of the assembly mix depending upon the cells and method of transformation.

DNA Re-hydration – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
This protocol is used for re-hydration of DNA. We used this mainly for our newly arrived primers.
Materials required
Oligonucleotide Specification Sheet (OSS)
OD260 values
Sterile Pure Lab Distilled Water
Method
To begin, read the Oligonucleotide Specification Sheet to find the OD260 values and number of DNA bases for each primer/DNA.
Once those values are found, divide the (OD260 value) by (number of DNA bases x10).
Multiple the result by 1000
Divide 10 by the previous result, and multiple all by 100: the end result is the volume of primer DNA (in µl) required to prepare 100µl at 10pM concentration.
Rehydrate lyopholsed primers in 1ml sterile water
Remove the appropiate volume of rehydrated primer and add Pure Lab Distilled Water to make the solution up to 100 µl in total.
Please note:

Extra care must be taken when following this protocol, i.e. there must be NO contamination.
The newly arrived primer tubes have to be handled with extra caution, because they will be the main stocks which working stock solutions will be made from. Therefore, gloves have to be worn, as well as preventing any contamination. Water will be used in order to liquefy the primers and the water used will be from Pure Lab Distilled Water.

PCR Purification – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials
1.5 ml microcentrifuge tubes
2 ml collection tube
QIAquick columns
Qiagen Buffer PB
Qiagen Buffer EB
Qiagen Buffer PE
DNA mixture from PCR
Protocol
Add 5 volumes of Qiagen Buffer PB to 1 volume of PCR product.
Put a QIAquick spin column into a 2ml collection tube.
Apply the mixture to the QIAquick column to bind the DNA and centrifuge for 30 to 60 seconds at 13,000 rpm.
Discard flow-through. Add 0.75 ml of Buffer PE to the spin column to wash and centrifuge for 30 to 60 seconds at 13,000 rpm.
Discard flow-through and centrifuge for another 1 minute at 13,000 rpm.
Place QIAquick spin column into a clean 1.5 ml microcentrifuge tube.
Add 50 µl Buffer EB to the center of the membrane of spin column and centrifuge for 1 minute at 13,000 rpm.
If analysing purified DNA using gel, add 1 volume of Loading Dye to 5 volumes of DNA.
Store the PCR product either at 4°C or at -20°C.

GoTaq PCR – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
Add the following as mentioned below to make up to a final volume of 50 µl in the PCR tube:
32.5 µl of distilled H_2O
10 µl of 5x GoTaq Buffer
1 µl of dNTPs
2.5 µl forward primer
2.5 µl backward primer
1 µl template DNA
0.5 µl of GoTaq polymerase
Conditions for ThermoCycler
After putting the PCR tubes in the thermocycler, set the thermocycler's condition as mentioned below:
Initialise - 95°C for 2 minutes.
Denature - 95°C for 30 seconds.
Anneal - ~°C for 30 seconds (depends upon the melting temperature of template)
Extension - 75°C for 30 seconds
Extension finish - 75°C for 5 minutes
Hold - 4°C

Steps 2 to 4 are repeated for 30 cycles before continuing to step 5.
Some important points to note while performing PCR are as following:
Extension time depends on size of DNA and time used is 30 seconds/kb.
PCR is best when everything is kept on ice!
Melting temperatures for annealing step of primers is important and can have a big effect on the product formed.

Phusion PCR – Newcastle – 2010 http://2010.igem.org/Team:Newcastle/Protocol_list
Materials required
Add the following as mentioned below to make up to a final volume of 50 µl in the PCR tube:
27.5 µl of distilled H_2O
10 µl of 5x Buffer
1 µl of dNTPs
5 µl forward primer
5 µl backward primer
1 µl template DNA
0.5 µl of Phusion polymerase
Conditions for ThermoCycler
After putting the PCR tubes in the thermocycler, set the thermocycler's condition as mentioned below:
Initialise - 98°C for 30 seconds.
Denature - 98°C for 10 seconds.
Anneal - Depends on the melting temperature, Tm of the primer and it lasts for 20 seconds.
Extension - 72°C (The extension time depends on the the size of the fragment which is to be amplified).
Extension finish - 72°C for 5-10 minutes
Hold - 4°C
Some important points to note while performing Phusion PCR are as following:
Annealing temperature of the primer depends on the GC content and thus before setting up the thermocycler, check the melting temperature once again online or on the notes provided by the company which made the primers.
Rate of extension by Phusion polymerase is 1Kb/ 30 seconds and thus always set extension time based on the size of the fragment you are planning to amplify. Note that the least extension to be set is 10 seconds for the Phusion polymerase to work.

Steps 2 to 4 are repeated for 30 cycles before continuing to step 5.

PCR Purification – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

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MATERIALS

- Heating block
- Microcentrifuge

- PCR Purification Kit

- Binding Buffer

- Wash Buffer

- Elution Buffer

- Water nuclease free
- Purification columns
- 1.5 or 2 mL microcentrifuge tubes
- Ethanol 96-100 %.
- 3 M sodium acetate, pH 5.2

SOLUTIONS

- Prior to the initial use of the PCR Purification Kit, dilute the Wash Buffer (concentrated) with ethanol (96-100%)
- Examine the Binding Buffer for precipitates before each use. Dissolve the precipitate by warming the solution to 37 C and cooling to 25 C.
- Wear gloves when handling the Binding Buffer
- Check the color of the Binding Buffer solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 uL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

CHECK-LIST PROCEDURE

- Add a 1:1 volume of Binding Buffer to completed PCR mixture. Mix by pipetting gently up and down.
- If the DNA fragment is <500 bp, add a 1:2 volume of 100 % isopropanol [optional]. Mix by pipetting gently up and down.
- Transfer up to 800 uL of the solution from epp to the purification column.
- Centrifuge for 30-60 sec at >12000 g.
- Discard the supernatant from collection tube. [If the total volume exceeds 800 uL, the solution can be added to the column in stages. After the addition of 800 uL of solution, centrifuge the column for 30-60 sec and discard flow through. Repeat until the entire solution
has been added to the column membrane.]
- Add 700 uL of Wash Buffer (diluted) to the purification column.
- Centrifuge for 30-60 sec at >12000 g.
- Discard the flow through and place the purification column back into the collection tube.
- Centrifuge the empty purification column for an additional 1 min at >12000 g to completely remove any residual wash buffer.
- Transfer the purification column to a clean 1.5 mL microcentrifuge tube.
- Add 50 uL of Elution Buffer to the center of the purification column membrane and centrifuge for 1 min

at 12000 g.
- Discard the purification column and store the purifi ed DNA at -20 C.

TROUBLESHOOTING

- For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 uL does not significantly reduce the DNA yield. However, elution volumes less than 10 uL are not recommended.
- If DNA fragment is >10 kb, prewarm Elution Buffer to 65 C before applying to column.
- If the elution volume is 10 uL and DNA amount is >5 ug, incubate column for 1 min at room temperature before centrifugation.

Agarose Gel Electrophoresis – METU-Turkey – 2010
http://2010.igem.org/Team:METU_Turkey/Procedures

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MAIN STEPS/TIME TABLE

- 1% gel preparation [30 min]
- Running [1 h]
- Visualization [15 min]

MATERIALS

- Electrophoresis tank
- Power supply
- Transilluminator
- Appropriate comb
- P10 and P100
- DNA Ladder 100-10 kb (Fermentas #SM0331)
- Agarose
- Loading dye
- TAE Buffer (1X)
- Distilled water [M14]
- Sterile dH2O [M14]
- EtBr
- Parafilm

SOLUTIONS

TAE buffer
- Stock TAE electrophoresis buffer (50X)
- Use 1X TAE
- 20 mL TAE
- 980 mL dH2O
1% Electrophoresis gel
- 0.5 gr Agarose
- 50 mL 1X TAE buffer (1%)

CHECK-LIST PROCEDURE

- Mix 2 uL DNA + 3 uL sterile dH2O + 1 uL loading dye on the parafilm
- Load sample to the wells of 1% gel
- Adjust the voltage of power supply to 75 V
- Adjust the time of power supply to 65 min
- Check transilluminator
- After running of the samples record the gel image [go to SOPs-experimental for application of camera and record of image]

NOTES

- Before adding of EtBr make sure the temperature of gel solution, its temperature should not be too high.
- Make sure the voltage is at the correct settings at all times and always use enough buffering solution to cover the gel in order to prevent inconsistent readings.

TROUBLESHOOTING

Missing Bands
- Use a lower voltage or decrease the electrophoresis time if smaller bands are missing since this may indicate that they were pushed off of the gel. However, increase the electrophoresis time if larger bands are missing which means that the components have not separated yet.

Smear
- Check sample for nuclease contamination, buffering conditions and excess salt or protein. If you still see smeared results, decrease the amount of sample used.

Non-existent or Faint Bands
- Increase amount of sample or time of electrophoresis at a lower voltage.

LIGATION – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

CHECK-LIST PROCEDURE

Add;
- uL nuclease free water
- uL rapid DNA ligation buffer
- uL T4 DNA ligase
- uL from 25uL insert1(downstream) elute (elution must be done with nuclease free water)
- uL from backbone
- uL from 25uL (upstream) elute (elution must be done with nuclease free water) to end up in 15 uL T4 DNA ligase solution
- Keep the T4 DNA ligase solution in room temperature for 5 min.

NOTES

- After gel extraction, treatment with nuclease free water is needed.
- Total mixture shouldn't exceed 15uL.
- Ligation ratios are calculated by comparing the molar ratios. Molar ratios of insert to vector is calculated by applying vector as reference.
Example: Vector ~ 2000 bp - Molar ratio is given as 1x

Insert 1 ~ 500 bp - Molar ratio is 4x compared to vector

Insert 2 ~ 100 bp - Molar ratio is 20x compared to vector

- At ligation, all inserts are put in 1x concentration
- Before ligation, nanodrop results are very significant. By these results, we can estimate the insert amount after gel extraction.
Example: Vector ~ 2000 bp at 2400 bp

Insert 1 ~ 600 bp at 2500 bp

Insert 2 ~ 100 bp at 2400 bp

If we start ligation with 5 ug each, after gel extraction, we will have appr. 4.0 ug Vector, 1,2 ug insert 1 and 200 ng insert 2.

- While estimating recovery from gel, always take account 10% loss of sample.
- With increasing restriction digestion volume, add 0.5 uL FD enzyme for 30-40 uL increasing of total volume. But add 1 uL enzyme for 1 ug plasmid DNA until total volume reaches to 50 uL.

PCR – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

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MAIN STEPS/ TIME TABLE

- Each step is carried out for 1 tube
- PCR solution preparation [30-45 min]
- Thermocycler [2.5 h]

- Denaturation

- Annealing

- Primer extension

- PCR purification [30 min]
- DNA concentration determination with Nanodrop [5 min]
- Electrophoresis [2 h]

MATERIALS

- Thermal Cycler [M08-Techne TC-512 Gradient PCR]
- Spin [M03]
- P10 and P100 micropipetes
- DNase-free micropipet tips
- Microfuge tubes (0,5 mL, thin-walled for amplification reactions)
- Electrophoresis [MO6]
- Nanodrop [M08]

- PCR kit (MasterMix)
- Forward primer
- Reverse primer
- Template DNA
- Nuclease-free water
- PCR purification kit

SOLUTIONS

- PCR MasterMix Kit [Fermentas]

CHECK-LIST PROCEDURE

Sample Preparation
- Calculate the amount of your samples.
- Design the tubes.
- Label the microfuge tubes.
- Transfer the PCR reaction to a pre-heated block.

- Adjustment of thermocycler [changeable parameters]
- Lid Temp: 105 C (to prevent evaporation)
- Step 1 (initial denaturation): 94 C, 5 min. Press select to go.
- Step 2 (cycle denaturation): 94 C, 30 sec.
- Step 3 (cycle annealing): primer specific, 30 sec.
- Step 4 (cycle extension): 72 C, 1 min. Press select two times.
- Step 5 (cycle loop): Go to step 2, 30 to 35 cycles.
- Step 6 (final extension): 72 C, 15 min.
- Step 7 (store): 4 C, forever.

Follow-Up Steps

(It is recommended that you use PCR products for RE digestion and ligation in the same day to prevent the damage to DNA ends)

Agarose gel electrophoresis
- PCR product purification is NOT needed for gel loading. You can directly load the PCR product to check the results.

NOTES

- To reduce the chance of contamination with exogenous DNAs, bake all glassware for 6 h at 15 C and autoclave all plasticware.
- If the thermal cycler is not equipped with a heated lid, use either mineral oil or paraffin wax to prevent evaporation of liquid from the reaction mixture.
- High concentrations of of dNTPs (> 4mM) are inhibitory, because of sequestering Mg2+.
- Pfu is more suitable, error rate of it per nucleotide is the lowest of any DNA polymerase.
- Inhibition of PCR can be caused by proteinase K, phenol, EDTA, ionic detergents, heparin, gel-loading dyes (bromophenol blue, xylene cyanol)

TROUBLESHOOTING

No bands
- PCR kit or polymerase enzyme is not working: Include positive control (ex. a control DNA sequence with primers)
- Primers are not working

- Secondary structure: Use DMSO for helping denaturation of DNA secondary structures

- Melting temperatures are different: see annealing temperature

- Annealing temperature is not right: Use 50 C. It is used as a generic temperature by sequencing center to amplify all different samples.

- Problem with template and primer working solutions:

- Measure concentration.

- Include positive control (ex. a control DNA sequence with primers)

- Increase the amount of template

Non-specific bands
- If you see no PCR product then decrease annealing temp 5-10 C; if you see non-specific products, then increase it by 5-10 C.

Alternative Procedure

- Master Mix

- 3 uL-10X Taq Buffer

- 3 uL-2mM dNTP mix

- 3 uL-25mM MgCl2

- 18 uL Nuclease-free water

- For 30 uL PCR mix

- 27 uL Master mix

- 3 uL-5uM forvard primer

- 0.25 uL-5 u/uL Taq

- 3 uL-250 ng/uL Template

Alternative Procedure with Pfu DNA polymerase

- 10 uL 10X Pfu Buffer
- 8 uL dNTPs (2.5 mM stock)
- 2.5 uL primer 1 (100 ng/uL)

- 2.5 uL primer 2 (100 ng/uL)
- 1 uL Pfu enzyme
- x uL template (around 10 ng)
- Complete to 100 uL with nuclease-free water

- 94 C 1 min
- 53 C 1 min
- 72 C 1 min 30 sec for 30 cycles
- End on a 10 min 72 C extension and keep at 4 C.

- Extension times are longer for Pfu than Taq.
- If amplification does not work for first time, add extra $MgCl_2$.

Isothermal Titration Calorimetry (ITC) – METU-Turkey – 2010
http://2010.igem.org/Team:METU_Turkey/Procedures

I) Sample Preparation

- thaw protein stock (10 min)
- centrifuge protein stock for 5 min @max speed (5 min)
- prepare solutions

- adding components (5 min)

- temperature equilibration (10 min)

- pH adjustment (5 min)

- degassing solutions 10 min
- total: 45 min

Calculations and Controls

- prepare recipies for protein and ligand solution preparations. Also estimate the acid/base addition quantities for pH adjustments. concentrated acid/base additions may denature protein and/or ligand. Therefore pH adjustments will be done at two stages.
A) buffer level using concentrated acid/base
B) protein/ligand solution level using less concentrated acid/base

- if using relatively old enzyme stocks or different buffer components, first be sure that the protein is still active.

II)Sample loading

Sample Cell Loading
- Wash the hamilton syringe 2 times w/ W1 and 2 times w/ W2
- Unload water in the sample cell. DO NOT leave residual water in the cell.
- Wash the sample cell w/ 5 times W1 and 5 times w/ W2

- Wash the sample cell w/ buffer which will be used in the experiment
- Slowly fill the syringe with macromolecule solution. Purge the trapped air in the needle. Tap the syringe.
- Load the sample cell. (2 bursts after 1.0 ml). DO NOT inject trapped air in the syringe. Take the excess solution in the sample reservoir.

Injection Syringe loading
- Purge the water in the Injection Syringe (purge-refill without putting the syringe in any solution or water)
- Attach the filling syringe. Open the fill port. Press "Up" once.
- Pass 1-2 ml of air through the syringe to purge residual water in the syringe.
- Wash the injection syringe w/ W3 before change the ligand (btw two experiments)
- Purge buffer in the syringe once
- Put the syringe into ligand solution
- VERY SLOWLY withdraw the plunger of the filling syringe until you see ligand solution exit through the filling port. IMMEDIATELY press the close fill port.
- Purge-refill while the injection syringe is in the ligand solution
- Insert the injection syringe into the sample cell and press gently to fit.

III) During the Experiment
- Bring syringe and falcon rack to lab. DO NOT leave them in ITC Room
- Record the sample preparation details of ongoing run into the excel file.
- Analyze the data of previous run and record the results into the excel file.
- Check the ongoing run, if signal is not good, stop the experiment

IV) After The Experiment
- Record set / initial / final values of baseline
- Put post-titration mix into post-titration vial.

- Check and record turbidity

- Set thermovac temp to experimental temp of the mix. Thermostat mix (half speed stirring) for 10 min, measure
and record the pH

- Label and keep the mixture for enzyme recycling. Take a sample of post-titration mix and conduct activity assays of pre and post titration solutions.

Cleaning Sample Cell
- Use W1 and wash sample cell 7 times and use W2 and wash sample cell 7 times. Fill sample reservoir halfway each time. Rinse the syringe once in between each wash

Cleaning Injection Syringe
- Attach the filling syringe. Open the fill port. Press "Up" once.
- Use W3 to pass 1-2 ml dH2O through the injection syringe. Keep the syringe in W3 and close the fill port. Detach the filling syringe.
- Insert the injection syringe into the port.

Glycerol stock preparation – METU-Turkey – 2010
http://2010.igem.org/Team:METU_Turkey/Procedures

MATERIALS

- SOB/LB Media
- Glycerol (100%)
- Sterile Milli-Q dH2O
- Liquid nitrogen

CHECK-LIST PROCEDURE

- Choose colony put into 5 mL of LB medium in 15 mL falcon.
- Incubate at 37 C @ 240 rpm not longer than 12-14 h.
- Centrifuge 5000 rpm 5min.
- Discard 4 mL solution from falcon.
- Add 176 uL glycerol into falcon to 15% final concentration.
- Vortex!
- Aliquot 150 uL samples.
- Quick-freeze in liquid nitrogen
- Place in -80 C deep freeze

Plasmid purification – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MAIN STEPS/TIME TABLE

- Incubation [12-14 h]
- Plasmid purification [30-45 min]

MATERIALS

- FERMANTAS GeneJET™ Plasmid Miniprep Kit (#K0502 or #K0503)
- Centrifuge (M03)
- Nanodrop
- Spectrophotometer
- Incubator with shaker (M15)
- Heat block
- P1000, P200 and P10 micropipettes

- sterile dH2O
- LB broth with appropriate antibiotic
- 15 mL falcon
- 1.5 mL epp

CHECK-LIST PROCEDURE

Important
- Check availability of LB broth with appropriate antibiotic
- Check presence of sterile dH2O
- Check incubator with shaker and centrifuge to be worked properly
- Look plasmid. Is it high-copy or low-copy?
- Prepare 5 mL of E.coli culture in LB media for purification of high-copy plasmids.
- For low-copy plasmids use up to 10 mL of culture.
- All purification protocol should be carried out at room temperature.
- All cenrifugations should be carried out @ 10000-14000 rpm. (depending on rotor type) (We used @ 13000 rpm.)
- Check the Lysis Solution and the Neutralization Solution for salt precipitation use.
- Redissolve any precipitate by warming the solution at 37 C, then cool back down to 25 C before use. Do not shake the Lysis Solution too vigorously.
- Pick a single colony
- Inoculate in 5 mL LB medium for high-copy or 10 mL for low-copy of LB medium supplemented with the appropriate selection antibiotic.
- Incubate @ 225 rpm not longer than 12-14 h at 37 C .
- Spin @ 4000 rpm for 5 min at 25 C .
- Discard the supernatant and keep pellet.
- Resuspended the pellet with 250 ul Resuspension solution. (Bacteria should be resuspended completely by vortexing until no cell clumps remain)
- Transfer the cell suspension to epp.
- Add 250 uL Lysis Solution
- Mix throughly by inverting the tube 4-6 times until the solution becomes slightly clear.
- Do not vortex!
- Do not incubate for more than 5 min. (To avoid denaturation of supercoiled plasmid DNA!)
- Add 350 uL Neutralization solution
- Mix immediately and throughly by inverting the tube 4-6 times. (The neutralized bacterial lysate is cloudy and viscous) ("throughly" to avoid localized precipitation of bacterial cell debris)
- Spin @ 13000 rpm for 5 min to precipitate cell debris and chromasal DNA.
- Transfer the supernatant to spin column (600 uL civari). (Avoid disturbing or transferring the white precipitate)
- Spin @ 13000 rpm for 1 min.
- Discard the flow-through.
- Place the column back into same collection tube.
- Add 500 uL Wash solution to spin column.
- Spin @ 13000 for 1 min
- Discard the flow-through.
- Repeat this step with using 500 uL Wash solution.
- Discard the flow-through
- Spin @ 13000 for an additional 1 min to remove residual Wash solution. (This step is essential to avoid residual ethanol in plasmid preps)
- Transfer spin column into a fresh 1.5 mL epp.
- Add 50 uL Elution buffer into center of the spin column membrane to elute the plasmid DNA
- Do not contact the membrane with pipette tip!
- Incubate for 2 min at 25 C. >> To increase yield incubation is done for 2 min at heat block at 42 C

- Spin @ 13000 for 2 min.
- Discard the column and store the purified plasmid DNA at -20 C.

- OPTIONAL= additional elution step with elution buffer or water. This step increases the yield by 10-20 %.
- NOTE: For elution of plasmids ≥ 20 kb, prewarm Elution buffer to 70 C before applying.

Quality Check / Validation
(Concentration / DNA purity / Target purity check)

- Alpha UV Spec
- Restriction Enzyme Digestion (single digestion)
- Agarose gel electrophoresis

SOLUTIONS

TROUBLESHOOTING

Low yield of plasmid DNA
- Bacterial culture too old

- Inoculate a fresh batch of antibiotic-containing growth medium with a freshly-isolated single bacterial colony from
an overnight plate.

- Cultivate the cells for no more than 16 h at 37°C while shaking in LB media.

- Reduce the cultivation time to less than 12 h when using rich media like TB.

- Incomplete lysis of bacterial cells

- It is essential that the cell pellet is completely resuspended in the Resuspension Solution prior to lysis. No cell clumps should be visible before the addition of the Lysis Solution.

- Check the Lysis Solution for salt precipitation before each use.

- Redissolve any precipitate by warming the solution to 37°C, then mix well before use.

- Cell cultivation in LB media is recommended.

- Reduce culture volume when using a rich cultivation media like TB.

- Inefficient elution of DNA

- The Elution Buffer must be dispensed to the center of the membrane for efficient elution.

Contaminated DNA preparation
- Residual ethanol

- Ensure that step 9 of the protocol is performed.

- RNA in the eluate

- Ensure that RNase A is added to the Resuspension Solution before the first use.

- Genomic DNA in the eluate

- To avoid shearing and liberation of genomic DNA, do not vortex or shake the cells during lysis and neutralization
(steps 2 and 3), mix by gentle inversion of the tube.

- Do not lyse the cells (step 2) for more than 5 min.

- Do not cultivate cells longer than 16 h in LB media or 12 h in TB media.

- Additional band of denatured plasmid

- Denatured plasmid DNA migrates ahead of supercoiled DNA. It is not suitable for following enzymatic manipulations
such as restriction digestion.

- To avoid denaturation, do not lyse the cells (step 2) for more than 5 min.

Cell Imaging – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

CLSM IMAGING

- Take 10mL from the samples (OD=1-1.9) that are dissolved in phosphate buffer solution.
- Spread the samples on the slide with the help of an inoculation loop.
- Wait for the sample to dry.
- Pass the slide through the flame 3-4 times, being careful to have the cells on the upper part of the slide.
- Observe the slides under Brightfield 100X

- Settings for GFP:

HFT 488

Ch 1

LP 505

Line 8

543 nm excitation

Laser power 5%

- Settings for RFP:

HFT 488/543

Ch 1

LP 560

Line 8

543 nm excitation

Laser power 10%

Gel Extraction – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MATERIALS

UV lamp - B58
Heat block - M3
Centrifuge - M8

CHECK-LIST PROCEDURE

GeneJET™ Gel Extraction Kit #K0692 procedure

NOTES

- Gel is cut under UV lamp.
- While the gel is being cut, it shouldn't be exposed to UV much otherwise DNA mutations may occur and mutated DNA wouldn't be appropriate to use in cloning.
- While cutting the gel, excess amount of gel should be removed.
- Before putting the gel piece into the eppendorf, empty eppendorf should be weight so that it can be omitted during the weighing of the gel.
- Before starting gel extraction, heat block should be set at 55C.

Restriction Digestion – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MAIN STEPS / TIMETABLE

- Restriction Digestion [1-16 h]
- PCR purification [1 h]
- Agarose Gel Electrophoresis [2 h]

MATERIALS

- Heat Block
- Spin
- Nanodrop

- Nuclease Free Water
- Vector
- Buffers
- Restriction Enzymes (EcorI, XbaI, SpeI, PstI)

- 0.2 mL eppendorf
- P10 micropipette
- Parafilm
- Ice
- Ice box

CHECK-LIST PROCEDURE

Experiment Preparation
- Nuclease free water and eppendorfs are autoclaved (1 day before)
- Heat block is set to 37 C

Preparing the Total Mix (for 20 uL total mix)
- Add uL water nuclease free to 0.2 mL PCR tube
- Add 2 uL appropriate 10x buffer(depending on enzyme type) (thawed before)
- Add uL DNA solution to the tube (ugr)
- Add 15 unit restriction enzyme to the tube
- Spin the tube for 5 sec
- Incubate the tube for h at 37 C at heatblock

Enzyme Inactivation (EcorI and XbaI)
- Spin the enzyme solution at 100 g for 15 sec
- Incubate at 65 C for 15 min at heatblock
- Store at -20 C in the freezer

Enzyme Inactivation (SpeI and PstI)
- Spin the enzyme solution at 100 g for 15 sec
- Inactivate the enzyme with Fermentas PCR Purification kit
- Store at -20 C in the freezer

Enzyme Inactivation (Fastdigest NotI)
- Spin the enzyme solution at 100 g for 15 sec
- Incubate at 80 C for 5 min at heatblock
- Store at -20 C in the freezer

Agarose Gel Electrophoresis
- Look at procedure for agarose gel electrophoresis

- Load 2 uL sample for confirmation of the restriction
- Load whole sample for insert extraction

Double Digestion

>>> EcorI-SpeI Double Digestion >>>
- Buffer R
- 4-fold excess of BcuI
- EcoRI
- Incubate at 37C

>>> XbaI - PstI Double Digestion
- Buffer O
- PstI
- 4-fold excess of XbaI
- Incubate at 37C

>>> EcorI - PstI Double Digestion
- Buffer O
- PstI
- EcorI
- Incubate at 37C

Genomic DNA purification – METU-Turkey – 2010
http://2010.igem.org/Team:METU_Turkey/Procedures

CHECK-LIST PROCEDURE

- Harvest up to 2x10^9 bacterial cells in a 1.5 or 2 mL microcentrifuge tube by centrifugation for 10 min at 5000 x g. Discard the supernatant.
- Resuspend the pellet in 180 uL of Digestion Solution. Add 20 uL of Proteinase K Solution and mix thoroughly by vortexing or pipetting to obtain a uniform suspension.
- Incubate the sample at 56C while vortexing occasionally or use a shaking water bath, rocking platform or thermomixer until the cells are completely lysed (~30 min).
- Add 20 uL of RNase A Solution, mix by vortexing and incubate the mixture for 10 min at room temperature.
- Add 200 uL of Lysis Solution to the sample. Mix thoroughly by vortexing for about 15 sec until a homogeneous mixture is obtained.
- Add 400 uL of 50% ethanol and mix by pipetting or vortexing.
- Transfer the prepared lysate to a GeneJET™ Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 1 min at 6000 x g. Discard the collection tube containing the flow-through solution. Place the GeneJET™ Genomic DNA Purification Column into a new 2 ml collection tube (included).
- Add 500 µl of Wash Buffer I (with ethanol added). Centrifuge for 1 min at 8000 x g. Discard the flow-through and place the purification column back into the collection tube.
- Add 500 µl of Wash Buffer II (with ethanol added) to the GeneJET™ Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥12000 x g). [Optional. If residual solution is seen in the purification column, empty the collection tube and re-spin the column for 1 min. at maximum speed. Discard the collection tube containing the flow-through solution and transfer the GeneJET™ Genomic DNA Purification Column to a sterile 1.5 ml microcentrifuge tube (not included).]

- Add 200 µl of Elution Buffer to the center of the GeneJET™ Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 2 min at room temperature and centrifuge for 1 min at 8000 x g.
- Discard the purification column. Use the purified DNA immediately in downstream applications or store at -20°C.

NOTES

- For maximum DNA yield, repeat the elution step with additional 200 µl of Elution Buffer.
- If more concentrated DNA is required or DNA is isolated from a small amount of starting material.
- The volume of the Elution Buffer added to the column can be reduced to 50-100 µl. Please be aware that smaller volumes of Elution Buffer will result in smaller final quantity of eluted DNA.

TROUBLESHOOTING

Low yield of purified DNA
- Excess sample used during lysate preparation.
Reduce the amount of starting material. Do not use more tissue or cells than indicated in lysis protocols.
- Starting material was not completely digested.
Extend the Proteinase K digestion at 56°C until complete lysis occurs and no particles remain.
- Ethanol was not added to the lysate.
Make sure that the ethanol was added to the lysate before applying the sample to the Purification Column.
- Ethanol was not mixed with the lysate.
After the addition of ethanol to the lysate mix the sample by vortexing or pipetting.
- Ethanol was not added to Wash Buffers.
Make sure that ethanol was added to Wash Buffer I and Wash Buffer II before use. Follow the instructions for Wash
Buffer preparation on p.3.

Purified DNA is degraded
- Sample was frozen and thawed repeatedly.
Avoid repeated freeze / thaw cycles of the samples. Use a new sample for DNA isolation. Perform extractions from fresh material when possible.
- Inappropriate sample storage conditions. Store bacteria at -20°C until use.

RNA contamination
- RNase A treatment was not carried out.
Carry out RNase A treatment step described in the purification procedure.

Column becomes clogged during purification
- Excess sample was used during lysate preparation.
Reduce the amount of starting material. A maximum of 2x10^9 of bacteria cells, 5x10^6 of suspension cells is recommended for lysate preparation.
- Tissue was not completely digested.
Extend the Proteinase K digestion at 56°C until complete lysis occurs and no particles remain.

Inhibition of downstream enzymatic reactions
- Purified DNA contains residual ethanol.

If residual solution is seen in the purification column after washing the column with Wash Buffer II, empty the collection tube and re-spin the column for an additional 1 min. at maximum speed (≥12000 x g).
- Purified DNA contains residual salt.
Use the correct order for the Washing Buffers. Always wash the purification column with Wash Buffer I first and then proceed to washing with Wash Buffer II.

Competent Cell Preparation – METU-Turkey – 2010
http://2010.igem.org/Team:METU_Turkey/Procedures

MAIN STEPS/TIME TABLE

- Incubation [12-14 h] >>> Spread plate
- Incubation [12-14 h] >>> Liquid culture
- Competent cell preparation

MATERIALS

- Centrifuge
- Autoclave
- P1000, P10 and P2
- Incubator with shaker [M15]
- pH meter [M08]

- Stock competent cells
- LB
- 2 mL epp
- Strep
- Ice
- Liquid nitrogen
- Bacto yeast extract
- Bacto tryptone
- Magnesium sulfate
- Potassium hydroxide
- Potassium acetate
- Rubidium chloride
- Calcium chloride
- Manganese chloride
- Glycerol
- Dilute acetic acid
- Filter
- MOPS
- Dilute NaOH

CHECK-LIST PROCEDURE

- Inoculate streak plates from liquid stock competent cells and incubate overnight at 37 C
- Put 10 mL LB + strep + colony into 3 eppendorf. Incubate overnight at 37 C (- Inoculate 200 ul to 1000 ul from overnight culture into 100-500 ml Psi broth (scale up or down as needed). Incubate at 37 C with aeration to A600=0.6-0.7
- Ice 15 min. From this step onward the cells must remain COLD. (4C or on ice)
- Pellet cells in appropriate centrifuge tube 3-5000 x g 5 min (~5000 rpm in a Sorvall SS-34 rotor)
- Discard supernatant and add 0.4 volume (ie of original volume, here it is 40-400 ml) TfbI, resuspend and ice 15 min.
- Pellet cells in appropriate centrifuge tube 3-5000 x g 5 min (~5000 rpm in a Sorvall SS-34 rotor)
- Discard supernatant and resuspend in 0.04 volume TfbII, ice 15 min and either use immediately or quick freeze at
-70C for storage. I usually save these in 100ul to 200ul aliquots. Quick freeze in ethanol-dry ice or liquid nitrogen prior to storage in a -70 to -80 C freezer. Thaw on ice just before using in a transformation experiment.

SOLUTIONS

Preparation of Psi broth (per liter)
- 5 g bacto yeast extract
- 20 g bacto tryptone
- 5 g magnesium sulfate
- PH 7.6 with potassium hydroxide
- Autoclave 40 min

Preparation of TfbI (per 200 ml)
- 0.588 g potassium acetate (final molarity/conc= 30 mM)
- 2.42 g rubidium chloride (final molarity/conc= 100 mM)
- 0.294 g calcium chloride (final molarity/conc= 10 mM)
- 2.0 g manganese chloride (final molarity/conc= 50 mM)
- 30 mL glycerol (15% v/v)
- Adjust PH 5.8 with dilute acetic acid
- Sterilize with filter

Preparation of TfbII (per 100 ml)
- 0.21 g MOPS (final molarity/conc= 10 mM)
- 1.1 g calcium chloride (final molarity/conc= 75 mM)
- 0.121 g rubidium chloride (final molarity/conc= 10 mM)
- 15 mL glycerol (15% v/v)
- Adjust PH 6.5 with dilute NaOH
- Sterilize with filter

NOTES

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typically transform 50-100 ul cells with 2-10 ul of a ligation reaction, and you should get between 1x10exp8 to 1x10exp9 cfu's/ug DNA.

Retrived from http://130.15.90.245/e__coli_competent_cells.htm

- All steps should be done in ice.
- Pellet centrifuge steplerinden sonra, once bir miktar TFB solution ile suspend edilmeli sonra gereken degerde dilute edilmeli for enhance the bacterial yield.
- 3 ayri kapta hazirlanacak bacterial culture lar icin ilk once totalde culture hazirlanmali sonra bolusturulmeli for control the total bacterial yield.
- Aeration of culture is critical for competence. For 200mL or 100mL culture keep in 1000mL flask.
- 600 nm deki absorbance olcumlerinde bacterial growth absorbance inin 0.6 olmasi icin gereken zamani yaklasik olarak tayin etmek icin Hamdi Hoca bir equation onerdi

$\ln(A2) - \ln(A1) = k.\ t(min)$

A2= Last measured absorbance value
A1= Previous measured absorbance value
t= time between these two measurements

For medium and buffer preparations different from above procedure we prepared Psi Broth 600mL
- 3 g bacto yeast extract
- 12 g bacto tryptone
- 6 g magnesium sulfate

TfbI 250 ml
- 0.705 g potassium acetate (final molarity/conc= 30 mM)
- 2.9 g rubidium chloride (final molarity/conc= 100 mM)
- 0.352 g calcium chloride (final molarity/conc= 10 mM)
- 3.3 g manganese chloride (final molarity/conc= 50 mM)
- 36 mL glycerol (15% v/v)

TfbII per 30 ml
- 0.063 g MOPS (final molarity/conc= 10 mM)
- 0.33 g calcium chloride (final molarity/conc= 75 mM)
- 0.0363 g rubidium chloride (final molarity/conc= 10 mM)
- 4.5 mL glycerol (15% v/v)

Alkaline Phosphatasen – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MATERIALS

- Heat block
- Spin

- Linear DNA
- 10X reaction buffer for AP used in reaction
- FastAP™ Thermosensitive Alkaline Phosphatase
- Nuclease-free water

- P10 micropipette

CHECK-LIST PROCEDURE

- Prepare the following reaction mixture: Linear DNA (~3 kb plasmid) 1 µg (~1 pmol termini) 10X reaction buffer for AP used in reaction 2 µl FastAP™ Thermosensitive Alkaline Phosphatase 1 µl (1 u) Water, nuclease-free (#R0581) to 20 µl Total volume 20 µl
- Mix thoroughly, spin briefly and incubate 10 min at 37°C.
- Stop reaction by heating for 5 min at 75°C.

NOTES

- For efficient dephosphorylation plasmid DNA should be free of RNA and genomic DNA.
- This protocol is suitable for removal of 3' and 5' -phosphate groups from DNA and RNA.

SDS-Page – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MAIN STEPS/TIME TABLE

- Solution preparation [2 hours]
- Cleaning of aparatus [20 min]
- Seperating gel preparation and powing into glasses [30 min]
- Waiting for polymerization of seperating gel [1 hour]
- Stacking gel preparation and powing onto polymerized gel [30 min]
- Waiting for polymerization of stacking gel [1 hour]
- Sample preparation [20 min]
- Running conditions
- for 145 min at 30 mA 120 V through gel
- amper is constant
- Keeping gel in fixing solution [1.5 hour]
- Silver staining and visualization [2 hours]
- Coomassie Blue Staning [3 hours]

MATERIALS

- Vertical Electrophoresis apparatus
- Power supply
- pH meter
- Balance
- Silver staining shaker platform
- Transilluminator

- Graduated cylinder

- Spatula (plastic and metal)
- Filter paper
- Bottle
- Pipette (200 uL, 10 mL)
- Gloves
- Mask
- Aluminum foil

For Gel preparation
- Acrylamide
- Bisacrylamide
- Deionized Water
- Tris base
- SDS
- APS
- TEMED

For Silver Stanning
- Fixer
- %50 ethanol
- Pretreatment solution
- Silver nitrate
- Devoloping solution
- Stop solution

- Marker: Fermentas / Page Ruler Protein Ladder SM0661 (10-200kDa)

SOLUTIONS

Running Buffer (10L, 1X)
- 25mM Tris, pH 8.3
- 250mM Glycine
- 0.1% SDS
or
- 60 ml 10X stock + 6 ml 10% SDS + 534 ml dH2O

- 30.3g Tris
- 187.7g Glycine
- 10g SDS
- Final volume 10 L in demijon

Staining Solution (Coomassie Blue) (1 L, 1 X)
- 2 g brilliant blue (R-250)
- 450 ml methanol
- 450 ml dH2O
- 100 ml acetic acid
- store @ 24 C

Destaining Solution (4 L, 1 X)

- Methanol:Acetic Acid:dH2O 40:10:50
- Prepare 4.0 L (1.6 L MeOH, 0.4 L AcA, 2.0 L dH2O) in 1 gallon amber bottle, cap tightly
- store @ room temp.

4X Sample Loading Buffer
- 400 mM DTT
- 40 mM Tris
- 10% Glycerol
- 4% SDS
- 0.4% Bromophenol blue

- prepare 15 ml
- 925.2 mg DTT
- 72.6 mg Tris
- adjust pH to 6.8
- 1500 ul Glycerol
- 600 mg SDS
- 6 mg Bromophenol blue
- aliquot 50 x 600 ul
- store at -20 C
- vortex well

30% Acrylimid 1% Bis-acrylimide
- prepare in laminar flow
- 30 g acrylimide and 1 g bis-acrylimide in 100 ml (toz stock kimyasal icin)
- 75 mL acrylimide solution (40% stock, Apllichem) and 25 mL bis-acrylimide solution (2% stock, Apllichem)to final volume 100 ml (sıvı stock kimyasal icin)
- store at 4 C, stable for 1 month

Seperating Gel Buffer
- 1.5 M Tris pH 8.8

- for 100 mL Buffer solution
- 18,15 g Tris pH 8.8
- store at room temp.

Stacking Gel Buffer
- 0.5 M Tris

- for 50 mL Buffer solution
- 3 g Tris pH 6.8
- store at room temp.

APS (Ammonium Persulfate) Stocks (100 mg/ml)
- dissolve 0.6 g in 6 ml dH2O
- aliquot 10 x 600 ul and store @ -20 C

1% Bromophenol Blue
- 0.01 g in 1 ml 1M Tris, pH 7.0
- store at room temp. in amber bottle

CASTING 13% GEL

- set heater to 100 C for sample prep step

Seperating Gel (30 ml)
- 13 ml 30% Acrylimide 1% Bis-acrylimide
- 7.5 ml seperating gel buffer
- 8,45 ml dH2O
- 500 ul %10 SDS
- 250 ul APS (initiator of polymerization)
- 25 ul TEMED (catalyst of polymerization)

- Load 5.4 ml separating gel between glasses

Stacking Gel (10 ml)
- 1.6 ml 30% Acrylimide 1% Bis-acrylimide
- 2.5 ml stacking gel buffer
- 5.85 ml dH2O
- 100 ul %10 SDS
- 15 ul 1% Bromophenol Blue
- 50 ul APS (initiator of polymerization) [add after resolving gel is casted]
- 10 ul TEMED (catalyst of polymerization) [add after resolving gel is casted]

- Load 1.7 ml stacking gel between glasses
- Inset the comb
- make sure the comb has not been inserted in a tilted way. check from behind the apparatus
- load seperating gel
- add some butanol or isopropanol before resolving gel solidifies
- make sure gel stays on flat surface while solidifies to prevent tilted surface
- load stacking gel

- if bubbles form in the stacking gel after polymerization, press the plates between hands to push them out

SAMPLE PREPARATION AND LOADING

- Do not overload the the samples, purity check is difficult with overloaded samples.
- Sample volume: 5 ul sample+ 5 ul loading buffer + 10 ul dH2O
- vortex loading buffer before use
- put samples in heating block (100 C) for 5 min
- if possible, do not load into the first and last lanes
- load 5 ul marker
- load 17 ul samples

PREPERATION
- check the wire on running apparatus, clean and test

RUNNING

- never terminate the run early, lighter bands dont separate
- 600 ml running buffer is required for each run

Running Standards
- 5 mA > 3/4 of gel > 25hrs >>> overnight running
>> bizim icin 2mA
- max: 80 mA for both old and new gel systems
- For Lab 103 Tankı
- for 145 min at 30 mA 120 V through seperating gel
- amper sabit

After Run
- wash electrophoresis unit after each use
- weekly cleaning of power connections recommended to prevent oxidation

STAINING
- Stain the gel for 30 min

DESTAINING
- load the tray fully with destaining buffer
- do not put too many (over-destaining) or too less (under-staining) paper sheets
- destaining takes 2-3 hrs

NOTES

SEPERATİNG GEL %13 (30 ML)

Load 5.5ml from seperating gel between glasses

13 ml Acrylamide\bisacrylamide
7,3 g acrylamide

0,2 g bisarylamide 25 ml d.H2O

8.45 ml dH2O

7,5 ml 1,5 M Tris-Base pH=8,8

18,15 g Tris-Base
100 ml d.H2O pH=8,8 HCI

500 ul SDS

0,3 g SDS

3 ml d.H2O

250 ul APS

50 mg APS

500 ul d.H2O

25 ul TEMED

Stacking Gel (10 ml)

Load 1.7 from stacking gel between glasses

1.6 ml Acrylamide/bisacrylamide

7,3 g acrylamide (T=%30, C=%2,67)

0,2 g bisarylamide 25 ml d.H2O

5.85 ml dH2O

2,5 ml 0,5 MTris-Base pH=6,8

3 g Tris-Base

50 ml d.H2O pH=6,8 HCI

100 ul SDS

0,3 g SDS

3 ml d.H2O

50 ul APS

50 mg APS

500 ul d.H2O

10 ul TEMED

TROUBLESHOOTINGS

Smear / GuHCl / Urea
Normally having 6M GuHCl or Urea will cause a lot of band smearing and will not produce sharp bands on SDS -PAGE.
Alcohol or Acetone ppt. is suggested for proteins denatured by GuHCl or Urea for running on gels.

SDS
SDS should be added to all gels and the tank buffer at 0.1%. You can replace half the SDS in the Cathode buffer with 25 mg/l CBB-G250, then you can watch the protein bands during electrophoresis (Schägger et al., Anal. Biochem. 173 (1988) 201-5)

Separation of proteins with small MW difference

- Increase the gel % to upto 20%
- A longer SDS-PAGE apparatus, that will be of assistance as you can actually run the proteins out to a greater extent.
- A gradient gel is usefull if you want to separate small and large proteins on the same gel.
- Prepare the samples as usual, i.e. add Laemmli buffer or whatever you use, but leave out the SDS from the gel while preparing it. Include SDS in the running buffer however. I have seen some appr. 10-kDa proteins that differ by 2 amino acids separate quite well under these conditions. The gel % around 15-20...

Ghost (Double) Bands

Degradation
PMSF related > protease action > degradation
PMSF is not a very efficient protease inhibitor and if I am not mistaken it only works against serine proteases.
As a remedy, we can purchase and use a better protease inhibitor...

Oxidation Related
Reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol cleave disulfide bonds into free sulfhydryl (SH) groups to allow proteins to unfold completely. However, the reducing agent can be oxidized during sample heating which may allow these disulfide bonds to reform, leading to the appearance of ghost bands in the high molecular weight area or precipitation at the sample application point.

Blocking the reduced SH groups can prevent disulfide bonds from reforming. One common way to do this is to alkylate with iodoacetamide. Iodoacetamide also alkylates residual DTT to prevent point-streaking and other artifacts in horizontal flatbed gels. The recommended amount of iodoacetamide is 2.0-2.5% (w/v) in the sample. The iodoacetamide should be added after boiling the reduced sample, but prior

to loading the sample onto the SDS-PAGE gel. Alternatively, for first-dimension IPG strips, perform a second equilibration step for the IPG strips with an iodoacetamide solution in SDS equilibration buffer (without DTT). The alkylation reaction should take 15-20 minutes at room temperature.

Ethanol precipitation – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MAIN STEPS/TIMETABLE

Total time required to complete the procedure is 1h 40min.

MATERIALS

- Incubator
- Centrifuge

- Absolute ethanol stored at -20°C
- DNA sample
- 95% ethanol stored at room temperature
- Water

CHECK - LIST PROCEDURE

- Measure out 2 volumes absolute ethanol into DNA sample. (The sample is generally in 1.5mL eppendorf.)
- Incubate at -80°C for 1 hr.
- Centrifuge at a speed of at least 10000 Xg for 30 mins at 0°C, gently aspirate out the supernatant and discard it.
- Measure out 750 - 1000 µl of 95% ethanol into the eppendorf tube that is used at first step.
- Centrifuge at a speed of at least 10000 Xg for 10 mins at 4°C, gently aspirate out the supernatant and discard it.
- Dry the pellet in air until white pellet appears.
- Add appropriate volume of water to pellet.
- Resuspend pellet by vortexing/by shaking vigorously.

NOTES

- Storing the absolute ethanol in -20 is recommended.
- Long incubation time is critical for small fragments.
- Another critical step for small fragments under 200 base pairs is the fourth step. Generally, washing involves adding the ethanol and inverting several times.
- The pellet in the 6th step is air dried so that it turns white, showing that all ethanol is eliminated.)
- Many protocols recommend resuspending in 10 mM Tris-HCl or TE. The advantage of TE is that EDTA chelates magnesium ions which makes it more difficult for residual DNases to degrade the DNA. I generally prefer H2O and don't seem to experience problems of this sort. If you plan to ultimately use electroporation to transform your DNA then resuspending in H2O has the advantage of keeping the salt content of your ligation reaction down.

- Incubation in - 80C is done in deep of deep freezer.
- ALL STEPS ARE DONE IN ICE.

Protein expression – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

Main Steps/Time Table

- Pre-culturing/5 hours
- Culturing/5 hours
- IPTG induction/15 hours

Materials

- LB medium with a suitable antibiotic
- TB medium with a suitable antibiotic
- IPTG

- 2-liter flask
- Incubator with shaker
- Centrifuge

Check List Procedure

- Culture transformed E.coli BL21 for 5 hrs at 37 C in 10 LB medium containing antibiotic
- Inoculate 4 ml pre-cultured cells into 400 ml of TB medium containing antibiotic in a 2-liter cultivation flask
- Culture it for 5 hrs at 37 C with a rotary shaker at 180 rpm!
- Add 0.5 mM IPTG in a final concentration
- Continue the cultivation for 15 hrs at 22 C.
- Harvest the cells with centrifuge.

Alternative Check List Procedure

- Inoculate single colony for 5 hrs at 37 C in 10 mL LB medium containing antibiotic
- Inoculate 4 ml pre-cultured cells into 400 ml of TB medium containing antibiotic in a 2-liter cultivation flask
- Culture it for 5 hrs at 37 C (OD600 to 0.5 - 0.6) with a rotary shaker at 180 rpm
- Add 1 mM IPTG in a final concentration (0.5- 2.0 mM recomended for pT7)
- Continue the cultivation for 3-4 h at 37 C (15 hrs at 22 C.)
- Harvest the cells with centrifuge.

Solutions

- TB (Terrific Broth) see Procedure [Media]

Notes

Incubation temperature incubation time
15°C overnight

20°C overnight
25°C overnight
30°C 5-6 h
37°C 3-4 h

reference: http://www.embl.de/pepcore/pepcore_services/protein_expression/ecoli/

Cell Lysis

Materials:

- water bath
- vortex
- pipet 10 ul
- ice

Check List Procedure

- For 1 ml cell culture;

- resuspend the cell pellets in 1 ml 2-5% SDS
- vortex 1 minute
- incubate in 50 c water bath for 15 min
- put in ice for 1 min
- add 10 ul from each of
- 1 mg/ml RNAse A stock (final concentration: 10 ug/ml)
- 0.5 mg/ml DNase I stock (final concentration: 5 ug/ml)
- 100 mM MgCI2 stock (final concentration: 1mM)
- incubate on ice for 15 min

Solutions

- 1 mg/ml RNAse A stock
- 0.5 mg/ml DNase I stock
- 100 mM MgCI2 stock
- 2-5% SDS

Transformation – METU-Turkey – 2010 http://2010.igem.org/Team:METU_Turkey/Procedures

MAIN STEPS/TIME TABLE

- Fresh o/n bacteria
- LB Agar plate preparation[30 min]
- Transformation [2.5-3.0 h]
- Incubation of plates [14-17 h]

MATERIALS

Heat Block
Incubator
p10, p100 and p1000

SOC medium
Ice
Ice box
Bent
desultory
Parafilm
Spreader
LB Agar plates with antibiotic
Vector DNA
Competent cells

CHECK-LIST PROCEDURE

- Check availability of LB Agar
- Check availability of SOC medium and preheat
- Check presence of sterile dH2O
- Check incubator with shaker and heat block to be worked properly

- Mix (spin) 1 uL vector DNA and 50 uL competent cells.(1-10 ng vector)
- Incubate in ice bath for 30 min.
- Heat shock for 20 sec. in 42C at heatblock.(not exceed 30 sec)
- Ice bath for 30 sec.
- Add 600ul of SOC medium on bacteria culture.
- Incubate in 200 rpm shaker for 1h
- Inoculate into plates

SOLUTIONS

Preparation of LB
- 4 gr LB Broth
- 2.7 gr Agar
- 200 mL dH2O
- Autoclave the bottle.
- After cooling, add 200 uL antibiotic (The LB agar solution should be cool enough not to damage to antibiotic).
- Pour the plates .
- Keep the plates +4 C

Preparation of Commercial LB Agar (LB agar-Xgal- IPTG-Amp/Fermentas)
- Mix 200 ml dH2O + 1 paket LB agar

- Heat in Microwave 360 watt; 6-8 min
- Pour to plate

Preparation of SOC medium (1L)
- 900 mL dH_2O
- 20g Bacto tryptone extract
- 5g Bacto yeast extract
- 2 mL of 5M NaCl
- 2.5 mL of 1M KCl
- 10 mL of 1M $MgCl_2$
- 10 mL of 1M MgSO
- For maximum effectiveness, the SOC medium should be adjusted to a pH of 7.0
- Autoclave the solution
- Add 20 mL of 1M glucose (autoclaved solution)
- Adjust to 1L with dH_2O

NOTES

- Time intervals of heat shock and ice bath should be strictly obeyed.
- For the competent cells and vector DNA, inoculation to media must be performed to either 1.5 mL or 2.0 mL eppendorfs.
- Always bacteria should be added onto the vector solution.
- Media should be added into the bacteria solution(not bacteria on media).
- Incubation at heat shock must not exceed 30 seconds.
- Preheating of plates to 37 C before inoculation is recommended(20 min)
- Competent cells should be thawed in ice
- All eppendorfs should be cold before addition of vector and bacteria.
- Shaking of eppendorfs in the incubator must be horizontal (not vertical)

TROUBLESHOOTING

- If transformation is not completed, firstly control the competent cells whether they are working or not. If competent cells are working, control whether the plasmid concentration is enough or not (10 ng plasmid is enough).

Low transformation efficiency or low colony number
- Ensure that excess mineral oil is not transferred into the transformation reaction when pipetting the enzyme-treated DNA. Using the smallest pipet tips available, insert the pipet tip completely below the mineral layer overlay and clear the pipet tip while submerged beneath the mineral oil overlay before collecting the sample.
- Ensure that sufficient mutant DNA is synthesized in the reaction. Increase the amount of the enzym-treated DNA used in the transformation reaction to 4 uL.
- Visualize the DNA template on a gel to verify the quantity and quality. Nicked or linearized plasmid DNA will not generate complete circular product. Verify that the template DNA is at least 80% supercoiled.
- It is not uncommon to observe low numbers of colonies, especially when generating large mutations. Most of the colonies that do appear, however, will contain mutagenized plasmid.

Low mutagenesis efficiency or low colony number with the control reaction
- Ensure that supercompetent cells are stored at the bottom of a –80 C freezer immediately upon arrival (see also Transformation Guidelines).
- Verify that the agar plates were prepared correctly.
- For best visualization of the blue (beta-gal+) phenotype, the control plates must be incubated for at least 16 h at 37 C.

Transformation efficiency is too low (evaluated in control transformation with supercoiled DNA)
- Old bacterial cultures were used to prepare competent cells.
- Seed overnight culture from a freshly streaked bacterial culture plate. Refresh bacterial strains weekly. For seeding of overnight E.coli DH5αlpha culture, use only <24 hours fresh culture plates.

Low number or no transformants
- Volume of ligation reaction mixture too large.
- Do not use more than 5 uL of ligation reaction mixture per 50 uL of competent cells.

DNA amount is too high
- Do not use more than 100 ng of plasmid DNA for transformation of 50 uL of competent cells.

Inefficient ligation
- Simple sticky end ligation reactions should yield 50-200 colonies. If the efficiency of competent cells was acceptable, but transformation of ligation mixture yielded no or only few transformants, repeat cloning experiment.
- Use high quality DNA, enzymes and follow the recommended ligation protocol.
- Background colonies without plasmid.
- Insufficient amount of antibiotic in agar medium.
- Use recommended amount of appropriate antibiotic in LB agar plates. Antibiotics are heat-sensitive therefore allow the LB medium to cool to 55 C before addition of the antibiotic to it.

Satellite colonies
- Some fast growing strains (e.g. C600) lead to formation of smaller satellite colonies around transformants after
>16 hours of incubation. Use shorter incubation times and do not involve such small colonies into clone analysis.

Competent Cell **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

- Inoculate streak plates from liquid stock competent cells Top10(plate with strep) and BL21 DE3(antibiotic free plate) ; incubate overnight at 37 C
- Put 10 mL LB + strep(100ug/ml) + colony into 3 eppendorf. Incubate overnight at 37 C
- Inoculate 200 ul to 1000 ul from overnight culture into 100-500 ml Psi broth. Incubate at 37 C with aeration to A600=0.6-0.7
- Ice 15 min. From this step onward the cells must remain COLD. (4C or on ice), pellet cells in appropriate centrifuge tube 3-5000 x g 5 min
- Discard supernatant and add TfbI 0.4 volume of initial volume, resuspend and ice 15 min.
- Pellet cells in appropriate centrifuge tube 3-5000 x g 5 min
- Discard supernatant and resuspend in 0.04 volume TfbII, ice 15 min and either use immediately or quick freeze at -70C for storage.

Transformation **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

- Mix 1 uL vector DNA (synthesized genes) and 80 uL competent cells.(1-10 ng vector)
- Incubate in ice bath for 30 min.
- Heat shock for 20 sec. in 42C at heatblock.(not exceed 30 sec)
- Ice bath for 30 sec.
- Add 600ul of SOC medium on bacteria culture.
- Incubate in 200 rpm shaker for 1h
- Inoculate into plates with appropriate antibiotics.

Glycerol Stock Preparation **– METU-Turkey – 2010**
http://2010.igem.org/Team:METU_Turkey/Procedures

- Pick at least 5 single each big colonies
- Inoculate each colony into 10 mL subculture incubate at 37 C @ 240 rpm not longer than 12-14 h
- Centrifuge cultures in 5 ml aliquots, discard 4ml of supernatant, resuspend pellet into 1 mL
- Add 176 uL glycerol into each epp to 15% final concentration
- Vortex, aliquot 100 uL samples to cryotube
- Quick-freeze in liquid nitrogen
- Place in -80 C deep freeze

Plasmid purification **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

Procedure is followed with respect to the instructions of The GeneJET™ Plasmid Miniprep Kit
- Pick a single colony from transformants
- Inoculate in 5 mL LB medium for high-copy or 10 mL for low-copy of LB medium supplemented with the appropriate selection antibiotic.
- Incubate @ 225 rpm not longer than 12-14 h at 37 C .
- Spin @ 4000 rpm for 5 min at 25 C, discard the supernatant and keep pellet.
- Resuspended the pellet with 250 ul Resuspension solution.
- Transfer the cell suspension to eppendorf, add 250 uL Lysis Solution
- Mix throughly by inverting the tube 4-6 times until the solution becomes slightly clear.
- Add 350 uL Neutralization solution
- Mix immediately and throughly by inverting the tube 4-6 times.
- Spin @ 13000 rpm for 5 min to precipitate cell debris and chromasal DNA.
- Transfer the supernatant to spin column 600 uL
- Spin @ 13000 rpm for 1 min, discard the flow-through.
- In same collection tube add 500 uL Wash solution to spin column.
- Spin @ 13000 for 1 mini discard the flow-through.
- Repeat this step with using 500 uL Wash solution then discard the flow-through
- Spin @ 13000 for an additional 1 min to remove residual Wash solution.
- Transfer spin column into a fresh 1.5 mL epp.
- Add 50 uL Elution buffer into center of the spin column membrane to elute the plasmid DNA
- Incubate for 2 min at 25 C spin @ 13000 for 2 min.
- Discard the column and store the purified plasmid DNA at -20 C.

Before digesting the plasmids, the purity and concentartions of plasmids are required. We analyzed these values by Alpha UV spectrophotometer. According to the values in some cases as for small promoter

fragments, the isolated plasmids should be concentrated. We processed two methods to precipitate small DNA fragments. One of them was to vacuum the plasmids after isolation in elution buffer. Other method was ethanol precipitation.

Ethanol Precipitation **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

- Measure out 2X volumes absolute ethanol into DNA sample.
- Incubate at -80°C for 1 hr.
- Centrifuge at a speed of at least 10000 Xg for 30 mins at 0°C, gently aspirate out the supernatant and discard it. (All steps should be done in ice)
- Measure out 750 - 1000 µl of 95% ethanol into the eppendorf tube that is used at first step.
- Centrifuge at a speed of at least 10000 Xg for 10 mins at 4°C, gently aspirate out the supernatant and discard it.
- Dry the pellet in air until white pellet appears.
- Add appropriate volume of water to pellet.
- Resuspend pellet by vortexing/by shaking vigorously.

Restriction Digestion **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

Prepare total mix in following order with respect to purity values.
- Add water nuclease free to 0.2 mL PCR tube
- Add appropriate 10x buffer (depending on enzyme type fast digest or conventional enzymes)
- Add DNA solution to the tube (ugr)
- Add required unit restriction enzyme for DNA ugr to the tube
- Spin the tube for 5 sec
- Incubate the tube for h at 37 C at heatblock
- For enzyme inactivation incubate tube additional 80 C for 5 min (for fast digest enzymes)
- Purify cut plasmid using instructions of Fermentas PCR purification kit

Validation- Agarose Gel Electrophoresis **– METU-Turkey – 2010**
http://2010.igem.org/Team:METU_Turkey/Procedures

-Mix 5 uL DNA + 1 uL loading dye on the parafilm (5:1)
- Load sample to the wells of 1% gel with EtBr
- Adjust the voltage of power supply to 75 V (changes)
- Adjust the time of power supply to 65 min (changes)
- Check transilluminator
- After running of the samples record the gel image

Gel Extraction **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

The GeneJET™ Gel Extraction Kit instructions are proceeded while extracting the digested DNA fragments from agarose gel.
Before ligating we had tried some more protocols to enhance the efficiency of ligation For efficient ligation of digested fragments, there should not be the phosphates at the end of sites. Then for efficient dephosphorylation of sites of plasmid we treated the fragments with alkaline phosphatase method.This protocol is suitable for removal of 3’ and 5’ -phosphate groups from DNA and RNA.

Alkaline phosphatase treatment **– METU-Turkey – 2010**
http://2010.igem.org/Team:METU_Turkey/Procedures

- Prepare the following reaction mixture:
Linear DNA (~3 kb plasmid) 1 µg (~1 pmol termini)
10X reaction buffer for AP used in reaction 2 µl
FastAP™ Thermosensitive Alkaline Phosphatase 1 µl (1 u)
Water, nuclease-free (#R0581) to 20 µl
Total volume 20 µl
- Mix thoroughly, spin briefly and incubate 10 min at 37°C.
- Stop reaction by heating for 5 min at 75°C.

Ligation **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

Prepare ligation mix in following order and in 1:1 ratio (vector:insert)
- Add nuclease free water
- Add rapid DNA ligation buffer
- Add the calculated amounts of insert elute (1 or more) (elution from gel extraction procedure must be done with nuclease free water)
- Then add from backbone (calculated amount in 1:1 ratio)
- Add T4 DNA ligase), end up T4 DNA ligase in 10 to 15 uL
- Keep the ligation mixture in room temperature for 5 min.

Transformation **– METU-Turkey – 2010** http://2010.igem.org/Team:METU_Turkey/Procedures

- Mix 1 uL vector DNA (ligated parts) and 80 uL competent cells.(1-10 ng vector)
- Incubate in ice bath for 30 min.
- Heat shock for 20 sec. in 42C at heatblock.(not exceed 30 sec)
- Ice bath for 30 sec.
- Add 600ul of SOC medium on bacteria culture.
- Incubate in 200 rpm shaker for 1h
- Inoculate into plates with appropriate antibiotics.

Restriction Digests – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
Method:
Determine the concentration of the DNA sample by running both the vector and insert on a 1% agarose gel and comparing the bands intensity with the ladder (concentration known).
Calculate how much solution is needed to obtain desired total amount of DNA for digestion.
The volume of DNA solution can be no more than 70% of the total solution. Therefore calculate the total volume of digestion (probably around 20µl or 30µl).
Transfer the DNA, BSA, the appropriate buffer and ddH2O into a microcentrifuge tube. Finally, add the enzymes to the solution. N.B. The enzymes should be kept on ice before being added to the digestion.

Incubate for 60-90min at 37°C. Put in the freezer or on ice immediately after to stop further digestion. Especially important for EcoRI and other enzymes with star activity.
Use gel electrophoresis to confirm correct digestion.
Gel purification can be used to obtain the desired digestion product from the gel.
Reaction mixtures:

Required components	Example
1/20 Enzyme 1	1.5µl EcoRI
1/20 Enzyme 2	1.5µl PstI
1/10 BSA	3µl BSA
1/10 Buffer* (x 10)	3µl Buffer 4 (x 10)
X/10 DNA solution	20µl DNA (pSB1C3)
7-X/10 ddH2O	1µl ddH2O
Total: 10/10	Total: 30µl

Or you can use this as a guide:

20µl reaction volume unless digesting large amounts of DNA (use 30µl)
4µl DNA (if from Midi-preps, use 8µl Mini-Prep DNA)
2µl Buffer * (1 in 10µl total volume)
2µl 10xBSA (1 in 10µl total volume)
1µl Enzyme 1 (use 1.5µl for 30µl digests)
1µl Enzyme 2 (most Bio-Brick REF assembly protocols require a second enzyme) (use 1.5µl for 30µl digests)
The buffer depends on the restriction enzymes used.

Prefix Insertion:

	Enzymes used:	Required buffer:
Prefix (insert)	EcoRI & SpeI	Buffer 2
Suffix (vector)	EcoRI & XbaI	EcoRI buffer

Suffix insertion:

	Enzymes used:	Required buffer:
Prefix (vector)	SpeI & PstI	Buffer 2
Suffix (insert)	XbaI & PstI	Buffer 3

SpeI doesn't cut particularly at the end of PCR products particularly well as there are few flanking bases. Can leave overnight and add the second enzyme as a second 90 minute cutting step.

Ligations – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
A typical ligation reaction mixture is around 10 µl and contains
1 µl DNA T4 ligase
1 µl DNA T4 ligase buffer (check to ensure it contains ATP) (10x)
Purified, linearised vector*
Purified, linearised insert*
ddH2O
There should be a ratio of 6:1 for moles of insert to vector. This can be calculated using the following equation:

Insert mass (ng) = 6 x (Insert length (bp)/vector length (bp) x Vector mass (ng) Once the solution is made up, the tubes are vortexed and then spun down for around 10 seconds in a microcentrifuge. The ligation is done at 14°C in a water bath in the cold cabinet, and is left overnight.

E. coli Transformations – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
One 15ml tube for each sample, in addition to one for a negative control, is put on ice.
Tubes containing 1ml LB were incubated in a water bath is set to 42°C .
Between 25μl and 40μl of competent cells is transferred to each tube.
The cells are left on ice for 10min.
5μl of the DNA sample are transferred into each tube, but ddH2O is added to the control tube(s). The liquids are added directly into the cell culture.
N.B. During pipetting the sides of the tube should not be touched to avoid contamination. Bubbles should be avoided because they can cause the cells stress.

The tubes are transferred into the 42°C water bath for exactly 45 seconds and then put on ice for 2 minutes. Timing must be exact.
The tubes are put on a rack and 1ml LB is added to all of them. This levels the temperature of the solution at about 37°C.
The tubes are then put into a shaking incubator at 37°C for 1 hour.
The solution from the 15ml tubes is then transferred to a microcentrifuge tube and spun at 13500 rpm for a few seconds.
The supernatant is discarded and the remaining LB is mixed with the pelleted cells. This increases the concentration of the cells in the LB.
50 – 100μl of this solution is then pipetted onto chloraphenicol plates and left overnight at 37°C.
The next day colony PCR can be used to examine if the transformation was successful.
PCR – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
PCR Reaction Mix
25μl Total Reaction Volume
18.75μl ddH2O
2.5μl Buffer (Barns for any enzyme or Taq, Pfu buffer depending on enzyme used)
1μl Forward primer
1μl Reverse primer
1μl Template
0.5μl dNTPs
0.25μl Enzyme (Taq, Pfu etc)
PCR programme – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

Heated lid - 110°C
35 cycles
95°C for 1.5 mins - Denature the template
--°C anneal primers
t°C optimal / time optimal to extend (depends on enzyme used)
(-t°C optimal: 72°C for Taq // time optimal: 2-3kb/60 sec) (-t°C optimal:68°C for Pfu // time optimal: 1kb/15 sec)

Final step 68/72°C for 10/5mins – to allow full extension of any oligonucleotides

Single Colony PCR A master mix is generally used for SCP, as well as Taq polymerase because the high error rate is not an issue here as it is purely confirmatory. Cells from an individual colony are first spread onto a replica plate, and the same loop is then used to inoculate a microcentrifuge tube containing 100µ ddH20 which will later be heated to 95°C for 5 minutes to be used in the SCP (the same loop is finally used to inoculate LB for the overnight cultures). The protocol for the first SCP was as follows:

19.75µl ddH20
2.5µl Barnes buffer
1µl template (this comes from the tube that contains 100µ ddH20 and was inoculated with cells.
0.5µl dNTPs
0.5µl forward primer
0.5µl reverse primer
0.25µl Taq polymerase.
We also used a positive control (other DNA to which the primers will definitely anneal) and a negative control (ddH20).

The temperature cycle was as follows:

95°C for 30 seconds
30 cycles of: 95°C for 30 seconds, 62°C for 90 seconds, 68 °C for 30 seconds
68°C for 10 minutes
Hold at 4°C
Overnight Cultures
Tubes containing 5ml of LB medium are inoculated with cells from one colony and then 5µl of antibiotic (for example chloramphenicol) is added. They are then left at 37°C overnight.
SDS-PAGE – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
Short for: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Prepare gel : Two glass plates are cleaned with ethanol and are fitted into a holder. The separation layer of the gel is prepared first, following the recipe but before and after addition of the last two substances the solution should be inverted. The mixture, that now starts to polymerize, in now pipetted between the glass plates until it reaches the green bar. Around 700µl of ethanol are than added on top of the gel, which is left to solidify. The separating gel contains 10% acrylamide (toxic!) that has been polymerized by TEMED. Stacking gel contains less acrylamide for wide pores. After the gel has solidified take out the comb. Once solidified the stacking gel can be prepared using a different recipe but same method. Once the ethanol has been removes the solution is poured onto of the gel and the comb inserted. The gel is now left to solidify.
Load samples : The proteins, which have been denatured by SDS, are loaded into the wells.
Run gels : The gel is run at 100V for around 2-3 hours until dye front has reached the bottom of the gel.
Analysis of results : The gel can be analyzed by staining with Coomassie blue or Western Blot.
Catechol Assay – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
Catechol assay is performed in the plate reader on a 96 well plate
Each well must be filled with 100um of solution
Usually use 90ul of cell culture and 10um of catechol solution
Catechol stock solution is at 100mM concentration. And when added to the well we have a 10fold dilution. For example if an aliquot concentration of 1mM catechol is made, which would be used for assay, when the well is added catechol drops to a concentration of 0.1mM.
Always dilute catechol with H2O.

Always have a blank of 90ul medium (the one which you grew the cells overnight) with 10ul catechol solution
Always have a negative of 90ul growing cells and 10ul of H2O.
Other Useful Information
PCR purification – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol
Used to purify DNA to remove primers, salts and enzymes. It can also be used to purify away small fragments from restriction digests, for example when cutting a vector open. We used the E.Z.N.A.® Cycle Pure Kit and protocol (Omega bio-tek) (ddH2O instead of Elusion Buffer used in last step).

Gel purification – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

We used the QIAquick® Gel Extraction Kit (250) and protocol (ddH2O instead of Elusion Buffer used in last step).

Excise DNA from the gel and put into BF falcon clip-top tube (Blue Box) (Sybr Safe performs better under blue light)
Check excision of the right band and weigh the slice (gel can be frozen at -20°C to be extracted at a later date)
Add 3xvolume of buffer QG
Incubate at 50°C for 10 minutes or until completely dissolved, vortex every 2-3 minutes
Check colour – consult kit protocol if not orange
Add 1xvolume Isopropanol (crucial step to ensure that the DNA binds the column
Put 800µl into the QIA quick spin column with 2ml collection tube
Centrifuge for 1 minute and discard the flow through, repeat if necessary.
Add 500µl buffer QG to the quick spin column and centrifuge for 1 min
Add 750µl buffer PE to the quick spin column and centrifuge for 1 min
Dry the column by centrifuging for 1 minute
Place QIA quick spin column into 1.5ml collection tube (eppendorf tube)
Elute the DNA with 35µl of ddH2O

Minipreps – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

The E.Z.N.A.® Kit and protocol was used.

Midipreps – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

The QIAGEN HiSpeed Plasmid Midi Kit and protocol was used.

Agarose gels – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

1% Agarose gel (for DNA 1g Agarose for each 100ml 1xTAE buffer)
Marker – dilute invitrogen 1kb plus DNA Ladder (1 in 10 Loading Buffer (LB))
Diluting Primers – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

Add a volume of H2O equivalent to the yield of primer on the information sheet 1µg=1µl ddH2O

Leave to stand for 20 minutes
Mix thoroughly (pipette up and down)
Store at -20°C
Dilute 1 in 10 before use.
Oligo annealing – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

20µ total reaction volume
2µl of each single stranded primer
16µl ddH2O
2µl Buffer 2 (1 in 10 total volume)
Sequencing reaction mix – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

15µl total reaction volume
3µl of sequencing primer (amplifies the relevant DNA fragment)
50-100ηg of DNA
Label with sequencing labels and make sure that the codes from these are entered on MWG website.
DpnI Digests – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

DpnI digests methylated DNA, such as template DNA extracted from cells (colony PCR/Mini/Midi-prep), while leaving non-methylated PCR products uncut.

Can use 1µl of DpnI straight in the 25µl PCR product (PCR buffer is sufficient)
Alternatively use Buffer 4 – does not require BSA.
Incubate for 1 hr at 37°C
Rapid Alkaline Phosphatase

Dephosphorylation useful to prevent vector self re-ligation. – Imperial College London – 2010 -
http://2010.igem.org/Team:Imperial_College_London/Protocol

10µl Total Reaction Volume
--µl DNA vector (concentration depends on relative concentrations of the parts to be ligated)
1µl Phosphatase buffer
1µl Alkaline Phosphatase
-µl make up 10 µl with ddH2O

Antibiotics and Concentrationas – Groningen – 2010 http://2010.igem.org/Team:Groningen#/protocols
Antibiotics
Ampicillin
100 mg/ml Ampicillin (1000x) Stock
1 g of Ampicillin sodium salt in 10 mL of demiwater (or 50% EtOH)
Add NaOH or KOH to allow the Ampicillin to dissolve
Filter sterilize 0.2 µm filter and aliquot
Store -20 °C
Chloramphenicol
35 mg/ml Chloramphenicol (1000x) Stock
0.35 g in 10 mL 100% EtOH

Filter sterilize 0.2 µm filter and aliquot
Store -20 °C
Kanamycin
50 mg/ml Kanamycin (1000x) Stock
500 mg in 10 mL demi water
Filter sterilize 0.2 µm filter and aliquot
Store -20 °C

Protocols for Lactococcus – Groningen – 2010 http://2010.igem.org/Team:Groningen#/protocols
Preparation of cells
10 ml ON culture (SMGG) in 100 ml SMGG
Grow to OD600=0.2-0.7
Wash three times with 50 ml icecold wash buffer
Resuspend in 1 ml wash buffer
Electroporation
1 µl DNA in 40 µl cell-suspension in ice cold cuvette
Electroporate at 2.5 kV, 25 µF, 200 Ohm.
Add 4 ml SMG17MC
Incubate 2 hours (Ery induction=50 ng/ml)
Concentrate cells to 0.5 ml.
Plate on GSM17- Agar.

Protocols for Streptomyces – Groningen – 2010 http://2010.igem.org/Team:Groningen#/protocols
Growth condition
-Make plates with a thick layer of medium, 100 ml medium per big Petri dish.Medium of Streptomyces is mannitol soya flour medium (MS or SFM) 300 ml per 1L bottle to avoid boiling over in autoclave
Agar 6 g
D-mannitol 6 g
Soya flour 6 g
Demiwater 300ml
Plating ΔrdeAB spores 10^5 /plate
-Incubate for 7 days at 30 °C
-Harvest mycelium with razorblade

Cell wall isolation
-Scrape the mycelium with a razor blade and resuspend in 15 mL demi-water.
-Run this suspension throught the cell disrupter at 13 kpsi 6X:
Clean all the parts off the cell distrupter with 70% ethanol, including the tower, make sure the nozzle is not blocked by running some ethanol through it.
Let the loading cilinder and the nozzle rest in ice on aluminium foil for 5/10 minutes to cool them off(prevents protease activity later on).
Filtrate your samples using a 10 mL surringe and the filter device, catch your filtrate in a clean greiner tube.
Press the loading cilinder on the tower tightl (don't be to careful)
Load **no more than 15 mL** of your **filtered** suspension
Put on the nozzle and the lit of the nozzle, tightly

The big cilinder can be placed on top of the nozzle/ loading cilinder, twist and turn to get it on and align the dent at the bottom of the cilinder with the dent on the french press
Adjust your pressure to 13 kpsi
Use the pin to start the machine, after two loud klblam!'s remove it
Collect the distrupted cells(they are in the nozzle), using a surringe of 10 mL
Run it through another 5X
Collect your severely distrupted cells using the surringe, collect them in a greiner tube and put that on ice
Clean all parts of the french style cell distrupter with 70% ethanol
-Cook in 2% SDS (spin off your French press samples- 5000 rpm and 5 min- then add 2% SDS)
-Spin off, 4000 rpm, 5 minutes: Use pellet
-Cook in 2% SDS
-Spin off,4000 rpm, 5 min: Use pellet -Wash 10X with demi-water:
Resuspend
Spin off: Use pellet
Etc.
-Freeze pellet (For freeze drying: free it well)
-Freeze dry your pellet
Freeze the jar in which you will freeze dry your sample as well
Turn on the machine, it needs to get on temperature and suck vacuum
Make sure all the valves are closed
The big red valve needs to be open
The machine is ready when temperature reaches -80 and stays stable
Your greiner tube will be put in the jar with either a loose tube hood or parafilm to seal it off (prick 4 holes), seal the jar of with the rubber top
Place the jar on one of the valves and open it up slowly
When the sample is dry-freezed
Close the valve and remove the jar
Turn off the machine
Close the red valve

Chaplins – Groningen – 2010 http://2010.igem.org/Team:Groningen#/protocols
Expression Chaplins in NZ8900
-Grow O/N culture in Ty with antibiotics (Km5 and Cm5)
-Measure OD600 (with 0.9 ml with 100 µL of O/N culture, number *10=OD)
-Inoculate 15ml TY (with ab) in 100 ml flask to OD=0.1
-Grow till OD600 is approx. 0.5
-Take 2 ml t=0 sample
-Induce with 1% subtiline
-Continue growing
-Take 2 ml samples every hour
Chaplin purification
-5mg cell wall in 1.5 mL tube. Do not use gloves because static electric interaction
-Add app. 1 mL of TFA with a glass pasteur pipette.Do this in fumer and never leave TFA open for more than 30 sec!
-Vortex 1 min
-Spin down insoluble material for 10 min at 13000 rpm
-Transfer supernatant to new 1.5 mL eppendorf tube or 5 mL Greiner tube
-Dry TFA for 1-2 hours
-When tube is dry, resolve in water or Tris buffer

Sample treatment
-Take 2ml sample and spin down
Supernatant
-1.5 ml of supernant in separate cup, rest of supernatant can be discarted (at this point you can store in freezer)
-TCA precipitation until drying pellet after washing with aceton
-Continue with TFA treatment
Cell pellet
-TFA treatment
-Pellet of TFA treatment with cells should be airdried
-Lysed for half and out at 37 °C (P-buffer with 20 mg/ml lysozyme)
-Speedvacum
-TFA treatment

Protocols for Bacillus subtilis 168 – Groningen – 2010
http://2010.igem.org/Team:Groningen#/protocols
2-step transformation procedure for *Bacillus subtilis* 168
Cells are grown O/N at 37 °C in supplemented medium contains Spizizen's salts (without antibiotics)
Dilute cells 10X in fresh medium (10 ml) and grow for 3 h at 37 °C, shaking
Make agar plates
Cells are then diluted 1:1 in starvation medium(prewarmed, no antibiotic)
After 2 hours, approx. 1 µg DNA (BRB689; Cmr *amyQ+*) is added to 100 µl cells in a 2 ml tube, and the mixture is incubated for 30 min at 37 °C (200 rpm)
Dilute 3-fold in prewarmed TY medium and plate after another 45 min (37 °C;200 rpm)
Dilutions (in starvation medium) for plating are:
* 10^0 and 10^-1 on selective media
* 10^-5 on non-selective plates (viable count)

Normal SDS-PAGE using Tris-Glycine Gels and Electrophoresis Buffer – Groningen – 2010
http://2010.igem.org/Team:Groningen#/protocols
Wear gloves at all times!
Requirements:
30% acrylamide(Biorad) **NEUROTOXIN!**
4X separation gel buffer: 1.5M Tris.HCl, 0.4% SDS pH6.8
4X stacking gel buffer: 0.5M Tris.HCl, 1.92M glycine, 1% SDS water saturated isobutanol
TEMED(in yellow cabinet)
10% APS (Ammonium persulfate) ; prepare 10 ml and keep cold in fridge
Hoeffer Mighty gel system Casting of the gel:
Per gel take one small glass plate with 'ears', one big white ceramic plate, two grey spacers with perpendicular plastic bits (0.75 mm), and one white comb (0.75 mm)
Clean the plates etc with soap, rinse with demiwater and ethanol, and dry
Assemble the system in the gel casting holder. Mark the line of separation/stacking
Mix the separation gel in 10 ml plastic tube:
for 2 gels

Seperation gel (0.75 mm)	**12.5%**	**16%**
30% acrylamide	4 ml	5.1 ml
4X separation buffer	2.4 ml	2.4 ml

MQ	3.2 ml	2.1 ml
10% APS	28 µl	28 µl
TEMED	28 µl	28 µl

Pipet the separation gel mix immediately in between the glass plates until the marked line is reached
Pipet water-saturated isobutanol on top of the polymerizing separation gel
Let the separation gel polymerize completely before preparing the stacking gel
When the gel is polymerized, discard the isobutanol and wash the gel with water
Mix the stacking gel in a 10 ml tube:

for 2 gels

mQ	1.92 ml
4X stacking buffer	0.83 ml
30% acrylamide	560 µl
10% APS	14 µl
TEMED	7 µl

Pipet the stacking gel mix immediately on top of the separation gel and place the comb without air bubbles. Mark the teeth of the comb with a marker
Let the gel polymerize (5-30 min); check also the remaining gelmix in the tube
Disassemble the gel casting holder and take out the gel/plates
Use the gel immediately or seal the gel in plastic seal bags and store at 4 °C

Assembly of the Tris-Glycine gel in the electrophoresis unit

Take the electrophoresis unit of the Hoeffer system and place it next to a power unit
Dilute 10X and pour 1X electrophoresis buffer in the container(roughly 1 cm high)
Place gel in the buffer without air bubbles under the gel
Use the red clamps to place the gel tightly in the unit
Pour 1X electrophoresis buffer in the chamber so that the top of the gel is immersed
Take the comb out of the gel and rinse the wells using a hooked needle and syringe

Runninge of the gel

Prepare the samples in 1X SDS sample buffer (NOT nucleic acid loading buffer)
Boil the samples for 5 min and spin down
Pipet the samples and protein marker carefully into the wells
Place the electrode cap on the unit and press lightly.Put the other side of the electrode cables in the correct holes of the power unit
Switch on the power unit and run until the blue front is appr. 1 cm from the end of the gel. Alternatively, use the prestained protein marker to identify the exact point of stopping
Disassemble the electrophoresis unit and take the gel
Take one plate off and cut one corner away for positioning purpose
Carefully bring the gel into a clean staining container using some demi water and/or spacers
Stain the gel with CBB or silver

Transformation to *E. coli* – Groningen – 2010 http://2010.igem.org/Team:Groningen#/protocols

Add 10 µL of ligate to 100 µL competent cells
Put cells for 30 minutes on ice
Heat shock the cells for 45 seconds at 42 °C
Put cells on ice for 2 minutes
Add 400 µL LB and grow for 1 hour at 37 °C
Plate 100 µL of cells on LB agar (2%) plates

BioLector – DTU Denmark – 2010 http://2010.igem.org/Team:DTU-Denmark/Lab_protocols

Preparation
Make a plate design specifying which strains will be in each of the wells in your BioLector plate. It's highly recommended to run all strains in duplicates. The following controls should be included: strain used for transformation, strains expressing the GFP and/or RFP that will be measured in the other wells.
Make overnight cultures of the strains you want to run in the BioLector.
Materials
Growth media (LB)
Overnight cultures
Adhesive Gas Permeable Seal
Adhesive Seal for Evaporation Reduction for 48-well plates
Procedure
Measure OD of the overnight cultures.
Dilute the overnight cultures down to an OD of 0.05.
Add 1.5 mL of diluted culture to each of the 48 wells.
Apply the Adhesive Gas Permeable Seal to the plate and make sure it is stuck on tightly. The seal must be put on precisely, as putting it on crookedly will result in some of the wells not being fully covered.
Apply the Adhesive Seal for Evaporation Reduction on top of the Gas Permeable Seal.
Place the BioLector plate carefully into the BioLector in the specified slot until it clicks in.
Using the Start Assistant in the BioLector, set the temperature and humidity to be held during the experiment. The filters to be used for measurements of each well in the experiment should also be set, as well as the sampling time (3 mins per filter used).
Run the BioLector as long as needed. The measurements of the biomass and amount of FPs in each well can checked while the BioLector is still running. It is recommended to run the experiment at least until the cells in each well reach stationary phase.
Use the following settings:
37 °C
Fluorescence gain of 80
Biomass excitation 620 nm (light scattering)
GFP filter was 486nm (ex) / 510nm (em)
Ligations – DTU Denmark – 2010 http://2010.igem.org/Team:DTU-Denmark/Lab_protocols
Preparation:
Calculate your ligase concentration by estimation of DNA concentration from your restriction gel.
Which vector to use?
Material:
Prepare ligation mix with a total volume of 20 µl
2 µl 10x Buffer
Parts to be ligated: vector+insert 1:5, DNA conc. less than 50 ng
fill up with water
1 µl ligase
control (no insert)
Add ligase and keep mix at RT for 1h.
To inactivate the ligase place your mix at 65ºC for 10 min.
Store on ice.
Before starting on the ligation(s), always run your digestion products after you've performed a clean-up/purification on a gel to verify you actually have been successful in your digestions.
If you want to be very precise with your DNA-content calculations, when running the gel you can load the DNA-ladder sequentially with a difference of 2-fold dilution.
If your digestions were successful and you are happy with the way your gel looks, you are set to start your ligation calculations.
Firstly, make sure you have a DNA-ladder illustration indicating the mass of DNA at different positions

on the gel, then compare the intensity of bands of your digested products with the intensity and position of the bands from the DNA-ladder(s).
The same intensity of bands indicates they must have the same mass of DNA.
After you have determined how much DNA (mass) you have of each band, you can calculate the DNA content on a mole basis by dividing the mass with the length of the fragment. In general a ligation-mix should look like this:
Usually a ligation mix should have a total volume of 20 µl, although if you require additional amounts of the same ligation, make a duplicate of the ligation you are trying to make.
The buffer needed is simply called “10x ligation buffer”, and being 10 times concentrated, you need to add 1/10th of the total volume of buffer. Hence if the total volume is 20 µl, you need to add 2 µl of your 10x ligation buffer.
The amount of DNA added should conform to the calculations you made from the gel you ran with your digested products. A rule of thumb should be that you take more insert than vector, this way increasing your chances of actually getting a ligation. The ratio of insert to vector should be 5:1 and the total amount of DNA should not exceed 50 ng.
The two remaining items to be added into your mix will be ddH2O and Ligase. The amount of Ligase enzyme to be added is only 1 µl, so you know how much DNA (insert+vector), Ligase and the buffer to add; the rest of the volume (→ 20 µl) should be accounted for with ddH2O.
Remember though that Ligase should only be added in the end, because once added into your mix its going to get to work and you don’t want it playing around with your dna before you have added everything else. Another important thing to remember is that the Ligase enzyme cannot be taken from the freezer to your lab-bench; you have to instead add your Ligase into your mix by working at the freezer.
Controls are always important and good indications if things are working the way they should be, so a good control with ligations is a negative control, that being just vector with no insert, the rest of the recipe being the same. This should hopefully give you no colonies as it would indicate your digested vector is in fact digested and didn’t re-ligate. If there are colonies on your negative control plate, it will give you a good indication of the background noise on your actual ligation plates.
PCR product purification – DTU Denmark – 2010 http://2010.igem.org/Team:DTU-Denmark/Lab_protocols
Materials
1 vol PCR product
2 vol NT Buffer
600 µl NT3 Buffer
300 µl Elution Buffer NE
Procedure
Mix 1 volume of sample with 2 volumes of NT buffer in an 1,5 ml Eppendorf tube.
Place a column into a 2 ml collection tube and load the sample.
Centrifuge at 11.000 g for 1 min.
Discard flow through and place the column back into the collection tube.
Add 600 µl NT3 buffer and centrifuge at 11.000 g for 1 min.
Discard flow through and place the column back into the collection tube.
Centrifuge at 11.000 g for 2 min to remove NT3 buffer. Discard flow through.
Place the column into a clean 1,5 ml Eppendorf tube.
Add 30 µl Elution Buffer NE and incubate at RT for 1 min to increase the yield of eluted DNA.
Centrifuge at 11.000 g for 1 min.
Plasmid purification by miniprep (Zymo Research Group) – DTU Denmark – 2010
http://2010.igem.org/Team:DTU-Denmark/Lab_protocols
Materials
2 ml cell culture
600 ul TE Buffer
100 µl 7X Lysis Buffer

350 µl (cold) Neutralization Buffer
200 µl Endo-Wash-Buffer
400 µl Zyppy Wash Buffer
50 µl Zyppy Elution Buffer
Procedure
Spin down 2 ml of cell culture in a 2 ml Eppendorf tube at 11.000 g for 5 min.
Remove supernatant, spin again down for 10 seconds, remove supernatant (if the pellet is not sufficient, repeat step 1 in the same tube).
Resuspend your pellet in 600 ul TE buffer.
Add 100 µl 7X Lysis Buffer (blue) and mix by inverting the tube 4-6 times. Proceed to the next step within 2 minutes.
Add 350 µl cold Neutralization Buffer (Yellow) and mix carefully. The sample will turn yellow and a yellowish precipitate will occur, then the reaction is finished. Invert the tube an additional 2-3 times to ensure complete neutralization.
Centrifuge at 11,000 g for 5 min.
Transfer the supernatant (ca. 875 µl) into a column. Avoid disturbing the cell debris pellet.
Place the column into a collection tube and centrifuge for 30 seconds.
Discard the flow-through and place the column back into the same collection tube.
Add 200 µl Endo-Wash-Buffer to the column and centrifuge for 30 seconds.
Add 400 µl of Zyppy Wash Buffer to the column and centrifuge for 30 seconds.
Transfer the column into a clean 1,5 ml Eppendorf tube and then add 50 µl Zyppy Elution Buffer directly to the column. Let the product(s) stand for 1-15 minutes on the table at RT.
Centrifuge for 30 seconds to elute the plasmid DNA.
Restriction/Digestion – DTU Denmark – 2010 http://2010.igem.org/Team:DTU-Denmark/Lab_protocols
Materials:
25 µl DNA (depending on your DNA conc)
10 µl FD-buffer
1 µl (2.5 µl can also be used) restriction enzyme (1 & 2) **NOTE:** REs should be kept on ice.
63 ul (60 ul when 2.5 µL RE is used) ddH2O
Enzyme selection for BioBricks digest
A BioBricks part:
-----E--X---Part---S--P-----
Vector with upstream part - S,P
Downstream Insert - X,P
Upstream Insert - E,S
Vector with downstream part- E,X
E= EcoRI X= XbaI S= SpeI P= PstI
Procedure
Turn the thermo block on 30 min before use.
Mix everything - the enzymes should be added last, giving a total volume of 100 µl.
Leave for 2 hours at 37°C in thermo block/incubator.
Deactivate the enzyme by leaving it at 65°C in a thermo block for 10 min.
Keep on ice until its cooled down.
IMPORTANT: If you only use one RE on your plasmid, SAP treatment is required:

Mix 50µL of the restricted DNA with 2.5µL SAP and 5µL SAP-buffer.
Put it in the incubator 37°C for 60 min.
15 minutes on heat block 65°C to deactivate enzyme.
Ice.

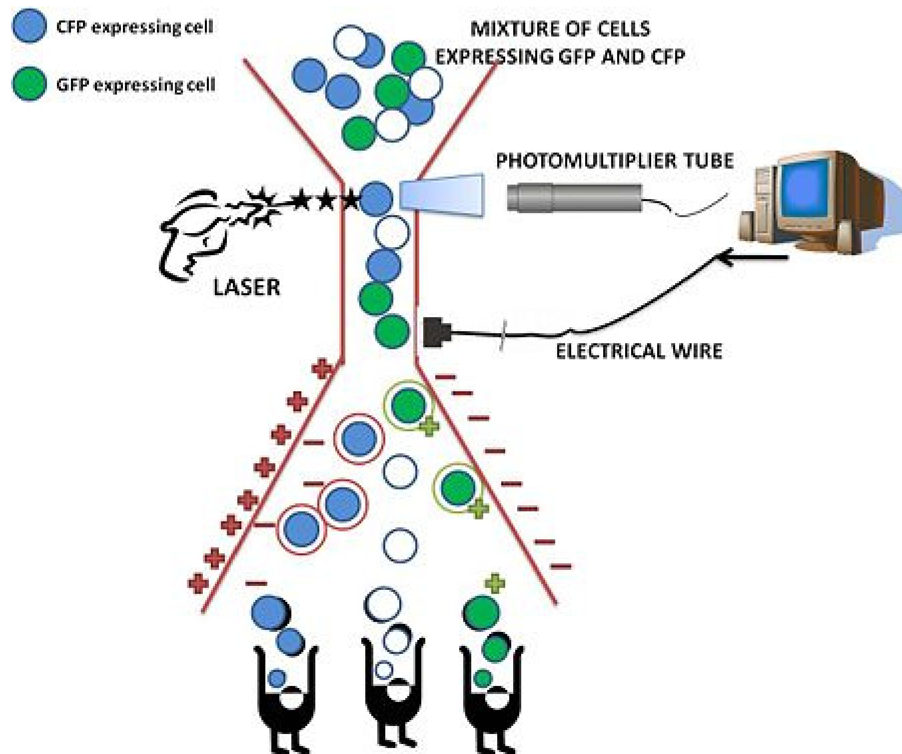
Transformation protocol – DTU Denmark – 2010 http://2010.igem.org/Team:DTU-Denmark/Lab_protocols
Materials
NOTE: The materials should be kept on ice.
5X 1 ml LB media
Cuvettes
50 µl electrocompetent cells
1 µl ligated plasmid with insert(s)
Procedure
Add 50 µl electrocompetent cells to the cuvette.
Add 1 µl ligated plasmid to the cuvette and make sure that it is mixed thoroughly with the cells without creating bobbles.
Have 1 ml LB media ready in a pipette.
Wipe the cuvette with tissue to ensure that the metal are free of water.
Insert the cuvette in the electroporator and press ‘ Pulse’.
Quickly add the LB media to the cuvette and transfer as much as possible back to the 5 ml tube.
Incubate for 1 hour at 37°C.
Plate out in duplicates (20 and 200 µl) on LB-plates containing your favorite antibiotic.

Flow cytometry Analysis of fluorescence Proteins – Aberdeen – 2010 - http://2010.igem.org/Team:Aberdeen_Scotland/Protocols
The flow cytometer used by our team was the Becton Dickinson 'LSRII '
Please note that as a technique, flow cytometry was used in many of our experiments although this is frequently referred to in our wiki text as FACS (Fluorescent activated cell sorting) analysis . However, we stress that in fact no cell sorting was performed in our experiments.

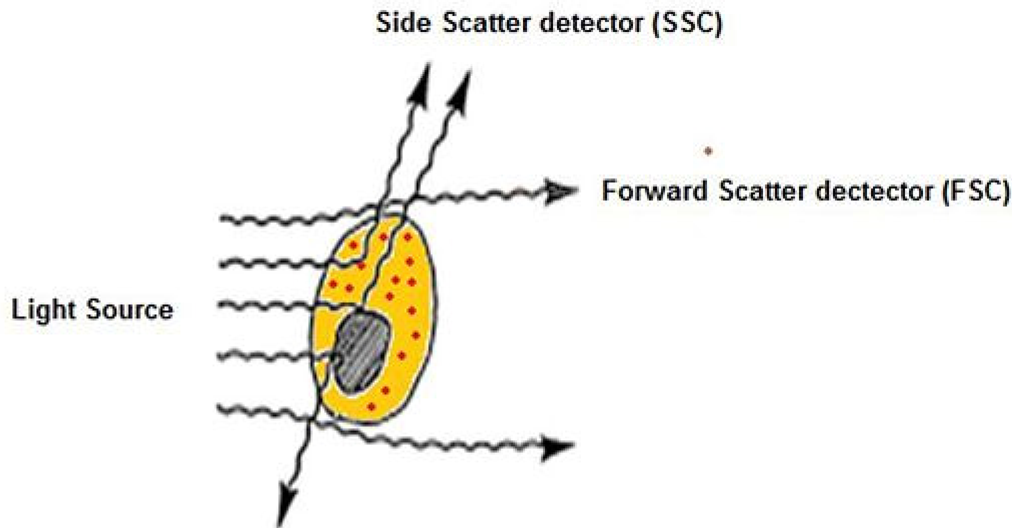
Flow cytometry and Its Advantages
Flow cytometry (FCM) is a technique used for counting and examining individual microscopic particles such as cells on the basis of their fluorescence. One of its unique features is that it measures fluorescence per cell or particle, contrasting with spectrophotometry which measures absorption and transmission of wavelengths as a bulk volume of the sample.

How Flow Cytometry Works

The sample is injected into the center of the sheath stream of flow cytometer in a liquid state; therefore the particles are distributed randomly. The fluidics system is then responsible for separating out the particles into an ordered stream of single particles.



After hydrodynamic focusing, the cells or particles of interest pass through the laser beam therefore intercepting and scattering the light which excites the fluorochromes to a higher energy state. The energy is then released as a photon of light with spectral properties unique to specific fluorochromes. Light scattered in the forward direction (as shown in the below diagram) is collected by a lens which is in line with the beam known as the forward scatter channel (FSC). The FSC intensity gives the particles size and can give information used to distinguish between cellular debris and living cells. The side scatter channel (SSC) is perpendicular to the beam and provides information about the granular content within a particle. Both FSC and SSC are unique for each particle and a combination of the two may be used to differentiate between different cell types in a heterogeneous sample.



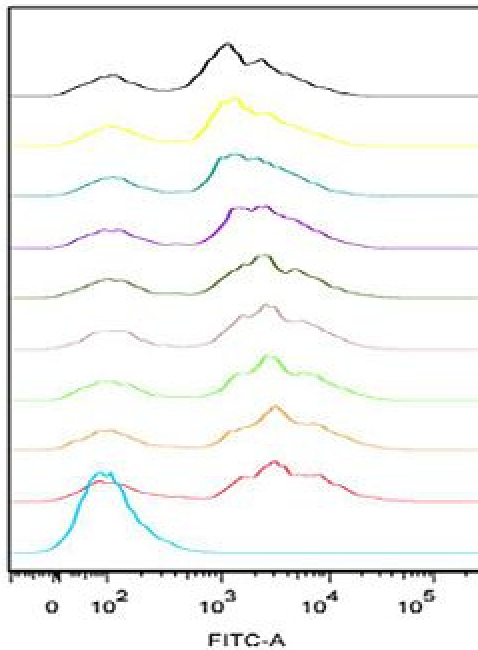
Cell Sorting
Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. The rate of flow sorting at 10 000 cells/second provides a method for sorting a heterogeneous mixture of biological cells into separate storage containers. It is based upon the specific light scattering and fluorescent characteristics of each cell. It is an extremely useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.

How Cell Sorting Works
After the cells have passed through the laser beam and the detectors, a vibrating mechanism causes the stream of cells to break into individual droplets. An electrical charge is placed at the point where the stream breaks into droplets immediately prior to the fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The droplets then travel through a strong electrostatic field and are deflected based on their charge into waiting sample tubes. The number of cells and level of fluorescence in each tube is then known.

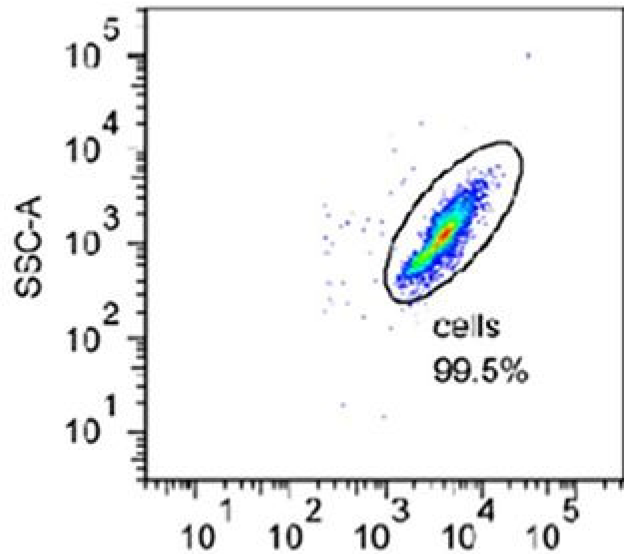
Variables Considered for Our Project When Using the Flow Cytometry.
During our experiment our choice of fluorochrome was restricted by the possibility of spectral overlap. When two or more fluorochromes are used during a single experiment there is a chance that their emission profiles will coincide, making measurement of the true fluorescence emitted by each particle very difficult. Therefore careful consideration of the excitation and emission wavelengths of the Green Fluorescent Protein and the Cyan Fluorescent Protein was carried out prior to the experiment to ensure there was no overlap.

Data Received From Flow Cytometry and Analysis

The graph shown below is an example of a single-parameter histogram obtained from the flow cytometry during our experiment. These graphs display a single measurement parameter; the relative fluorescence (as shown above) or light scatter intensity on the x-axis and the number of events (cell count) on the y-axis. This graph is very useful for evaluating the total number of cells in a sample that have the physical properties selected for or which express the marker of interest (as is the case with our project). The graph involves flow analysis on a mixed population of cells (some expressing GFP and some are not) this results in several peaks on the histogram. In order to identify the positive dataset, a positive and a negative control is used for positive identification of the peak corresponding to the cells which were and which were not expressing GFP.



Below is an example of a density plot taken during one of our experiments. In this plot, the particle counts are shown by dot density. Each cell recorded i.e. one of the dots shown above, is referred to as an event. The green colour represents larger number of events and the red one even more. The different colours are used to create a three-dimensional feel.



In preparation of the Flow cytometry analysis we;
1. Washed and resuspended samples in PBS at a density of 10^5-10^7 cells/ml.
2. Less than 1 ml was required for analysis and cells were stored on ice until analysed then vortexed before analysed.

Induction protocols for the GAL1, CUP1 and MET17 promoters – Aberdeen – 2010 - http://2010.igem.org/Team:Aberdeen_Scotland/Protocols
Yeast cells containing the relevant transformants were inoculated into 5ml SD medium cultures. Cultures contained 2% raffinose to provide the sugar source and a combination of amino acids depending on the plasmid and selection marker (Ura, His, Met, Trp at 0.2% and Leu at 0.6%).

Cultures were incubated on a shaker overnight at 30oC.

Samples were taken the following day and the OD600 was measured. Specific volumes of the yeast cultures were then taken and inoculated into new 5ml SD raffinose cultures that additionally contained the relevant concentration of inducer/repressor (galactose or copper sulphate or methionine).

The volumes used in these inoculations were calculated so that the OD600 reading obtained prior to testing was 0.6.

After cultures had reached an OD600 of 0.6 samples were washed and re-suspended in PBS

The induction of GAL1, CUP1 and MET17 was then tested by observing the expression of associated fluorescent proteins (GFP and CFP)

BioBrick construction – Aberdeen – 2010 - http://2010.igem.org/Team:Aberdeen_Scotland/Protocols

Introduction

For the iGEM 2010 project one of the team's aim was to contribute to the iGEM community via the testing and building of Bio-brick parts using standard plasmid parts. Here, we outline the process we used to construct Biobricks that were submitted to the Registry of Parts. The process consists of three steps: vector preparation (its purification and digestion), insert preparation (its amplification and digestion) and the final ligation step.

For the first step, plasmid pSB1C3 was chosen. It is a high copy BioBrick assembly plasmid (2072 bp) compatible with assembly standard 10.

We have successfully ligated four components of the toggle switch "AyeSwitch" to the pSB1C3 plasmid: Phage MS2 coat protein, Phage lambda N-peptide (and a tandem N-peptide variant) as well as B-box sequence encoding a regulatory mRNA stem loop. *Parts Submitted to Registry of Parts*

Protocol

Vector Preparation

Construction plasmid: pSB1C3 (High Copy BioBrick assembly plasmid) was provided by iGEM HQ as a PCR-amplified linear piece of DNA

1) The linear vector preparation was cut with EcoRI and PstI restriction enzymes

2) Cut vector was electrophoresed on an agarose gel to estimate size, quality and quantity (the latter in comparison to known amounts of molecular mass DNA ladders)

4) Restriction enzymes heat inactivation – 20 min at 65°C then pulse spin

5) In a normal ligation, at this point the vector would be treated with alkaline phosphatase to remove the 5' phosphate groups and prevent self ligation. However, with linear, PCR amplified vector as the starting material this was not necessary; from the *Registry of Parts;*

Short single stranded DNA fragments will not ligate to 4 bp overhangs. By creating a very short overhang on a PCR of a plasmid backbone, the remnant, when cut with EcoRI and PstI is sufficiently short that it will not anneal at ligation temperature, and will therefore not ligate.

RESULT: purified plasmid backbone with EcoRI and PstI cohesive ends

Insert Preparation

Selected part of the AyeSwitch such as MS2 coat protein.

1) PCR reaction to amplify the desired fragment for BioBrick construct i.e. MS2 coat protein from CUP1p - [MS2-CFP] plasmid (template) + forward and reverse primers of MS2 coat protein

2) Gel electrophoresis to assess whether desired fragment was amplified and to determine its concentration.

3) Digestion with restriction enzymes (EcoRI and PstI) to generate sticky ends

4) Restriction enzymes heat inactivation - 20 min at 65°C then pulse spin.

RESULT: purified selected insert with EcoRI and PstI cohesive ends.

Ligation Reaction

Vector + selected insert

1) Ligation in the molar ration of 1:3 (vector : insert).

Including a number of controls: a) vector alone (control for uncut vector presence) b) vector alone + ligase (control for unsuccessful alkaline phosphatase treatment) c) insert alone (control for template presence i.e. CUP1p - [MS2-CFP])

Ligation	Vector	Insert	Dilution buffer	Ligation buffer	Li
Vector backbone + selected insert	+	+	+	+	
Only vector	+	x	x	x	
Vector + ligase	+	x	+	+	
Only insert	x	+	x	x	

2) The ligation mix is then transformed into E. coli competent cells and grown overnight in LB plates + Chloramphenicol. It would be expected to see E. coli growing colonies only on vector backbone + insert plates.
3) PCR of E. coli colonies to amplify chosen fragment after successful ligation.
4) Gel electrophoresis to verify the lengths of fragments after successful ligation.
5) Getting DNA sequenced – final verification.
6) BioBrick submission.

Construction of Plasmids In Vivo using Yeast Homologous Recombination – Aberdeen – 2010 - http://2010.igem.org/Team:Aberdeen_Scotland/Protocols
Introduction
For the iGEM 2010 project the yeast shuttle vectors CUP1p - [MS2-CFP] and GAL1p-[Npep-GFP] were used to express the main constructs that form the AyeSwitch. In addition to this, in vivo modifications to these plasmids were also made to allow troubleshooting and testing of Bio-Brick parts. *See Results Section*

To carry out these modifications, in vivo homologous recombination was used to do this as it utilised the natural processes of the yeast involved whilst simultaneously transforming it with the modified plasmid.
Protocol

1.) The shuttle vector is linearised by cutting at unique restriction sites. In the case of CUP1p - [MS2-CFP] and GAL1p-[Npep-GFP]this can be done by checking its DNA sequence and the construct it carries to ensure there are no identical restriction sites.

2.) To check the desired cut has been obtained, the restriction cut products can be run on a gel to detect for the correct bands.

3.) In parallel, Primers are designed for the inserts that have to be homologously recombined. The essential feature is that the primers must also include 45bp from the end of the restriction cut site of the shuttle vector as part of the primer sequence. This applies for both forward and reverse primers and is

essential to allow homologous recombination of insert into shuttle vector.

4.) The primers are then used to PCR amplify the desired insert. Again, gel electrophoresis may be used to check the correct length of PCR product has been obtained.

5.) A transformation is carried out using yeast, the cut shuttle vector and PCR amplified products from previously. Transformed yeast is selectively cultured with suitable controls.

6.) To check that the appropriate modified plasmids have been transformed, the yeast can screened by using Colony PCR Screening.

7.) Colony PCR Screening involved taking a smallest amount of yeast from an individual colony, digesting its cell wall to release its DNA content. PCR primers for the homologous insert used previously are then used to see if any PCR amplification occurs.

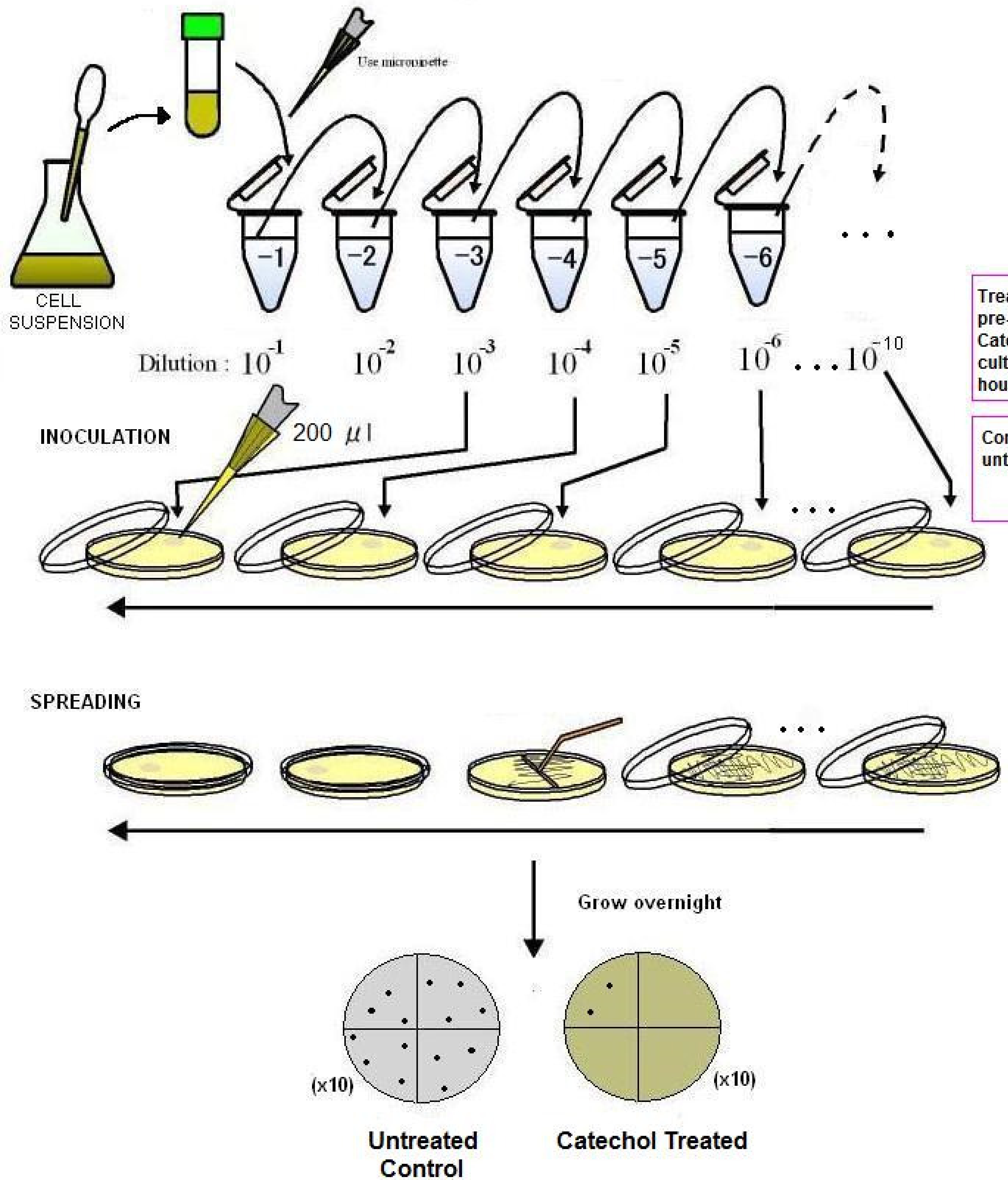
The presence of the desired PCR product indicates that the desired modified shuttle vector has been made and transformed.

References

Hinnen A, Hicks JB and Fink GR. Transformation of yeast. Proc Natl Acad Sci, 75 1978 (1929-33)

Tolerance Assay – Toronto – 2010 http://2010.igem.org/Team:Toronto/Protocols

A 0.5 M (10x) stock solution of catechol was made in a 5 ml volume of autoclaved, distilled water. This solution was added to 25 ml of sterile MM2 media to make up a volume of 30 ml. This was added to 20 ml of MM2 innoculated with E.coli DH5a (approximate density 1x10e8 cells per ml) for a final volume of 50 ml (catechol concentration 50mM). Control samples were created as above minus catechol. Treated and untreated samples were placed in a 37C shaker incubator and aliquots were removed every two hours. 0.5 ml aliquots were serially diluted in 4.5 ml of MM2 media and four dilutions representing 1×10^{-3}, 1×10^{-4}, 1×10^{-5} and 1×10^{-6} were plated by spreading 200 ul of the diluted sample on LB agar plates. After drying, plates were incubated in an inverted position in a 37C incubator overnight. Plates with between 30 and 300 colonies were counted to determine the number of colony forming units using the method described by Imperial College (iGEM 2009 OpenWetWare).



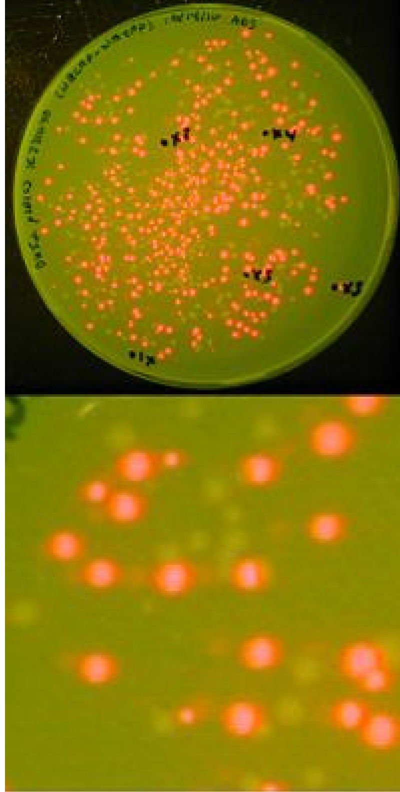
Making Chemically Competent Cells – Toronto – 2010 http://2010.igem.org/Team:Toronto/Protocols
Day 1

1. Start a 5 mL LB overnight culture in the 37C incubator from either a colony streaked plate or with one of the last frozen competent stocks of the strains you want to make competent.
Day 2
1. Inoculate the 5 mL overnight culture into a new flask with 200 mL of fresh LB.
2. Grow until O.D. 600 = 0.4.
a. To check O.D., take 1 mL of the bacterial culture and put it in a plastic cuvette.
b. Take another plastic cuvette and add in 1 mL of LB.
c. Change the wavelength of the spectrophotometer to 600 nm.
d. Blank using the LB cuvette.
e. Measure and record the absorbance of the cuvette containing the culture.
f. If the O.D. is below 0.4, place the flask back in the 37°C incubator and repeat 'e' at a later time point. Dispose of the cuvette with the culture but keep the cuvette with the LB as a blank.
3. Transfer 50 mL of the culture into a pre-chilled Falcon tube. Repeat for each of the remaining 3 Falcon tubes.
4. Place the Falcon tubes in ice for 15 minutes to cool the cultures.
5. Pellet the cells using a Beckman centrifuge at 4000 rpm for 10 minutes at 4°C.
6. Pour out the media in the sink and stand the Falcon tubes in an inverted position on paper towels for 1 min to drain away traces of liquid.
7. Re-suspend the pellets in each tube with 10 mL of ice cold 0.1M $CaCl_2$ by lightly pipetteing with a P1000.
8. Store on ice for 10 min.
9. Pellet the cells using a Beckman centrifuge at 4000 rpm for 10 minutes at 4°C.
10. Pour out the media in the sink and stand the Falcon tubes in an inverted position on paper towels for 1 min to drain away traces of liquid.
11. Re-suspend the pellets in each tube with 2 mL of ice cold 0.1M $CaCl_2$ by lightly pipetteing with a P1000.
12. Pool the aliquots and keep on ice for 1 hour.
13. Make a dry ice/ethanol bath.
a. Dry ice is on the 14th floor in the corresponding room where the incubators are at on our floor.
b. Ethanol is under the fumehood in a cabinet labelled "Flammables" beside the imaging room.
14. Add in 4.8 mL of ice cold 40% glycerol and very gently invert the tube to mix well.
15. Divide the mixture into the pre-chilled microfuge tubes in 400 µL aliquots.
16. Flash freeze the aliquots in the dry ice bath.
17. Store in the -80°C freezer.
Transforming Chemically Competent Cells – Toronto – 2010
http://2010.igem.org/Team:Toronto/Protocols
1. Thaw a stock of competent cells that you want to transform into on ice for 10 minutes. DO NOT REMOVE IT FROM THE ICE DURING THIS TIME.
2. If you are doing more than 1 transformation, take 100 µL of cells from the freezer stock and add it to prechilled microfuge tubes. If not, take 100 µL of cells to use as transformation controls.
3. Add 1 µL of DNA to the cells you want transformed. Stir gently with the pipette as any serious agitation will decrease transformation by a lot.
4. Leave mixtures on ice for 30 minutes.
5. Heat shock cells for 30s at 42°C. Different times and temperatures will decrease transformation efficiency.
6. Place mixtures on ice for 5 min.
7. Add LB to each tube until the total volume is 1 mL. (i.e. If there is 100 µL of cells, add 900 µL of fresh LB)
8. Place the tubes in the 37°C shaker for 1 hour.
9. Pre-warm and label (date, initials, what DNA is transformed with what cells, amount plated) selection plates in the 37°C incubator with the sign "BACTERIA ONLY".

10. Spread plate 2 amounts of transformants (10 µL and 50 µL) onto the corresponding selection plates.
11. Place overnight in the 37°C incubator with the sign “BACTERIA ONLY”.
Digesting DNA – Toronto – 2010 http://2010.igem.org/Team:Toronto/Protocols
1. Thaw the NEBuffer(s) that you will need to digest your DNA of choice.
a. The NEBuffer will have to be chosen carefully if digesting with 2 or more enzymes.
b. Always try to maximize the percent activity (indicated in the NEB info slips for each restriction enzyme).
c. Be on the lookout for star activity in different buffers.
d. If you can’t find a common NEBuffer, you can also digest sequentially.
2. Add your components in the following order (for a 25 µL reaction)
a. ddH2O
b. 10X NEBuffer (2.5 µL)
c. DNA
d. Restriction Enzyme (5 units)
i. If you scale up the reaction volumes, you won’t need to add more enzyme because 5 units is already way more than enough (unless you digesting 5+ µg)
3. Turn on the PCR machine and place the tubes inside one of the 48 well blocks. Select the program Kris > DIGEST, and the block your sample is in (A or B).
a. If you need to run the samples on a gel, add 5 µL of 6x Loading dye and either freeze the sample in -20°C.
4. If the enzymes can be heat inactivated (check the NEB slips), select the program in Kris > INACTIV.
a. If not, use the PCR purification kit to remove the restriction enzyme from the mixture.
b. Inactivation of enzymes is important if you want to ligate your DNA (otherwise, the enzyme will just digest right after the ligase works on it).
Ligating DNA – Toronto – 2010 http://2010.igem.org/Team:Toronto/Protocols
1. Thaw the 10X T4 DNA Ligase Buffer.
2. Add the components in the following order (10 µL reaction):
a. 10X T4 DNA Ligase Buffer (1 µL)
b. Vector DNA (10 ng)
c. Insert DNA (6 insert:1 vector molar ratio)
d. ddH2O (to make the volume 9.5 µL total, if necessary)
e. T4 DNA Ligase Buffer (0.5 µL)
3. Leave at room temperature for 30 minutes.
4. Freeze the mixture in the -20°C freezer for future use or use 1 µL in a transformation immediately.
Gel Electrophoresis – Toronto – 2010 http://2010.igem.org/Team:Toronto/Protocols
1. Based on what percentage gel you want to run, add half the percentage in grams to 50 mL of 1X TBE in a 125 mL / 250 mL flask.
a. For example, if you want to make a 1% gel, add in 0.5g of agarose into 50 mL of 1X TBE.
2. Place the flask in the microwave and heat it for 45 seconds. If it starts bubbling, immediately open the microwave door.
3. Take out the flask and swirl the mixture for 5-10 seconds. Use gloves or a folded dry paper towel to hold the flask to avoid getting burned.
4. Place the flask in the microwave and heat for 15-30 seconds. If it starts bubbling, immediately open the microwave door. Let the flask and mixture cool until it is warm when you touch it.
5. Add 2.5 µL of ethidium bromide into the cooling mixture and mix well.
a. Be VERY careful not to get any EtBr on your skin/clothes.
6. Take the small tray and tape both ends. Be sure to wrap the tape tightly around edges and corners so that no fluid will leak out.
7. Slowly pour the gel into the tray and see if it leaks. If it does, try to add more tape in the area to stop the leak.
8. Once the gel is poured, place the comb into preset ridges on the top of the tray.

9. Once the gel has solidified, carefully remove the comb and the tape.
10. Place the gel tray (with the gel still on it) into the electrophoresis cell. Place the wells facing the negative electrode (black).
11. Fill the electrophoresis cell with enough 1X TBE to cover the gel.
12. Add in 10 µL of to the side where the positive electrode (red) is located.
13. Load your samples and ladder(s); record what samples are in what wells.
14. Close the cover of the electrophoresis cell and plug it into the power supply box.
15. Set the voltage to 110V (you can push it to 120V, but don't go any higher as the gel will melt) and turn off the power supply when the dye reaches at least ¾ down the gel (usually 45 min – 1 hr).
Polymerase Chain Reaction – Toronto – 2010 http://2010.igem.org/Team:Toronto/Protocols
1. Let the dNTPs, primers, DNA template(s) and 10X reaction buffer thaw at room temperature
2. Keep the Taq polymerase on ice at all times.
3. Add the reagents in the following order for a 25 µL reaction:
a. 10X reaction buffer (2.5 µL)
b. ddH2O (filling volume up to 25 µL, following these values it would be 17.15 µL)
c. Forward primer (0.2 µL)
d. Reverse primer (0.2 uL)
e. 2 mM dNTPs (3.75 µL)
f. DNA template (1 µL)
g. Taq polymerase (0.2 µL)
4. Repeat step 3 for all DNA samples you want amplified. Make the appropriate controls.
a. For the negative control, replace the DNA template with 1 µL of ddH2O.
5. Place the tubes in the PCR machine (in the same block) and select the PCR program you want to run. If you don't have a pre-made program, make the following program in your own folder:
a. Set the lid temperature to 105°C
b. Step 1: 95°C for 15 min
c. Step 2: 94°C for 30 seconds
d. Step 3: (Primer Tm – 3) for 30 seconds
e. Step 4: 72°C for (1 min per kb of product)
f. Step 5: Repeat steps 2-4 39 times.
g. Step 6: 72°C for 20 min
h. Step 7: 4°C forever
6. Run it in a gel or use the PCR purification kit as necessary.

Assembly of BioBricks using the Red/White 3-Antibiotic Assembly Method – Lethbridge – 2010
http://2010.igem.org/Team:Lethbridge/Notebook/Protocols



This method is a variant of the 3-Antibiotic Assembly Method that has been developed by Ginkgo Bioworks and New England Biolabs.
In the 3-Antibiotic Assembly Method, the destination backbone (pSB1A3, pSB1C3, pSB1K3, or pSB1T3) is amplified via PCR, using DNA received in the 2010 Distribution.
We had difficulty generating a large quantity of plasmid backbone in this manner, and as a result were unable to assemble biobricks.

As an alternative, we exploited the ability of part BBa_J04450 (expressing red fluorescent protein - RFP) to create in its host cell a very strong red color following incubation; this has proven a useful and successful variant of the 3-Antibiotic Assembly Method.

We retained the selection advantage of the 3-Antibiotic Assembly Method (associated with having a destination plasmid containing a different antibiotic resistance than the upstream and downstream plasmid) as the expressing RFP biobrick is available in a wide variety of BBF plasmids.

With this variation, we can not only visually screen our colonies for the presence of our expected assembly product (as indicated by a lack of red fluorescence), we also have a built-in negative control in that a lack of assembly product will result in a re-ligation of the expressing RFP biobrick into the destination backbone, producing red colonies.
Step 1 - Restriction – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
Performed according to Ginkgo Bioworks/NEB BioBrick Assembly Kit, with several modifications.
Digestion of upstream part
5µL Upstream part plasmid†
0.5µL EcoRI-HF
0.5µL SpeI
2.5µL 10x NEBuffer 2
0.25µL 100x BSA

16.25µL MilliQ H_2O
Digestion of downstream part
5µL Downstream part plasmid†
0.5µL XbaI
0.5µL PstI
2.5µL 10x NEBuffer 2
0.25µL 100x BSA
16.25µL MilliQ H_2O
Digestion of destination plasmid
5µL Destination plasmid†‡
0.5µL EcoRI-HF
0.5µL PstI
2.5µL 10x NEBuffer 2
0.25µL 100x BSA
16.25µL MilliQ H_2O
Restriction digests were incubated at 37°C for 10 minutes.
Following each restriction digest, all samples were subjected to heating at 80°C for 20 minutes to irreversibly denature the restriction endonucleases.

† Typical concentrations obtained from our minipreps are approximately 50ng/µL, therefore adding 5µL of plasmid DNA gives the restriction reaction a concentration of 10ng/µL, as recommended in the NEB BioBrick Assembly Kit Literature.
‡ Destination plasmid is either pSB1A3, pSB1C3, pSB1K3, or pSB1T3, containing part BBa_J04450 which is the expressing red fluorescent protein.
Step 2 - Ligation – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
Performed according to Ginkgo Bioworks/NEB BioBrick Assembly Kit
Ligation of Upstream and Downstream parts into Destination Plasmid
2µL Upstream part digestion
2µL Downstream part digestion
2µL Destination Plasmid digestion
2µL 10x T4 DNA Ligase Buffer
1µL T4 DNA Ligase
11µL MilliQ H_2O
Ligation mixes were incubated on the bench top (~20°C) for 10 minutes, then transformed into competent DH5α cells.
Step 3 - Transformation – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
Thaw 50µL of aliquotted competent cells (DH5α) on ice
Gently pipet 2.0µL (~1ng) DNA (from ligation mix) into competent cells
Incubate the cells on ice for 30 minutes
Heat shock the cells in a water bath at 42°C for EXACTLY 45 seconds
Incubate cells on ice for 5 minutes
Add 400µL of sterile SOC media to the cells and incubate at 37°C for 90 minutes with shaking (250RPM)
Plate 200µL on LB agar plate containing the appropriate antibiotic (reserve remaining cells and re-plate if no growth)
Allow cell suspension to be absorbed into agar by leaving agar side down for 10-15 minutes
Incubate the plates in the 37°C incubator for approximately 36 hours (agar on top)
Step 4 - Selection of Colonies and Colony PCR – Lethbridge – 2010
http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
Following ~36 hour incubation at 37°C, plates were inspected, and white colonies were picked and subsequently subjected to Colony PCR.
Colony Picking

Pick a colony from a transformation plate using a sterile toothpick or micropipette tip
Transfer the cells to a 1.5mL microcentrifuge tube containing 50µL of sterile MilliQ H_2O
Place microcentrifuge tube in heat block at 99°C for 5 minutes to lyse the cells and denature DNases.
Centrifuge at max speed in a table-top microcentrifuge for 1 minute to remove cell debris
Use 5µL of supernatant as template for PCR
PCR to confirm length of newly assembled part
Reaction Conditions: – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols

Component	Concentration	Volume (µL)
MilliQ H_2O	N/A	33.8
Pfu Buffer with $MgSO_4$	10x	5
dNTP	10mM	2
VF2 Forward Primer	10µL	2
VR Reverse Primer	10µL	2
Template DNA	N/A	5
Pfu DNA Polymerase	2U/µL	0.2

Cycling Conditions:

Step	Temperature	Time
1-Initial Denature	98°C	3 minutes
2-Denature	98°C	30 seconds
3-Anneal†	58°C	30 seconds
4-Extend	72°C	30 seconds‡
5-Final Extend	72°C	15 minutes

† Annealing temperature for Phusion is MT +3°C
‡ Extend time is 15-30 seconds per kb; time adjusted accordingly
Step 5 - Plasmid DNA Purification – Lethbridge – 2010
http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
We followed Qiagen's QIAPrep Spin Miniprep protocol, as follows:
Pellet cells from 1-1.5mL of liquid culture in a microcentrifuge tube (remove and discard supernatant)
Resuspend cells in 250µL buffer P1
Add 250µL buffer P2 and mix by inverting 4-6 times
Add 350µL buffer N3 and mix immediately by inverting 4-6 times
Centrifuge for 10 minutes at 13000RPM at room temperature in a table-top microcentrifuge
Apply resulting supernatant to spin column
Centrifuge for 60 seconds (discard flow through)
Wash column with 750µL of buffer PE (discard flow through)
Centrifuge for an addition 60 seconds to remove residual PE buffer (discard flow through)
Transfer column to clean 1.5mL microcentrifuge tube
Add 50µL buffer EB, let stand for 1-5 minutes, and centrifuge for 1 minute (retain flow through)

Protocols prior to the development of Red/White 3-Antibiotic assembly
Competent Cell Transformation – Lethbridge – 2010
http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
Thaw 20µL of aliquotted cells (DH5α or BL21(DE3)) on ice.

Gently pipet 2.0µL of DNA into competent cells
ATTENTION:
Do not perform any additional mixing
Never use more DNA that 10% of the volume of the competent cells otherwise the cells get destroyed by osmotic shock
Incubate the cells on ice for 30 minutes.
Heat shock the cells in a water bath at 42°C for EXACTLY 45 seconds.
Incubate the cells on ice for 5 minutes.
Add 250µL sterile LB media to the cells and incubate at 37°C for 1 hour with shaking (200RPM).
Plate 100µL and 50µL on prewarmed LB agar plate containing the appropriate antibiotic.
For ligations, plate all 250µL.
Leave plate for 10-15 minutes to soak the cell suspension into the agar.
Flip plate over (agar on top)
Incubate the plates in the 37°C incubator overnight

Plasmid DNA Purification by Boiling Lysis (Small Scale AKA Miniprep) – Lethbridge – 2010
http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
Aseptically transfer 1.5mL of each overnight culture to a 1.5mL microcentrifuge tube (MCT) and pellet the cells by centrifugation in a benchtop microcentrifuge (2min at 13000RPM)
Remove and discard as much of the supernatant as possible by aspiration (e.g with a Pasteur Pipette). Do not suck up the cell pellet!!
Rinse the cell pellet by washing 1.0mL of sterile MilliQ H_2O gently down the inside wall of the MCT. This removes any traces of the supernatant adhering to the MCT wall while minimizing the disturbance to the cell pellet. (/li>
Resuspend the cell pellet in 350µL of STET.
Add 25µL of the Lysozyme solution and mix by inversion.
Place the MCT in the bioling water bath for EXACTLY 35 seconds, remove and incubate on ice for 5 minutes.
Pellet the cellular debris by centrifugation at 13000RPM for 15 minutes. Transfer the supernatant to a fresh MCT and discard the pellet.
Precipitate the plasmid DNA by adding 40µL of 3.0M sodium acetate (pH 5.2) and 420µL isopropanol. Mix by inversion. Mix by inversion and incubate for 5 minutes at room temperature.
Pellet the plasmid DNA by centrifugation at 13000RPM for 10 minutes at 4°C. A pellet of plasmid DNA should be visible at the base of the MCT when complete.
Being careful not to disturb the pellet, discard the supernatant and rinse the pellet with 500µL of ice cold ethanol.
Repeat above step.
Invert and tap the open MCT several times against a piece of paper towel on your bench to remove as much ethanol as possible.
Store the open MCT at room temperature for approximately 10 minutes to allow all remaining traces of ethanol to evaporate
Add 50µL of TE (pH 8.0) containing RNase A and resuspend the plasmid DNA by flicking the base of the MCT with your finger. The plasmid DNA is ready for use or can be stored long term at -20°C.
Restriction of Plasmid DNA (pDNA) – Lethbridge – 2010
http://2010.igem.org/Team:Lethbridge/Notebook/Protocols
In a 1.5mL microcentrifuge tube, add MilliQ H_2O to final volume of 20µL, 2µL of restriction enzyme buffer, 2µL of plasmid DNA, and 0.25µL of each restriction enxyme.
Incubate at 37°C for 1 hour.
Heat kill restriction enzymes on a heat block at 80°C for 20 minutes.
Store short-term on ice or long-term at -20°C.

Additional Protocols

Overexpression – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols

Inoculate two 5mL overnight cultures in LB media containing the appropriate antibiotic corresponding to the resistance of the plasmid backbone on which the gene is located; incubated at at 37°C in shaker.

Transfer the two 5mL cultures into an 2000mL Erlenmeyer flask containing 500mL of LB media containing the same antibiotic.

Measure and record initial read OD_{600} reading (Time = 0) blanking against the uninoculated LB media.

Begin incubation at 37°C in shaker.

Measure and record another OD_{600} reading 1 hour after (Time = 1) and continue record OD_{600} every 30 minutes (T + 0.5) until OD_{600} = 0.600 is reached.

When OD_{600} has reached 0.600, aliquot out a 2mL sample and induce the 500mL culture (ie. if promoter is pLacI, induce with IPTG) and continue incubating in 37°C shaker.

Aliquot 2mL samples and record the OD_{600} reading every hour for 3 hours following induction.

To run SDS PAGE, centrifuge the samples at 13,000 rpm for 5 minutes and discard the supernatant.

Resuspend the cell pellets with 100µL of 8M urea. Mix 10µL of the urea/cell sample with 5µL of 6X loading dye.

Plasmid DNA Purification by Alkaline Lysis (Large Scale AKA Maxiprep) – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols

Grow up a 500mL overnight culture in LB media containing the appropriate antibiotic corresponding to the resistance of the plasmid.

Centrifuge the cells at 5000 rpm for 10 minutes, discard the supernatant, transfer pellet to a 50mL falcon tube, record the weight of the pellet, and store at -20°C. (1- 2.5g cell pellets are expected)

Resuspend the cell pellet in 6mL Alkaline Lysis Solution I (ALS1). Vortex carefully and slowly; may use a clean glass stir rod. Add 20µL of 1mg/mL RNase A.

Add 1 mL of 10 mg/mL lysozyme (in 20mM Tris-HCl, pH 8.0)

Incubate at room temperature for 10 minutes.

Add 12mL of fresh ALS2, mix well, do not vortex, and incubate on ice for 10 minutes.

Add 9mL of ice cold ALS3, mix well, and incubate for 10 minutes on ice.

Centrifuge at 4°C and 5000 rpm for 15 minutes.

Decant supernatant filter within funnel into fresh 50mL centrifuge tube.

1:1 phenol:chloroform extraction: In the fume hood, add 4mL of phenol/chloroform (1:1 -- 2mL of phenol + 2mL of chloroform), vortex for 15 seconds, centrifuge at 4000 rpm and 12°C for 4 minutes, and collect the upper aqueous phase. To the aqueous phase, add 4mL of chloroform, vortex for 15 seconds, centrifuge at 4000 rpm and 12°C for 4 minutes, and save the upper aqueous layer.

Add 0.6 volumes of isopropanol to the saved aqueous layer and incubate on ice for 10 minutes. (Alternatively, precipitate overnight at -20°C)

Centrifuge at 4°C and 5000 rpm for 15 minutes.

Decant supernatant into a fresh falcon tube (can be saved for further plasmid DNA isolation)

Wash DNA pellet with 2mL 70% ethanol; centrifuge at 4°C and 5000 rpm for 5 minutes.

Air dry the DNA pellet; resuspend in 4mL 20mM Tris-HCl pH 8.0 by vortexing.

Add 200µL 1mg/mL RNase A and incubate at room temperature overnight.

Ethanol precipitation: Add 0.1 volumes of 3M Na-acetate pH 5.2, add 2 volumes of cold ethanol (-20°C), incubate 30 minutes on ice (or longer at -20°C).

Centrifuge at 4°C and 4500 rpm for 15 minutes; carefully remove supernatant with 200µL pipette.

Wash pellet with 750µL of 70% ethanol, centrifuge at 4°C and 4500 rpm for 2 minutes, remove supernatant, and air dry the pellet for 10 minutes.

Resuspend the pellet in 200µL of MilliQ H_2O (or TE).

Solutions – Lethbridge – 2010 http://2010.igem.org/Team:Lethbridge/Notebook/Protocols

ALS1: 50mM glucose, 25mM Tris-Cl, 10mM EDTA pH 8.0

ALS2: 0.2M NaOH, 1% (w/v) SDS

ALS3: 60mL of 5M K-acetate, 11.5mL glacial acetic acid, 28.5mL MilliQ H_2O
RNase A: 1mg/mL in 20mM Tris-HCl, pH 8.0 *Lysozyme:* 10mg/mL in 20mM Tris-HCl, pH 8.0

Taq polymerase chain reaction – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

Polymerase Chain Reaction Master Mix

Reagent	**1x Mix (µL)**	**5x Mix (µL)**
H_2O	28	140
10x Buffer	5	25
2 mM dNTP	5	25
Forward Primer (2 mM)	5	25
Reverse Primer (2 mM)	5	25
50 mM $MgCl_2$	1.5	7.5
Taq Polymerase	0.5	2.5
Total	**50**	**250**

DNA template is required. The quantity of water decreases in proportion to the volume of DNA template added so that the total volume remains the same.

Thermocycler PCR Program

Temperature (°C)	**Time (mins)**
95.0	5:00
95.0	1:00
55.0*	0:30
72.0	1:00
72.0	10:00
4.0	∞

NOTE: * indicates that the temperature of the step is primer specific. Steps 2 through 4 are repeated 30 times. The length of Step 4 is 1 minute for every 1000 base pairs of the template to be amplified.
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Making competent cells – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This procedure was done using Top10 Competent cells ordered from Invitrogen. 50 mL Falcon tubes were used for this protocol.
Innoculate 5-10 mL LB at 37°C while shaking
Subculture 1 mL of bacteria solution into 50 mL LB broth at 37° while shaking until OD600 is 0.4-0.6 (This step should require approximately 2.5 hours)
Centrifuge the subculture at 10 000 rpm at 4°C for 2 minutes
Resuspend pellet in 12.5 mL of cold $CaCl_2$ (50 mM) and leave on ice for 10 minutes
Centrifuge at 10 000 rpm at 4°C for 2 minutes and resuspend in 2 mL of cold $CaCl_2$ (50 mM, 15% glycerol solution)
Leave on ice for at least 30 minutes and then aliquot 200 uL and freeze at -80°C
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Bacterial transformation – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

Thaw Competent Cells
Add 10-100 ng of DNA
Ice solution for 30 minutes
Heat shock solution (5 minutes at 37°C or 2 minutes at 42°C)
Ice solution for 5 minutes
Recover with 250 µL of SOC (30 minutes for Ampicilin resistant plasmids and 60 minutes for kanamycin resistant plasmids)
Centrifuge for 5 seconds at 14 000 rpm and concentrate solution to 100 µL
Plate 20-50 µL onto each spread plate
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Restriction digest – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol is part of the Construction Protocol. Start by selecting one of the parts you wish to combine as the vector (the plasmid will be kept) and the other part as the insert. The two parts will need to be mixed separately at the beginning. The parts must be kept separate for the digestion period.

Add to the Insert Tube:
600 ng of DNA (Calculate this from the concentration of plasmid)
3.5 µL of 10x Buffer
0.5 µL of each restriction enzyme used (2 of EcoRI, XbaI, SpeI, or PstI)
H_2O such that the volume of water and DNA in the tube is 30.5 µL and the total volume in each tube is 35 µL
Add to the Vector Tube:
250 ng of DNA (Calculate this from the concentration of plasmid)
3.5 µL of appropriate 10x Buffer
0.5 µL of each restriction enzyme used (2 of EcoRI, XbaI, SpeI, or PstI)
HH_2O such that the volume of water and DNA in the tube is 30.5 µL and the total volume in each tube is 35 µL

Mix two tubes as indicated above
Put the tubes into a 37°C water bath for one hour
Place the tubes into an 80°C heating block for 20 minutes to heat-kill the enzymes in the tube
Freeze the parts until they are needed
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Ligation – Calgary – 2010 http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol is part of the Construction Protocol. The tubes from the Restriction Digest should be removed from the freezer and thawed on ice before beginning ligation.
Mix 5 µL of the insert and 5 µl: of the vector in a new tube
Clearly label the tubes as unligated, write the date and freeze the tubes in -20°C in case the transformation does not work
Add 10 µL of 2x Quick Ligase Buffer and 1 µL of Quick Ligase to the tube containing the mixed Insert and Vector
Let the tube sit at room temperature for 5 minutes
Transform this mix (all 21 µL) into Top10 Competent Cells
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Miniprep Plasmid Preparation (GenElute) – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol is taken from the Sigma Aldrich distributed GenElute Miniprep Plasmid Preparation Kits. We modified the elution portion of the procedure by using double distilled water to elute rather than using TE buffer. We also skipped the step with the optional wash solution. Instead, the step with the addition of Wash Solution in the Column Tube was done twice. Another company's kit was used occasionally to prep the plasmids but the two kits were never mixed.
Make overnight cultures from LB agar plate growth (The protocol for the making of overnight cultures can be found as a separate protocol)
After allowing approximately 16 hours of growth, pellet the cells using a centrifuge for 20 minutes at a speed of 4000 rpm at 4°C
Discard the supernatant, while being careful not to discard any of the pellet
Resuspend the pellet in 200 µL of Resuspension Solution (with RNase A added) which is provided from the kit
Transfer the solution from a Falcon tube to a 1.5 µL microcentrifuge tube
Add 200 µL of Lysis Solution and invert gently to mix. Allow the mixture to clear for less than 5 minutes
Add 350 µL of Neutralization Solution and invert the tube 4-6 times to mix
Pellet the microcentrifuge tubes at 14 000 rpm using a microcentrifuge for 15 minutes. The resulting solution will be known as the lysate
Add 500 µL of the Column Preparation Solution to a binding column inside a collection tube. Centrifuge this tube for 1 minute at 14 000 rpm and discard the liquid underneath the binding tube
Transfer the lysate into the binding column, being careful not to transfer any solid. Discard the microcentrifuge tube with the solid
Centrrifuge the collection tube at 14 000 rpm for 1 minute. DIscard whatever liquid flowed through the binding column into the collection tube

Add 750 µL of Wash Solution with concentrated ethanol added to the column and spin at 14 000 rpm for 1 minute. Discard the liquid that flowed through into the collection tube
Repeat Step 12 a second time with the same quantity of Wash Solution
Centrifuge the tube for 1 minute at 14 000 rpm to dry the column
Transfer the column to a new 1.5 µL microcentrifuge tube
Add 50 µL of double distilled water to the column and spin for 1 minute at 14 000 rpm
Use a spectrophotometer to measure the concentration and the purity of your plasmid
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Agarose Gel Electrophoresis – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This procedure is involved in tandem with a PCR. The first step involves the making of an agarose gel as indicated by the Making of Agarose Gel protocol.
Create an agarose gel tray
Place the gel tray into a gel dock where it will be run. Add TAE buffer such that the entire gel is covered
Make mixed tubes with 3 µL DNA, 2 µL Loading dye, and 15 µL water
Insert 10 µL of the mixture into each well with 5 µL of the 1KB ladder in the first hole
Place thecovering on top and set it to run at 90V
When the bands are approximately halfway through the gel, which should be around 35-40 minutes, turn off the electricity and remove the gel
Use a computer imager to take a picture of the gel
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Making Agarose gel – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

The agarose gel is made in order to do agarose gel electrophoresis. The procedure is modified by the quantity of agarose added to the solution. The procedure here will detail the making of a 1.5% gel.
Measure out 1.5g of agarose
Add the agarose to 100 mL of TAE buffer. COver the beaker with saran wrap with a hole punched in it
Microwave the solution for 30 seconds and then swirl. Then, microwave for 1 minute at high power and swirl. Finally, microwave for 1 minute further and swirl
Take this solution to the fume hood and add 3 µL of ethidium bromide. Ethidium bromide is a suspected carcinogen so handle with care
Swirl the solution to allow the ethidium bromide to mix
Pour the solution into the tray. Use a 10 µL pipette tip to pop any bubbles that may result and insert the comb
Allow the solution to solidify and remove the comb
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Rehydration of registry DNA – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

The open source Parts Registry is where all parts are sent by iGEM teams each year. The distribution is done in three 384-well plates in dry DNA form. The DNA must be rehydrated, transformed into Top10 Competent cells and then plasmid prepped using the Miniprep Plasmid Prep Protocol as listed above before they are in usable DNA form.
Use a 10 µL pipette tip to puncture the aluminium foil covering of the desired well of DNA
After 5 minutes to allow the DNA to thaw, add 10 µL of double distilled water to the well and pipette up and down 3-4 times until the liquid comes up red
Take 2 µL of DNA and transform these into Top10 Competent Cells using the Transformation Protocol
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Construction technique – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol combines the Restriction Digest with the Ligation protocol as well as adding Antarctic Phosphatase Protocol.

Restriction Digest

Add to the Insert Tube:
600 ng of DNA (Calculate this from the concentration of plasmid)
3.5 µL of 10x Buffer
0.5 µL of each restriction enzyme used (2 of EcoRI, XbaI, SpeI, or PstI)
H_2O such that the volume of water and DNA in the tube is 30.5 µL and the total volume in each tube is 35 µL
Add to the Vector Tube:
250 ng of DNA (Calculate this from the concentration of plasmid)
3.5 µL of appropriate 10x Buffer
0.5 µL of each restriction enzyme used (2 of EcoRI, XbaI, SpeI, or PstI)
HH_2O such that the volume of water and DNA in the tube is 30.5 µL and the total volume in each tube is 35 µL
Mix two tubes as indicated above
Put the tubes into a 37°C water bath for one hour
Place the tubes into an 80°C heating block for 20 minutes to heat-kill the enzymes in the tube
Freeze the parts until they are needed

Ligation Protocol with Antarctic Phosphatase
Mix 5 µL of the insert and 5 µl: of the vector in a new tube
Clearly label the tubes as unligated, write the date and freeze the tubes in -20°C in case the transformation does not work
Add 10 µL of 2x Quick Ligase Buffer and 1 µL of Quick Ligase to the tube containing the mixed Insert and Vector
Let the tube sit at room temperature for 5 minutes
Add 5 µL of 10x Antarctic Phosphatase Buffer, 4 µL of water, and 1 µL of Antarctic Phosphatase to the Vector while freezing the insert. Put the tube into a 37°C water bath for 30 minutes and then place into the 65 °C heating block for 10 minutes

Transform this mix (all 21 µL) into Top10 Competent Cells
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Overnight cultures – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This procedure is used before the Sigma Aldrich GenElute Plasmid Preparation. You will need a 10 mL culture tube, 5 mL of LB Broth, Antibiotic, and single colonies on a plate.
Pipette 5 mL of LB Broth into the culture tube.
Add Antibiotic (10 µL Ampicillin, 5 µL Kanamycin, or 3 µL Chloramphenicol)
Select a single colony using a 200 µL sterile pipette tip
Place the culture tube into the shaker and let it shake at 175 rpm at 37°C
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Glycerol stock preparation – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This procedure is used to make long term stocks of plasmid parts that will definitely be used later on. The procedure was given by our lab technician Deirdre Lobb.
Grow 5 mL overnight cultures for the bacteria containing the plasmid which you wish to create glycerol stocks of (This procedure is indicated by the Overnight Growth Protocol)
Take 1 mL of the culture and add it to 1 mL of autoclaved 50% glycerol
Divide this solution into two tubes holding 1 mL each and store these in 1.5 mL microcentrifuge tubes
Use dry ice to flash freeze the tubes and store the glycerol stocks in a -80°C freezer
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LB agar preparation – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols
Autoclave mixture of dH2O and agar
Add either 1 mL of Ampicillin, 0.5 mL Kanamycin or 0.35 mL Chloramphenicol
Pour plates, flame and mark plates
Let dry overnight
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QIAprep spin Miniprep kit – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol is taken from the Qiagen distributed QIAprep Spin Miniprep Kit. We modified the elution portion of the procedure by using double distilled water to elute rather than using TE buffer. We also skipped the step with the optional wash solution. Instead, the step with the addition of Wash Solution in

the Column Tube was done twice. Another company's kit was used occasionally to prep the plasmids but the two kits were never mixed.
Make overnight cultures from LB agar plate growth (The protocol for the making of overnight cultures can be found as a separate protocol)
After allowing approximately 16 hours of growth, pellet the cells using a centrifuge for 20 minutes at a speed of 4000 rpm at 4°C
Discard the supernatant, while being careful not to discard any of the pellet
Resuspend the pellet in 250 µL of Buffer P1 (with RNase A added) which is provided from the kit
Transfer the solution from a Falcon tube to a 1.5 µL microcentrifuge tube
Add 250 µL of Buffer P2 and invert gently to mix. Allow the mixture to clear for less than 5 minutes
Add 350 µL of Buffer N3 and invert the tube 4-6 times to mix
Pellet the microcentrifuge tubes at 14 000 rpm using a microcentrifuge for 15 minutes. The resulting solution will be known as the lysate
Add 500 µL of the Column Preparation Solution to a binding column inside a collection tube. Centrifuge this tube for 1 minute at 14 000 rpm and discard the liquid underneath the binding tube
Transfer the lysate into the QIAprep spin column, being careful not to transfer any solid. Discard the microcentrifuge tube with the solid
Centrrifuge the collection tube at 14 000 rpm for 1 minute. DIscard whatever liquid flowed through the binding column into the collection tube
Add 750 µL of Buffer PE with concentrated ethanol added to the column and spin at 14 000 rpm for 1 minute. Discard the liquid that flowed through into the collection tube
Repeat Step 12 a second time with the same quantity of Wash Solution
Centrifuge the tube for 1 minute at 14 000 rpm to dry the column
Transfer the column to a new 1.5 µL microcentrifuge tube
Add 50 µL of double distilled water to the column and spin for 1 minute at 14 000 rpm
Use a spectrophotometer to measure the concentration and the purity of your plasmid
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Gel extraction – Calgary – 2010 http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol is utilized in accordance to the manufacturer's protocol from Omega E.Z.N.A (EaZy Nucleic Acid Isolation)
Place gel on the UV box
Carefully extract the fragment suspended in the gel>/li>
Mass gel fragments
Place fragment into a 1.5 mL tube and add 4 µL of H_2O
Volume of water added to volume of gel is 200% however if fragment it small 1 mL of water will suffice
Remove H_2O
Add equal amounts of H_2O and Binding Buffer (XP2) to the gel
Incubate mixture at 55 degrees for 7 mins
Mix with vortex for 2 mins
Place in the HiBind DNA Mini Column in the 2 mL tube
Add 700 µL at 10,000xg for 1 min
Discard liquid
Add 300 µL Binding Buffer (XP2) into the HiBind DNA Mini Column and spin down at 10,000xg for 1 min
Discard liquid
Wash the column with 700 µL of SPW buffer with added ethanol and spin down at 10,000xg for 1 min

Discard liquid
Wash the column with 700 µL of SPW buffer again and spin down at 10,000xg for 1 min
Discard the liquid
Spin down the column at 13,000xg for 1 min to dry the column
Elute in 50 µL of H_2O and wait 1 min
Spin down the column at 13,000xg for 1 min to dry the column
Use a spectrophotometer to measure the concentration and the purity of your plasmid
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PCR purification (Vaccum) – Calgary – 2010
http://2010.igem.org/Team:Calgary/Notebook/Safety_And_Protocols

This protocol is utilized in accordance to the manufacturer's protocol from Qiagen PCR Vacuum Prep Kit
Add PCR product
Distribute liquid evenly by evenly tapping
Vacuum for 15-20 mins to dry
Add 20 µL H_2O
Tap lightly against the table to redistribute the H_2O on the surface of the well
Pipette contents and transfer to clean 1.5 mL tube
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PCR (total volume: 50 µl) – TzuChiU Formosa – 2010
http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
25 µl Green master mix (Promega)
2 µl each primer
2 µl template
19 µl ddH_2O
Cycle 95/2 min; 30x(95/1 min;56/1 min;72/2 min); 72/7 min
Gel Extraction- use Gel /PCR DNA Fragments Extraction Kit (Geneaid) – TzuChiU Formosa – 2010
http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
Loading the PCR products into agarose gel; run gel with 1X TAE buffer
Excise the agarose gel slice containing relevant DNA fregments
Transfer gel slice to a 1.5 ml microcentrifuge tube
Add 500 µl DF buffer; incubate at 56 ℃ until gel slice has been completely dissolved
Transfer 800 µl gel mixture to DF column
Centrifuge 13000 rpm, 1 min
Discard the flow-through and place the DF column back
Add 400 µl W1 buffer; Centrifuge 13000 rpm, 1 min; Discard the flow-through
Add 600 µl wash buffer; Centrifuge 13000 rpm, 1 min; Discard the flow-through
Centrifuge 13000 rpm, 5 min; air- dry
Add 50 µl ddH_2O; Centrifuge 13000 rpm,1 min
Storage at – 20℃
TA Cloning- use pGEM®-T Easy Vector (Promega) – TzuChiU Formosa – 2010
http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
1µl pGEM®-T Easy Vector
5µl 2X rapid ligation buffer

1µl T4 DNA ligase
3µl insert
4 ℃, overnight
Ligation on pSB1C3- use Elite T4 DNA ligase (Geneaid) – TzuChiU Formosa – 2010
http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
9 µl insert
3 µl vector
2 µl 10X ligation buffer A
2 µl 10X ligation buffer B
1 µl Elite T4 DNA ligase
3 µl ddH2O
16℃, overnight
Transformation – TzuChiU Formosa – 2010 http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
Take out XL-10 Gold competent cell from -80 ℃ refrigerator; put on ice
Add 2 µl GFP generator DNA solution into competent cell
Place on ice, 30 min
Heat shock, 42 ℃, 90 sec
Chill on ice, 5 min
Add 800 µl LB medium (without antibiotics)
Incubate at 37 ℃, 1 hr
For TA ligation product, smear 100 µl 50 mg /ml Ampicilin, 100 µl 0.1 M IPTG and 50 µl X-gal over LB agar plate.For pSB1C3 ligation product, smear 100µl 34 mg /ml Chlorophenicol over the plate.
Bacteria solution centrifuge 3000 rpm, 5min
Remove 700 µl supernatant
Mix bacteria solution; Smear 100 µl over LB agar plate
Incubate at 37 ℃, overnight
Plasmid Extraction – TzuChiU Formosa – 2010 http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
Pick single colonies; each colony incubate with 3 ml LB medium (add antibiotics); 37℃, overnight
Centrifuge 7500 rpm, 1 min; remove supernatant
Resuspend bacteria with 200 µl PD1 buffer; resuspend bacterial pellet
Add 200 µl PD2 buffer; gently mix
Add 300 µl PD3 buffer; gently mix
Centrifuge 13000 rpm, 15 min
Transfer supernatant to kit filter
Add 400 µl W1 buffer; Centrifuge 13000 rpm, 1 min; Discard the flow-through
Add 600 µl Wash buffer; Centrifuge 13000 rpm, 1 min; Discard the flow-through
Centrifuge 13000 rpm, 5 min; air- dry
Dissolve DNA with 50 µl ddH2O; Centrifuge 13000 rpm,1 min
Storage at – 20℃
Double Enzyme Digestion – TzuChiU Formosa – 2010
http://2010.igem.org/Team:TzuChiU_Formosa/Notebook
16 µl ddH2O
0.5 µl Enzyme 1
0.5 µl Enzyme 2
3 µl NEB 10X buffer
0.3 µl NEB 10X BSA
10 µl DNA
Incubate at 37 ℃, 3 hr

LB medium and LB agar gel Medium for cultivation of E. coli – Tokyo-NoKoGen – 2010
http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
LB medium (1 L)
1; Add about 900 mL of distilled water to beaker.
2; Add reagents (Table) and stir.
3; Add distilled water up to 1 L and take LB medium to media bottle.
4; Autoclave for 20 min at 120°C.

LB agar gel (1 L)
1; Prepare LB medium without autoclave (Steps 1-3 of 1L scale of LB medium).
2; Add 15 g of agar and stirrer bar.
3; Autoclave for 20 minutes at 120°C.
4; Stir and cool LB medium with agar, add appropriate antibiotic (50 μL).
5; Pour LB medium (Step 4) in plate and cool down in clean bench.

Transformation – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
Preparation of E. coli contain particular plasmid
1; Incubate frozen competent cell (DH5α) on the ice for a few minutes.
2; Add 1~5 μL of plasmid to competent cell (DH5α) on the ice.
3; Incubate for 20 – 30 minutes on the ice.
4; Incubate for 45 seconds at 42°C.
5; Add 1 mL LB medium and cultivate for 1 hour at 37°C.
6; Spread culture medium on LB agar plate with appropriate antibiotic.

Plasmid extraction – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
Preparation of plasmid extracted from *E. coli*
1; Pick up single colony from agar plate and cultivate it in 1.5 mL LB medium containing appropriate antibiotec (50 μg/mL) overnight at 37°C.
2; Move the culture medium to 1.5 mL tube.
3; Centrifuge for 5 seconds at 15,000×g and 4 °C and discard supernatant.
4; Add 100 μL Solution 1 to the pellet and resuspend and incubate for 3 minutes.
5; Add 100 μL Solution 2, invert tube gently 5 times and incubate for 3 minutes.
6; Add 100 μL Solution 3, invert tube 5 times and incubate for 3 minutes.
8; Add 200 μL Solution 4 and invert tube 5 times and centrifuge for 3 minutes at 15,000×g and 4 °C.
10; Take supernatant to new 1.5 mL tube and centrifuge for 3 minutes at 15,000×g and 4 °C.
11; Vortex Bind mix for 1 min and add 800 μL Bind mix to new 1.5 mL tube.
12; Add 400 μL supernatant after centrifugation (Step 10) to tube containing Bind mix (Step 11) and mix.
13; Incubate for 3 minutes, centrifuge for 3 seconds at 5,000×g and 4 °C, and discard supernatant.
14; Add 1 mL of 50% ethanol and resuspend.
15; Centrifuge for 3 seconds at 5,000×g and 4 °C and discard supernatant.
16; Repeat wash (Steps 14-15).
17; Dry pellet for a few minuet under a vacuum to remove residual ethanol.
18; Add 50 μL nuclease-free water or TE buffer and incubate for 3 minutes at 65°C.
19; Centrifuge for 3 minutes at 15,000×g and 4 °C.
19; 40 μL of supernatant into new 500 μL tube.

Restriction enzyme digestion of DNA – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
Cleavage of insert DNA from plasmid

1; Mix DNA and restriction enzyme (Table).
2; Incubate for 2 hours at 37°C.
3; Incubate for 10 minutes at 65°C.
4; Confirm the band of DNA by agar gel electrophoresis.
<="" h2="" style="color: rgb(0, 0, 0); font-family: sans-serif; font-size: 12.012011528015137px; font-style: normal; font-variant: normal; font-weight: normal; letter-spacing: normal; line-height: 19.026838302612305px; orphans: auto; text-align: start; text-indent: 0px; text-transform: none; white-space: normal; widows: auto; word-spacing: 0px; -webkit-text-stroke-width: 0px; background-color: rgb(255, 255, 255);">
Confirmation and separation of digested DNA – Tokyo-NoKoGen – 2010
http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
Preparation of agar gel
1; Add 1 g of agar to 100 mL of 1×TAE.
2; Boil and stir until solution is clear.
3; Cool down, pour to gel form and set gel corm.
4; Incubate until gel dry out.
5; Stare gel in 1×TAE.

Agar gel electrophoresis – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
1; Place agar gel and pour 1×TAE in electrophoresis chamber.
2; Load DNA ladder and DNA sample mixed with loading dye on agar gel.
3; Electrophorese for 20 minutes at 100 V.
4; Stain gel by Sybr SafeTM Gel Stain (Invitrogen).
5; Visualize the band of DNA using UV light.
6; Confirm the length of digested DNA.

Gel purification – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
Purification of DNA from agar gel
GENECLEAN® II Kit(NaI、glass milk、NEW Wash)/Qbiogene
1; Cut the objective band in the agar gel after electrophoresis and stain with SYBR Safe.
2; Put the gel including objective DNA into 1.5 mL tube and measure the mass of that.
3; Add 2.5-3 fold volume NaI solution into the tube (Gel: NaI =1 mg : 1 µL).
4; Incubate the gel at 50°C for 5 minute.
5; Add 10 µL of glass milk and vortex.
6; Incubate for 5 minutes and vortex per a minute.
7; Centrifuge for 5 seconds at 15,000×g and 4°C and discard the supernatant.
8; Add the 500µL of New Wash and resuspend.
9; Centrifuge for 5 seconds at 15,000×g and 4°C.
10; Repeat wash (Steps 8-9).
11; Dry the pellet for 5-10 minutes under vacuum.
12. Add 20 µL of nuclease-free water and resuspend.
13. Centrifuge for 5 seconds at 15,000×g and 25°C.
14. Transfer supernatant including objective DNA into new tube.

Ligation – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols

Ligation inset DNA and vector
DNA Ligation kit Ver 2.1(Solution Ⅰ)/Takara
1; Mix the insert DNA, vector and solution I (Table).
2; Incubation at 16°C for 30 minute.
3; Transform E. coli with ligation sample.

Colony PCR – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols

Confirm of insert DNA in plasmid, directly using E. coli at PCR.
1; Add 10 µL of reagent solution (Table 1) to PCR tube.
2; Pick up single colony from agar plate with tooth pick and sting replica plate (new LB agar plate).
3; Put and stir toothpick to reagent solution (Step 1).
4; Amplify insert DNA with PCR program (Table 2).
5; Electrophorese PCR sample with agar gel.
6; Check the band and length of insert DNA and decide the colony with insert DNA.

Sequence analysis – Tokyo-NoKoGen – 2010 http://2010.igem.org/Team:Tokyo-NoKoGen/experiments/Protocols
Identification of insert DNA
*Preparation of PCR product
Big Dye® Terminator Cycle Sequencing Kit Ver. 3.1 (Premix, Buffer) / Applied Biosystems
1; Add reagent solution (Table 1) to PCR tube and amplify insert DNA with PCR program (Table2). *Purification of PCR product and sequence analysis
Agencourt CleanSEQ® and 96 R ring Super Magnetic Plate® / Beckman Coulter
1; Add 10 µL of Agencourt CleanSEQ® 10 µL to PCR product.
2; Add 62 µL of 85% ethanol, mix and incubate for 3 minutes.
3; Incubate for 3 minutes on 96 R ring Super Magnetic Plate® and discard supernatant.
4; Add 100 µL of 85% ethanol and mix.
5; Incubate for 3 minutes on 96 R ring Super Magnetic Plate® and discard supernatant.
6; Repeat wash (Steps 4-5).
7; Dry for 10 minutes.
8; Add 40 µL nuclease-free water and mix.
9; Transfer 30µL of clear sample into a new plate for loading on the detector.
10; Load sample on sequencer and analyze.

Transformation Protocol – Peking – 2010 http://2010.igem.org/Team:Peking/Notebook/Protocols
Materials:
Plasmid samples or ligation product;
Commercially competent cells;
LB non-antibiotic liquid meium;
LB antibiotic agar plates

Procedure:
1. Get the competent cells from -70 degree, and wait for its fusion. 30-50 µl of competent E.coli cells for each sample. Put microcentrifuge tubes to chill on ice for at least 2 min.
2. Add 2 - 3 ul of each plasmid sample or all the ligation product into the competent cells in the microcentrifuge tubes. Mix and incubate on ice for 30 min.
3. Heat pulse for 90 sec, at 42 degree. Put back to ice and incubate for 5 min.

4. Add 200 uL LB non-antibiotic liquid medium into each microcentrifuge tube. Shake the microcentrifuge tubes in shaker, at 37 degree, for 30 min to recover.
5. Plate 150 uL of the liquid medium with transformed cells immediately, on prewarmed LB antibiotic agar plates. Incubate overnight at 37°C for 10-14h.

Tips:
All procedures are performed on ice.
Make sure the cells are not left at ambient temperature for more than 5 min as thiswill significa ntly decrease the transformation efficiency.
When got out from the shaker, the competent cells ma y form pellet in the microcentrifuge tubes. You need to resuspend the cells before plating.

References:
Current protocols in molecular biology.

Site-directed Mutagenesis Protocol – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
Materials:
Takara MutanBEST Kit with the following content
A pair of primers which juxtapose their 5' ends and have contrary directions of 3' ends to import the mutation;
Template plasmid;
Commercial competent cells.

Procedure:
PCR Reaction
1. Design and synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide "primers" prior to use in the following steps
2. Prepare PCR reaction system which has the following composition(total 50 uL)
10x Pyrobest buffer II 5uL
ddNTP Mixture (2.5 mM for each) 4uL
Primer 1 (20uM) 1uL
Primer 2 (20uM) 1uL
Template plasmid 0.01~1ng
Pyrobest DNA Polymerase(5 U/uL) 0.25uL
ddH2O up to 50 uL
3. Proceed the reaction at the following reaction conditions
94 degree 30 sec;
55 degree 30 sec;
72 degree 5 min;
Repeat the cycle above.
30 cycles in total
Cycle each reaction using the cycling parameters above (For the control reaction, use a 5-minute extension time).
4. Electrophoresis PCR reaction system in 1% agarose gel.
5. Excise the gel slice and extract the target DNA fragment.
Blunting Kination Reaction
1. Prepare the following reaction system in a microcentrifuge tube.
DNA Fragment around 1 pmol

10x Blunting Kination Buffer 2 uL
Blunting Kination Enzyme Mix 2 uL
ddH2O up to 20 uL
2. React for 10 min at 37 degree.
3. React for 10 min at 70 degree.
Ligate Reaction
1. Add about 0.25 pmol (5 uL) of solution marked No.3 into a new microcentrifuge tube.
2. Add 5 uL of ligation solution, mix gently and thoroughly.
3. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parentalReact for 1 hour at 16 degree.
4. Transfer the whole reacted system into 100 ul of competent cells to transform. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes to be followed by subsequent steps. When the transformation is finished, plate the appropriate volume of each transformation reaction on agar plates containing the appropriate antibiotic for the plasmid vector.

Notes:
1. To maximize temperature-cycling performance, we strongly recommend using thinwalled tubes, which ensure idea l contact with the temperature cycler's heat blocks.The following protocols were optimized using thin-wa lled tubes.
2. Set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping the primer concentration constant.

Protocol for Preparation of Competent Cells for Transformation – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
For two transformations
Materials:
0.1 M Calcium Chloride chilled on the ice;
Overnight bacteria l culture or bacteria l colonies;

Procedure:
1. Add 20 µl of the overnight bacteria l culture or pick a colony to 1 ml of LB antibiotic liquid medium, Incubate at 37 degree in a shaker till the OD600 value reaches 0.4-0.6.
2. Put the tubes on ice to incubate for 5 min.
3. Pellet bacterial cells by 5 min centrifugation at 5000 rpm, discard the supernatant.
4. Resuspend cells in 600 µl of ice-chilled 0.1 M Calcium Chloride solution. Incubate on ice for 30 min.
5. Centrifuge for 5 min at 5000 rpm in a microcentrifuge tube , discard the supernatant.
6. Resuspend the pelleted cells in 100 ul of ice-chilled 0.1 M Calcium Chloride solution. Incubate on ice.
7. Add 50 µl of the prepared cells to each tube containing DNA sample , mix and incubate on ice for 30 min.
8. Transform subsequently as the transformation protocol.

Notes:
1. Make sure the cells are not left in the centrifuge at ambient temperature for more than 5 min as this will significantly decrease the transformation efficiency.
2. The rpm at centrifugation is not higher than 5000, as a high rpm ma y cause the lysis of cells.

Protocol for Ligation of Insert DNA into Plasmid Vector DNA – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
Materials:
Materials:
DNA sample(s) in water or TE buffer
10x ligation buffer
T4 DNA Ligase, 5 u/µl
ddwater

Procedure:
1. Test the concentration of the DNA sample(s).
2. Pipet the following into a microfuge tube:
a. Linearized vector DNA: around 100ng
b. Insert DNA (at 3:1 molar excess over vector): variable
c. 10x ligation buffer: 1uL
d. T4 DNA Ligase: 1uL
e. ddwater: Rest of volume
Total volume: 10 uL
3. Vortex and spin briefly to collect drops.
4. Incubate the mixture at 16 degree for 45-60min.
5. Use the ligation mixture for transformation.

Tips:
Thoroughly mix the 10x ligation buffer before use.
The optima l insert/vector molar ratio is 3:1.
To minimize recircularization of the cloning vector, dephosphorylate linearized plasmid DNA with Alkaline Phospha tase(CIAP) prior to ligation. Heats inactivate the phosphatase or remove from the mixture after the dephosphorylation step.
DNA purity is an important factor for successful ligation. Plasmids should be purified using a method that will ensure isolation of high quality DNA. Use only high quality agarose and fresh electrophoresis buffers for gel-purification of DNA fragments.

Protocol for DNA Purification from Reaction Mixture – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
Here is a suggested protocol; this protocol can be used to purify a wide range of DNA fragments with recoveries of >80%. The bolded should be noticed for a nice DNA extraction.
1. Put EB (elution buffer) or ddwater at 65 degree water bathing.
2. Add a 3:1 volume of Binding Buffer to the reaction mixture (e.g., for every 100 ul of reaction mixture, add 300 ul of Binding Buffer). Mix thoroughly.
Check the color of the solution. A yellow color indica tes an optima l pH for DNA binding. If the color of the solution is orange or violet, add 10 ul of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
3. Pour the solution to a fresh adsorption column. Centrifuge at 13000rpm for 1 min.
Pour off the liq uid in the collection tube. For critical samples, repeat the operation above.
4. Add 600 ul washing buffer (WB) before centrifugation at 13000 rpm for 1 min. Pour off the liq uid into beaker.
5. Centrifuge at 13000rpm for 10 min to spin the ethanol down.

6. Put the column into a fresh EP tube. If necessary air-dry the pellet for 10-15 min to avoid the presence resid ual ethanol in the purified DNA solution. Resid ua l of ethanol in the DNA sample ma y inhibit downstream enzymatic reactions.

7. Add 30-50 ul elution buffer (EB) to elute the DNA.
8. Get 5 ul of the eluted sample to identify with electrophoresis.

Note:
1. If a large amount of DNA is purified or if the volume of the binding reaction is greater tha n 1.5 ml increase the incubation time of the binding step to 15 min.

References:
Current protocols in molecular biology

Protocol for Chemical Inducible Expression of GFP – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
Materials:
4 groups of induce solution with a concentration gradient of 10^-7, 10^-5, 10^-3, 10^-2;
Overnight bacterial culture or bacterial colonies;
Phosphate Buffered Solution (PBS).

Procedure:
1. Add 20 µl of the overnight bacteria l culture or pick a colony to 5ml of LB antibiotic medium, Incubate at 37 degree in a shaker till the OD600 value reaches 0.4-0.6.
2. Add 0.5 mL of the fresh bacteria l culture and appropriate volume of inducer solution to prepare induction system with the concentration gradient of 10^-9, 10^-8, 10^-7, 10^-6, 10^-5, 10^-4, 10^-3, 10^-2.
3. Place the induction system at 37 degree for 2 hours.
4. Pellet bacteria l cells by 4 min centrifugation at 4000 rpm, discard the supernatant.
5. Resuspend the pelleted cells in 500 µl of PBS.
6. Transfer 100 uL of bacteria l resuspention into each well of 96-well plate to test the expression of GFP by flow cytometry or Microplate Reader.

Notes:
If desired, time sequential expression of GFP can also be tested, through verifying the incubating time of induction system at 37 degree.

Miniprep Protocol & Hints – Peking – 2010 http://2010.igem.org/Team:Peking/Notebook/Protocols
Here is a suggested protocol; the yield of the plasmid should be approximately 0.2-0.3ug/ul. The bolded should be noticed for a nice miniprep.
Procedure:
1. Inoculate 5ml LB medium (containing antibiotic) with a bacteria l clone, culture with vigorous shaking at 37 degree for 12-16 hrs.
2. Put EB (elution buffer) or ddwater at 65 degree water bathing.
3. Harvest bacteria by spinning at 13000rpm (~12000g) for 1 min. Aspirate supernatant. Add additional 750 ul culture media, respin and aspirate supernatant for several times .

4. Resuspend bacterial pellet by complete vortexing in 250ml resuspension buffer(RB, with 10ul RnaseA in it), which should be stored at 4 ℃. The bacteria should be completely resuspended - noclumps should be visible.
5. Add 250ul freshly lysis buffer (LB) and mix gently by inverting 5-6 times at room temperature. The mixture should appear translucent and mucous-like. The time of lysis will never be longer than 5 min.
6. Add 350ul neutraliza tion buffer (NB) and mix gently by inverting 5-6 times, incubate at room temperature for 3 min. The mixture should contain flocculent white precipitate at this point.
7. Remove bacterial debris by centrifugation at 13000rpm for 10 min; pour supernatant to a fresh adsorption column which can avoid the transfer of precipitate to the new column causing the precipitate is "sticky".
8. Centrifuge at 13000rpm for 1 min. Pour off the liquid in the collection tube. For critical samples, repeat the operation above.
9. Add 650 ul washing buffer (WB) before centrifugation at 13000 rpm for 1 min.Pour off the liquid into beaker.
10. Centrifuge at 13000rpm for 10 min to spin the ethanol down.
11. Put the column into a fresh EP tube. Air dries DNA for 10 min.
12. Add 30-50 ul elution buffer (EB) to elute the DNA.

Tips:
1. Typica l yield of high-copy-number plasmids, such as PSB1AK3, prepared by this method is about 0.2-0.3 ug of DNA per ul of original bacteria l culture, and 0.1 ug of DNA per ul for low-copy-number plasmids such as PSB3T5.
2. To analyze the DNA by cleavage with restriction enzyme(s) remove 2 µl of the DNA solution and add it to fresh microfuge tube that contains 5 µ l of water. Add 1µl of the appropriate 10 x restriction enzyme (s). Incubate the reaction for 2 hr at the appropriate temperature. Store the remainder of the DNA preparation at -20 degree. Analyze the DNA fragments in the restriction digest by gel electrophoresis.
3. For tetracycline , notice its photolysis .
4. Resuspension buffer (RB) should be stored in the refrigerator. RNAse should be in the -20 degree freezer.

References:
Current protocols in molecular biology

DNA Gel Extraction Protocol – Peking – 2010 http://2010.igem.org/Team:Peking/Notebook/Protocols
Protocol:
Here is a suggested protocol; this protocol can be used to purify a wide range of DNA fragments with recoveries of >80%. The bolded should be noticed for a nice DNA extraction.
1. Excise gel slice containing the DNA fragment using a clean scalpel or razor blade.
Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 ml tube and weigh. Record the weight of the gel slice.
2. Put EB (elution buffer) or ddwather at 65 degree water bathing.
3. Add a 3:1 volume of Solution Buffer to the gel slice (volume:weight) (e.g., add 300 ul of Binding Buffer for every 100 mg of agarose gel). Incubate the gel mixture at 65 degree for 5 min at least until the gel slice is completely dissolved.
Mix the tube by inversion every few minutes to facilitate the melting process. Check the color of the solution. Ayellow color indica tes an optima l pH for DNAbinding. If the color of the solution is orange or violet, add 10 ul of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.

4. Pour the solution to a fresh adsorption column. Centrifuge at 13000rpm for 1 min.Pour off the liquid in the collection tube. For critical samples, repeat the operation above for 2 or 3 times.
5. Add 650 ul washing buffer (WB) before centrifugation at 13000 rpm for 1 min.Pour off the liquid into beaker.
6. Centrifuge at 13000rpm for 10 min to spin the ethanol down.
7. Put the column into a fresh EP tube. If necessary air-dry the pellet for 10-15 min to avoid the presence residual ethanol in the purified DNA solution. Residual of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
8. Add 30-50 ul elution buffer (EB) to elute the DNA.
9. Get 5 ul of the eluted sample to identify with electrophoresis.

Notes:
1. Extract the gel as soon as you excise the gel slice.
2. If the purified DNA will be used for cloning, avoid UV damage of the DNA by minimizing the UV exposure to a few seconds or keeping the gel slice on a glass or plastic plate during UV illumination.
3. If a large amount of DNA is purified or if the volume of the binding reaction is greater than 1.5 ml increase the incubation time of the binding step to 15 min.

References:
Current protocols in molecular biology

DNA Double Digestion Protocol – Peking – 2010 http://2010.igem.org/Team:Peking/Notebook/Protocols
Materials:
DNA sample(s) in water or TE buffer
10x digestion buffer
Restriction enzymes (EcoRI or SpeI or XbaI or PstI)
DNA loading buffer (if electrophoresis is subsequent)
Agarose gel 1.5% (or different depending on expected band sizes)

Procedure:
1. Test the concentration of the DNA sample(s).
2. Pipet the following into a microfuge tube:
20uL reaction system 50uL reaction system
DNA around 1ug around 2.5ug
10x Digestion buffer 2uL 5uL
1st Enzyme 1-1.5uL 2.5-4uL
2ndEnzyme 1-1.5uL 2.5-4uL
ddWater Rest of volume Rest of volume
3. Incubate at recommended temperature (37.0 degrees) for 2 or 4 hours (0.5~2hfor enzymes of NEB, 4h for enzymes of Takara).
4. Take 2 to 5 uLof the digested sample, add loading buffer, and run it on the agarose gel to check the result, or take the entire sample to run to extract a wanted fragment).

Tips:
1. DNA:
For identification of DNA, use 0.4 ug/uL DNA; (or 2uL from a nice DNA mini prep)
For cloning, 1ug/uL DNA is enough.
2. Buffer: we’d better use the buffer that comes with the enzyme, which means buffers from other company may cause some abnormal results.

3. Enzyme: the maximum volume that an enzyme can be used is 1/10 of the total reaction volume (example: 2 uL for 20 uL reaction system). If you want to do overnight digestion, add less enzyme (example: 1 uL for 20 uL reaction system). It is necessary to point that too many enzymes will reduce the efficiency of enzyme digestion with glycerol in it.
4. Gel: make sure to run the uncut DNA as a control along with the digested DNA sample(s). And, always run a DNA marker!

References:
Current protocols in molecular biology (3.1.1-3.1.2)

Protocol for chemical inducible expression of MBP & sample preparation – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
Materials:
$HgCl_2$ store solution with the concentration of 10^{-3}M
Overnight bacterial culture or bacterial colonies;
fuming nitric acid
IPTG
1. Add 1ml of the overnight bacterial culture or pick a colony to 100ml of LB antibiotic medium, Incubate at 37 degree in a shaker till the OD600 value reaches 0.6.
2. Add 1ml IPTG to the 100ml bacterial to induce the expression of Metal Binding Peptide (MBP) or Lpp-Ompa-MBP or Dsba-MBP.
3. Incubate the culture at 30 degree for 0.5 hour.
4 Supply $HgCl_2$ to the culture to different concentrations of 10^{-6}M, 10^{-7}M, 10^{-8}M and control.
5. Incubate the culture at 30 degree for 40 hours.
6 Harvest cells by spinning at 5000rpm for 5 min. Discard the supernatant, Wash with 6ml of ddH_2O for two times.
7 Dehydration the bacteria with freeze dryer, followed by measurement of the weight of cell pellet.
8 Resuspend the pelleted cells in 8ml fuming nitric acid, followed by microwave digestion. Resuspend sample to 25mL. Then sample preparation is completed.

Cytosol MBP Sample Preparation – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols
1. Add 50 ul of the overnight bacterial culture to 50 ml of LB medium-containing ampicillin (100 g/ml). Cultures were grown at 37 °C until the A600 reached 0.6 and then induced with 0.5 mM IPTG at 30 °C for 3 h.

2. The cells were harvested by centrifugation at 6000 rpm for 10 min. The pelleted cells were suspended in 5 ml buffer A (40 mM Tris-HCl pH 8.0, 100 mM NaCl). Then the cell were lysed by ultrasonic, and the cell lysate was separated by centrifugation at 13000 rpm for 15 min.

3. Add 50 ul SDS-PAGE loading buffer into 50 ul supernatant, then heat it at 95℃ for 10 min.

4. The pellet were suspended in 5 ml buffer A. Add 50 ul SDS-PAGE loading buffer into 50 ul suspension, then heat it at 95℃ for 30 min.

Periplasmic MBP Sample Preparation – Peking – 2010
http://2010.igem.org/Team:Peking/Notebook/Protocols

1. Add 50 ul of the overnight bacterial culture to 50 ml of LB medium-containing Kanamycin (40 g/ml). Cultures were grown at 37 °C until the A600 reached 0.6 and then induced with 0.5 mM IPTG at 20 °C for 6 h.

2. The cell of 1 ml culture were harvested by centrifugation at 6000 rpm for 10 min. The pelleted cells were suspended in 100 ul buffer B (1 mg/ml lysozyme, 20%(m/v) sucrose, 30 mM Tris-HCl pH8.0, 1 mM EDTA), ice-bath for 20 min. The mixture were separated by centrifugation at 13000 rpm for 1 min.

3. Add 50 ul SDS-PAGE loading buffer into 50 ul supernatant, then heat it at 95℃ for 10 min.

4. The pellet were suspended in 5 ml buffer A. Add 50 ul SDS-PAGE loading buffer into 50 ul suspension, then heat it at 95℃ for 30 min.

SDS-PAGE Protocol – Peking – 2010 http://2010.igem.org/Team:Peking/Notebook/Protocols
1. Selection of a SDS-PAGE gel. Typically 10% acrylamide gels are used for high molecular weight (MW) proteins (>50 kDa), 12% gels for mid range MW proteins (15 - 50 kDa), and 15% gels for low MW proteins (<15 kDa). Load 5 ul marker or 20 ul protein sample each lane.

2. Run at 80V until samples enter the separation gel in gel running buffer (19.3 mM Glycine, 2.5 mM Tris base, 0.1% SDS), and then run at 120V. Electrophoresis is complete when the dye front migrates about 2 mm from the bottom of the gel..

3. Stain with Coomassie brilliant blue for 1 h, and then destain in destain buffer (50% H_2O, 20% AcOH, 30% methanol) for 1h.
Western Blot Protocol – Peking – 2010 http://2010.igem.org/Team:Peking/Notebook/Protocols
1. After loading 5 ul prestained protein marker or 20 ul His-Tag fusion protein sample each lane, run SDS-PAGE (without staining).

2. Cut a piece of PVDF membrane and wet in 10 ml methanol for 5 min. Then add 40 ml distilled water and shake for 10 min. Transfer PVDF membrane to western transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol)untill use.

3. Balance the gel and two holders in western transfer buffer for 10 min.

4. Assemble "sanwich":
Black (negative charge)-holder-gel-membrane-holder-red (positive charge)

5. Transfer at 20 mA/lane for 20 min.

6. Wash membrane for 10 min, with 15 ml 1X TBS (150 mM NaCl, 20 mM Tris-HCl pH7.5)

7. Incubate for 1 h in 27 ml blocking buffer (dissolve 5% skimmed milk powder in TTBS (dissolve 0.1% Tween20 in TBS)).

8. Wash three times, each for 10 min, with 20 ml TTBS.

9. Incubate for 1 h in His-Tag Monoclonal Antibody diluted 1:800 in blocking buffer.

10. Wash three times, each for 10 min, with 20 ml TTBS.

11. Incubate for 1 h with Rabbit anti-Mouse IgG AP Conjugate diluted 1:800 in blocking buffer.

12. Wash three times, each for 10 min, with 20 ml TTBS.

13. Prepare developing solution by adding 60 µl NBT solution and 60 µl BCIP solution to 15 ml AP buffer.

14. Place membrane in clean tray and add developing solution. Incubate membrane at room temperature until color develops. Strong purple signals should appear within 2–10 min.

15. To stop reaction, wash blot thoroughly in deionized water. Allow to air dry. Store dry blots at room temperature wrapped in plastic.

Preparation of Competent cells and Heat-shock Transformation – Osaka – 2010
http://2010.igem.org/Team:Osaka/Protocols
Preparation
SOB medium 1L (-> final conc.)
Bacto Tryptone 20g (-> 2.0%)
Bacto Yeast Extract 5g (-> 0.5%)
5M NaCl 2ml (-> 10mM)
2M KCl 1.25ml (-> 2.5mM)
Add miliQ water 990ml to all miture and sterilize by autoclave. Before using, add sterilized Mg2 solution (1M MgSO4, MgCl2) 10 ml.
SOC medium
Add sterilized 2M glucose 1 / 100 volume to SOB medium, and sterilize by filtration using 0.22 um microfilter. Store at 4℃.
Tranformation buffer, TB 1L (-> final conc.)
PIPES 3.0g (-> 10mM)
CaCl2 2H2O 2.2g (-> 15mM)
KCl 18.6g (-> 250mM)
After this mixture is suspended in about 950ml sterilized water, control the pH to 6.7-6.8 by 5N KOH (5M KOH). In low pH, this mixture cannot dissolve. Next, add MnCl2 4H2O (10.9g) to the solution, so as to be final concentration 55mM. Ajust it to 1L and sterilize by filtration using 0.22 um microfilter. Store at 4℃.
Liquid nitrogen
DMSO (dimethyl sulfoxide)

Preparation of competent cell – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
Streak E. coli appropriate strain on LB plate. Incuabte at 37℃ overnight.
Inoculate single colony (1-3mm in diameter) into 1ml SOB medium and incubate at 37℃ overnight. After that, inoculate overight culture into SOB 250ml provided in 3L conical flask.
Incubate at 18℃ for 19-50 hour with shaking (>200rpm).
If OD600 reach at 0.4-1.5 (anyphase may be possible to use), immediately put cell culture flask onto ice water for 10min.
Decant cell culture to 500ml centrifuge tube (or 250ml tube X2) and centrifuge in 3000rpm for 15min at 4℃.
Remove soup, suspend ppt by ice-cold TB 80ml, put it onto ice water for 10min.
Centrifuge in 3000rpm for 15min at 4℃.

Suspend ppt by 20ml ice-cold TB and add 1.5ml DMSO (final conc. 7%). Moreover, put on ice water for 10min.
Dispence 0.1-0.5ml each to 1.5ml tube and immediately transfer all tubes into liquid nitrogen. Cold shock is needed for competent cell.
Store at -80℃ until to use.

Transfomation method – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
Melt competent cell taken from freezer in hand and put on ice.
After dispencing 10-50μl each competent cell, add 1-20μl DNA. Put on ice for 30min.
Heat shock at 42℃ in water bath for 30sec and immediately put onto ice for 2min.
Add 40-200μl SOC medium and incubate at 37℃ for 1hour with shaking.
Plate the culture onto LB with appropriate antibiotics, for example ampicillin, kanamycin,chloramphenicol, etc. Incubate at 37℃ overnight.

Plasmid DNA miniprep – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
We use **GenElute™ Plasmid Miniprep Kit (SIGMA-ALDRICH)** and refer to **this accessory protocol (Japanese)** .

3A BioBrick assembly – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
We use **BioBrick™ Assembly Kit (NEB)** and refer to **OpenWetWare protocol**.

Extraction of genome DNA for PCR cloning – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
E. coli or *S. cerevisiae* are cultured overnigt.
Suspend about 10^5 cells into sterilized miliQ-water and place in boiling water for 5 min. When treating *S. cerevisiae*, wash cells once by PBS buffer. PBS buffer is 25mM sodium phosphate pH 7.0-7.2, 125mM $NaCl_2$.
Chill on ice for 2 min, then spin down for 3 min by microcentrifuge at 13,000 *g*.
Transfer the supernatant to a fresh tube. Use 2.5-10ul in one PCR.
Reference: M. J. McPherson et al., PCR: a practical approach, 1991.

Gene cloning – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
We perfome gene cloning as below.
PCR.
We use **TaKaRa Ex Taq®** for amplifying DNA and extracted genome DNA and gifted plasmids as template.
General cycle condition is 25 cycle; 94℃, 30sec, 55℃, 30sec, 72℃, 1min.
If non-specific products are heavy, annealing temperature (55℃) is changed higher.
If the length of gene is long or short, elongnation time (72℃ reaction time) is changed in that case.
Purification of PCR product.
We use **QIAquick PCR Purification Kit** for purification.
Restriction Digest.
Purified PCR products are digested by proper restriction enzymes; two of *Eco*RI, *Xba*I, *Spe*I and *Pst*I.
Ligation.
We use **T4 DNA Ligase** for ligation.
Refer to 3A BioBrick assembly.
Transfomation.
Refer to Preparation of Competent cells and Heat-shock Transformation.
Selection and Cultivation.
We select plasmid-introduced colony by discrimination of marker gene (*rfp*) and cultivate it in LB liquid medium at 37℃ overnight.
Miniprep and Check

Refer to Plasmid DNA miniprep.
Plasmid Check is performed by restriction digest.
Sequence.
Obtained Biobrick parts are sequenced.

PCR site-directed mutagenesis (Overlap extension PCR) – Osaka – 2010
http://2010.igem.org/Team:Osaka/Protocols
We refer to **introduction of site-specific mutagenesis (Japanese)** .
Design two mutagnesis primers, which are sense and antisense to target sequence.
First PCR. This is normal PCR as noted avobe with mutagenesis primer(sense or antisense) and flanking primer(antisense or sense). Reduce template to prevent comtamination of wild type template for second PCR.
Second PCR. This PCR introduces site-specific mutagenesis to target gene. Use first PCR product as template. To begin with, run PCR in 2 cycle without the ends of primers. Next, add those primers and perform normally PCR.

Congo red plate assay – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
Preparation
0.1% congo red solution
Congo red (KISHIDA) is water-soluble.
1% CMC medium
CMC medium is LB medium containing 1% CMC (carboxymethylcellulose, CMC).
1M NaCl
5% acetic acid

Plate assay
Spread 0.1% Congo red solution 5ml onto CMC plate entirely and stain it for 30min.
Discard fully-reacted solution and rinse the plate surface by distilled water.
Add 1M NaCl 5ml and leave for 5min.
Discard NaCl solution and rinse plate surface by distilled water.
Add 5% acetic acid and leave for 5min.
Observe and record the halo size.

Cellulase quantitative assay – Osaka – 2010 http://2010.igem.org/Team:Osaka/Protocols
Preparation
ASC, Acid Swollen Celulose
Cellulose powder is solved in concentrated phosphoric acid 50ml. After dissolved(at room temperature, about for 2hour), five volumes of distilled water are added, and the precipitated amorphouscellulose was centrifuged. Re-suspend it in ditilled water and re-centrifuge. Washing is repeated at least sixtimes, and the final suspended solution is adjusted to pH6.5 with 1M NaOH.

Solubilization of Antibiotics – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Mix the following (Final concentration is 50mg/mL).
Ampicillin:
Ampicillin
1.0g
MilliQ
20mL
Kanamycin:

Kanamycin
0.5g
MilliQ
10mL
Dispense 1.1mL of the solution into 1.5mL tubes.
Store in the -20℃ freezer.
Media – *Kyoto* – *2010* http://2010.igem.org/Team:Kyoto/Protocols
LB Media
Wash a graduated cylinder with MilliQ.
Add the following to about 180mL of MilliQ in the graduated cylinder:
Bacto-yeast extract
1.0g (0.5w/v%)
Tryptone
2.0g (1.0w/v%)
NaCl
2.0g (1.0w/v%)
1N NaOH
200µL
Seal the graduated cylinder by a Parafilm.
Dissolve the mixture by inverting the graduated cylinder.
Adjust volume to 200mL by adding more MilliQ.
Add the media solution to a 200mL Erlenmeyer flask.
(To prepare solid media, add 2.4g (1.2w/v%) of agar to the flask.)
Wrap the tops of the flasks with aluminum foil.
Place a small piece of auto clave tape on one of the flasks.
Autoclave the media on liquids.
Store at room temperature.
To pour plates, melt the solid media in the microwave, then place flask in water bath to bring media to 50℃.
Add 200µL of antibiotic
Ampicillin
100µg/mL
Kanamycin
50µg/mL
(Add 0.5 - 1.0w/v% Glucose for protein expression.)
Supplemented M9 Media
Wash a graduated cylinder with MilliQ.
Add the following to about 180mL of MilliQ in the graduated cylinder:
Na_2HPO_4
1.2g (0.6w/v%)
KH_2PO_4
0.6g (0.3w/v%)
NaCl
0.1g (0.05w/v%)
NH_4Cl
0.2g (0.1w/v%)
Seal the graduated cylinder by a Parafilm.
Dissolve the mixture by inverting the graduated cylinder.
Adjust volume to 200mL by adding more MilliQ.
Add the medium solution to a 200mL Erlenmeyer flask.
Wrap the tops of the flasks with aluminum foil.

Place a small piece of auto clave tape on one of the flasks.
Autoclave the media on a liquids.
Cool at room temperature.
When it become as cool as you can hold it,Add the following to it:
$MgSO_4$
1.0mM（final cocentration）
$CaCl_2$
0.1mM（final concentration）
thiamine hydrochloride
0.001%（final concentration）
casamino acids
0.2w/v%
Add 200μL of antibiotic:
Ampicillin
100μg/mL
Kanamycin
50μg/mL
Add 0.4w/v% Glucose.
Transformation – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Unfreeze conpitent cells on ice.
Dry a plate by letting the plate upside down and partly open in incubator.
Add 1μL DNA solution and 20μL compitent cells to 1.5mL tube, let stand for 30min on ice. If few colony is observed, increase the amount of the compitent cells or DNA, but make the amount of DNA not to get over that of the compitent cells.
Heatshock for 60s at 42℃.
Let stand for 2min on ice.
Culture for 1h in preculture medium (LB or SOC medium), and plate by using spreader. Do not heat spreader too much because e.coli will dead for heat.
Miniprep – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Use QIAprep Spin Miniprep Kit Cat. No. 27104 by QIAGEN
Pick a single colony from a freshly streaked selective plate and inoculate a culture of about 3mL LB medium containing the appropriate selective antibiotic.
Incubate at 170rpm for 8h at 37℃ with vigorous shaking.
Transfer a half of the culture to a tube.
Harvest the bacterial cells by centrifugation at 14,000g for 1min at 4℃. Remove the medium by decanting.
Transfer the half of the culture to same tube and harvest as same. Remove the medium by pippetting.
Resuspend pelleted bacterial cells in 250μL Buffer P1 and mix thoroughly by pippeting.
Add 250μL Buffer P2 and mix thoroughly by inverting the tube gently 4-6 times.
Add 350μL Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
Centrifuge for 10min at 14,000g at 4℃.
Apply the supernatants from step 10 to the QIAprep spin column by pipetting.
Centrifuge for 10s in a table-top microcentrifuge. Discard the flow-through.
Wash the QIAprep spin column by adding 0.5mL Buffer PB and centrifuging for 10s in a table-top microcentrifuge. Discard the flow-through.
Wash QIAprep spin column by adding 0.65mL Buffer PeE and centrifuging fo 10s in a table-top microcentrifuge.
Discard the flow-through, and centrifuge for and additional 1min to remove residual wash buffer.
Place the QIAprep column in a clean tube. To elute DNA, add 50μL water to the center of each QIAprep spin column, let stand for 1min, and centrifuge for 1min.

Discard the QIAprep spin column.
Measure the concentration of DNA by using eppendorf BioPhotometer plus.
Restriction Digestion.
Agarose Gel Electrophoresis for Confirmation.
Restriction Digestion – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Use EcoRI, XbaI, SpeI, PstI, DpnI（NEB）
Mix the following.
Sample
5µL
10xBuffer
1µL
Restriction Enzyme
0.1µL
MilliQ
3.9µL
Let stand for 2h at 37℃
PCR – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Standard PCR
Dilute template DNA. If the concentration of DNA is 2-100ng/µL, transfer 1µL to a clean tube and add 99µL MilliQ.
Dilute Primer. If the concentration of Primer is XµM, dilute primer X timers and transfer 1µL to a clean tube and add 99µL MilliQ.
Mix the following.
For use of KOD plus ver2:
25mM $MgSO_4$
3µL
2mM dNTPs
5µL
10xBuffer for KOD plus ver.2
5µL
Template DNA (5ng/µL)
5µL
Primer Forward (10µM)
1.5µL
Primer Reverse (10µM)
1.5µL
KOD plus ver.2
1µL
MilliQ
28µL
Total
50µL
For use of KOD FX:
2mM dNTPs
10µL
2xBuffer for KOD FX
25µL
Template DNA
5µL
Primer Forward (10µM)
1.5µL

Primer Reverse (10μM)
1.5μL
KOD FX
1μL
MilliQ
6μL
Total
50μL
(If amplification is not succeeded, try at 4.5 or 6.0μL 25mM $MgSO_4$.)
Let stand for 2min at 94℃.
25-40 cycles for 10s at 98℃, for 30s at Tm-5℃, and for 1min (1min for 1kb) at 68℃ (Tm is temparature at which primer will dissolve).
At 15℃ forever.
Agarose Gel Electrophoresis for confirmation.
Screening PCR ***– Kyoto – 2010*** http://2010.igem.org/Team:Kyoto/Protocols
Mix the following (Do on PCR Bench).
10x PCR buffer (TAKARA)
40μL
2.5mM dNTP
8μL
Primer-1 (10pmol/μL)
8μL
Primer-2 (10pmol/μL)
8μL
Ex Taq HS (TAKARA)
1.6μL
MilliQ
334μL (to total 400μL)
Dispense 25μL to 15 tubes.
Pick a single colony and transfer it to each tubes.
Suspend the colony.
Let stand for 10min at 90℃.
35 cycles for 30s at 94℃, for 30s 55℃, and for 1min at 72℃.
Let stand for 4min at 72℃.
Add 5mL Loading Buffer to the tubes.
Agalose Gel Electrophoresis for confirmation.
Negative Control: Use nothing.
Positive Control: Use a colony that will yield a product with this primers.
Mutagenesis (Point mutation, Deletion, Insertion) ***– Kyoto – 2010***
http://2010.igem.org/Team:Kyoto/Protocols
Mix the following.
10xBuffer
5μL
2mM dNTP
5μL
Primer Forward (10μM)
1.5μL
Primer Reverse (10μM)
1.5μL
Template Plasmid (50ng/μL)
1μL

KOD plus ver.2
1µL
MilliQ
35µL
Total
50µL
Prepare control: instead of KOD plus ver.2, add 1µL MilliQ.
Let stand for 2min at 94℃.
X cycles (1 cycle for 1kb) for 10s at 98℃ and for Ymin (1min for 1kb) at 68℃.
Hold at 4℃.
Take 25µL of the solutions into fresh tubes.
Add 1µL *Dpn*I (10U/µL).
Let stand for 1h at 37℃.
Agarose gel electrophoresis, using 5µL of the solution for confirmation.
Mix the following.
Sample
2µL
Ligation high
5µL
T4 Polynucleotide Kinase (5U/µL)
1µL
MilliQ
7µL
Total
15µL
Let stand for 1h at 16℃.
Transformation, using 10µL of the solution.
PCR Purification ***– Kyoto – 2010*** http://2010.igem.org/Team:Kyoto/Protocols
Use QIAquick PCR Purification Kit Cat. No. 28104 by QIAGEN
Add BufferPB about 5 times as much as the product of PCR.
Apply the solution to the column.
Centrifuge for 1min at 1300rpm. Discard the flow-through. If the amount of the sample is much, repeat this step by same column.
Add 750µL BufferPE and let stand for 2-3min. If the solution overflows, we decrease the amount of BufferPE.
Centrifuge for 1min and discard the through.
Centrifuge for additional 1min to remove residual buffer.
Place the column in a clean tube.
Add 10µL BufferEB or MilliQ to the center of each column, let stand for 1min, and repeat this step.
Centrifuge for 1min at 13000rpm.
Discard the column.
Electrophoresis – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Prepare 200mL of a 1.0% agarose solution:
Measure 2.0g agarose into a beaker.
Add 200mL 1xTAE buffer.
Wrap the top of the beaker with plastic wrap.
Punch a hole through the wrap with a pipette tip (To let out steam).
Dissolve the agarose by heating in microwave and swirling without boiling.
Allow the agarose to cool.
Pour the agarose solution into a gel tray on a gel maker.
If there is air bubbles, pushing them with a pipette tip.

Place comb in the maker.
Cover the maker with a plastic wrap.
Let stand for about 45min.
Remove the comb carefully.
Store in the Tupperware in the refrigerator.
Place the tray in electrophoresis chamber.
Cover the tray with 1xTAE buffer.
To prepare samples for electrophoresi, add 1µL of 6x Loading Buffer for every 5µL of DNA solution and mix well.
Load 6µL of the DNA solution per well.
Electrophorese at 100V for about 30min until Loading Buffer have migrated approximately three-quaters of the gel.
Stain the gel in 0.5µg/mL ethidium bromide for 20-30min.
Rinse the gel with MilliQ.
Place a plastic wrap on the transilluminator in the cabinet of Printgraph.
Place the gel on the transilluminator.
Turn on the transilluminator and confirm the position of the gel.
Shoot the picture.
Turn off the transilluminator.
Dispose of the gel.
Gel Extraction – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Use QIAquick Gel Extraction Kit Cat. No. 28704 by QIAGEN
Transfer cutted gel to a tube.
Add BufferQG about 3 times as much as the volume of the gel.
Stand the gel for 10min at 50℃ to Dissolve. If hard to dissolve, sometimes vortex.
Confirm the color of the solution. If the color is orange or purple, add about 10µL 3M sodium acetate to yellow.
Add isopropanol as much as the gel and mix.
Apply the solution to the column.
Centrifuge for 1min at 1300rpm. Discard the flow-through. If the amount of the sample is much, repeat this step by same column.
Add 500µL BufferQG and centrifuge for 1min. Discard the through.
Add 750µL BufferPE and let stand for 2-3min. If the solution overflows, we decrease the amount of BufferPE.
Centrifuge for 1min and Discard the through.
Centrifuge for additional 1min to remove residual buffer.
Place the column in a clean tube.
Add 10µL BufferEB or MilliQ to the center of each column, let stand for 1min, and repeat this step.
Centrifuge for 1min at 13000rpm.
Discard the column.
Ligation – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Make 2µL of Mixture (the vector and the insert at 1 : 5-10) and Control (only the vector).
Add 5µL Ligation High, 1µL T4 Kinase, and 7µL MilliQ to create a solution.
Incubate at 16℃ for 30 min. If the colonies of E.coli transformed with the Control,
Dephosphorylation – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Use Bacterial Alkaline Phosphatase (BAP, Takara)
Set the heating block at 65℃
Mix the following:
MilliQ
24µL
10x AP Buffer

5µL
DNA sample
20µL
BAP
1µL
total
50µL
Not need to purify the DNA sample.
Incubate the solution at 65℃ for 30min.
Add 50µL MilliQ to the solution (Total is 100µL).
Add 100µL mixed lipid of Phenol and Chloroform (at 1:1).
Vortex the solution sufficiently.
Centrifuge for 5min at 15,000rpm at room temparature and transfer the supernatant to a tube.
Repeat the previous step a few times.
Add 100µL Chloroform.
Vortex the solution.
Centrifuge for 5min at 15,000rpm at room temparature and transfer the supernatant to a tube.
Add 10µL 3M Sodium Acetate and 250µL 100% cold Etanol and mix them.
Let stand for 15min on the ice.
Centrifuge for 15min at 15,000rpm, 4℃ and discard the supernatant.
Add 500µL 70% Etanol.
Centrifuge for 5min at 15,000rpm, 4℃ and discard the supernatant.
Dry it.
Add appropriate quantiles (about 20µL) of TE Buffer to dissolve the DNA.
Stored at -20℃.
Sequence – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Use Big Dye Terminator 3.1（ABI）
Mix the following
5xBuffer
2µL
Primer (3.2µM)
1µL
Template Plasmid
200ng
Big Dye Terminator 3.1
0.5µL
MilliQ
up to 10µL
Let stand for 1min at 96℃.
35 cycles for 5s at 98℃, for 5s 50℃, and for 2.5min at 68℃.
Add 25µL 100% ethanol and 1µL NAOAC
Ethanol Precipitation – *Kyoto – 2010* http://2010.igem.org/Team:Kyoto/Protocols
Use Ethachinmate (NIPPON GENE、312-01791).
Add 3.3 µL of 3M Sodium Acetate (attached with Ethachinmate) into 100µL of DNA solution.
Add 1µL of Ethachinmate.
Vortex.
Add ethanol, 200-250µL.
Vortex.
Centrifuge at 12000xg for 5min.
Precipitation.

PCR Protocol – IIT Madras – 2009 http://2010.igem.org/Team:IIT_Madras/Protocols
Composition of PCR reaction:
PHusion Phusion® High-Fidelity DNA Polymerase(from Finnzymes) - 0.2 mul
5x Phusion HF Buffer (from Finnzymes) - 4 mul
dNTP – 1 mul
DNA Template – 1 mul
Primers(from Bioserve) – 1 mul each.
Water – 12.8 mul
Total volume for the reaction is 20 mul. Program used:
98 C for 2min.
98 C for 30sec.
(Tm+3)C for 30sec.
72C for x sec(15sec per kb).
GOTO 2 30 times.
72C for 30 min.
4C for storage.

Digestion Protocol – IIT Madras – 2009 http://2010.igem.org/Team:IIT_Madras/Protocols
Composition of the digestion mixture:
DNA - 4 mul
Enzyme 1 - 1.2 mul
Enzyme 2 - 1.2 mul
Buffer - 2 mul
Water - 11.6 mul
Total volume for the reaction is 20 mul.
Steps:
For EcorRI, PstI – The reaction mixture is kept at 37 degrees for 1.5 hours. For other Enzymes – The reaction mixture is kept at 37 degrees for 5 hours.
Inactivate enzyme by heating at 80 degrees for 20 mins.

T4 Ligation Protocol – IIT Madras – 2009 http://2010.igem.org/Team:IIT_Madras/Protocols
Composition of Ligation mixture:
T4 Ligase Buffer - 1mul
6:1 molar insert:vector (vector~10ng)
miliQ water - (8.5 - DNA volume) mul
T4 ligase – 0.5 mul
Steps:
Leave reaction at 22.5degC for 30min
Denaturate ligase at 65degC for 10min
Store at -20degC
Ultracompetent Cell Preperation Protocol – IIT Madras – 2009
http://2010.igem.org/Team:IIT_Madras/Protocols
Materials/Buffers * SOB SOLUTION FOR COMPETENT CELL PREPARATION
0.5% yeast Extract
2% Tryptone
10mM NaCl
2.5mM KCl
10mM $MgCl_2$

10mM $MgSO_4$.
Dissolve all in nanopure water and autoclave
* TRANSFORMATION BUFFER FOR COMPETENT CELL PREPARATION
10mM PIPES
15mM $CaCl_2$
250mM KCl
Dissolve in nanopure water and adjust pH to 6.7 with KOH or HCl. Then add $MnCl_2$ to 55mM and adjust final volume. Sterilize by filtration with 0.45 µm filter. Store at 4C
Cells were cultured on LB agar plate overnight at 37C.
10-12 colonies were cultured in 250ml SOB medium.
It was incubated at 37C for 1hour. Then the flasks were transferred to 19C. It was incubated till the OD600 reached 0.5
Flask was placed in ice for 10min.
The cells were pelleted by spinning at 4000rpm for 10min at 4C.
Cells were resuspended in 80ml ice cold TB(Transformation Buffer) and stored on ice for 10min.
It was centrifuged again at 4000rpm for 10min at 4C.
Pellet was resuspended in 20ml of TB with 1.5ml DMSO.
Final volume was aliquoted into microcentrifuge tubes (100-500µl) and stored at -80C
CAUTION!
* Caution: The whole procedure after the cells are pelleted out needs to be carried out in ice.
* Caution: TB buffer is heat sensitive, never take it out of ice.
Transformation Protocol – IIT Madras – 2009 http://2010.igem.org/Team:IIT_Madras/Protocols
100µl competent cells were thawed on ice
2 µl Plasmid DNA added to the tube and shaken gently.
Mixture left on ice for 30 min.
Heat shock given at 42C for 2min.
Incubated on ice for 3-5 min.
800 µl of LB broth added.
Flasks were shaken at 37C for 1hr.
They were centrifuged at 3000rpm for 5min and the pellet was resuspended into 100ul of the supernatant.
The 100 µl of the transformation mix was plated on LB agar plates.
Plates were incubated at 37C overnight.
Miniprep Protocol – IIT Madras – 2009 http://2010.igem.org/Team:IIT_Madras/Protocols
Overnight cultures were harvested (2-3ml broth cultures).
They were centrifuged at 13000rpm for 1min.
The pellet was resuspended in 250 µl of HP1 solution.
The cells were lysed by adding 250 µl of lysis solution i.e. HP2. Tubes were inverted 5-6 times.
350 µl of neutralization solution i.e. HN3 was added. Tbes were inverted 5-6 times to mix the solutions.
They were centrifuged at 13000rpm for 10 mins to get a white pellet.
The supernatant was carefully transferred to a HiElute Miniprep spin column.
It was centrifuged at 13000rpm for 1 min. Flow through was discarded.
500 µl of wash solution i.e. HPB was added to the column.
It was centrifuged at 13000rpm for 1 min. Flow through was discarded.
700 µl of wash solution i.e. HPE was added to the column.
It was centrifuged at 13000rpm for 1 min. Flow through was discarded.
It was centrifuged at 13000rpm for 1 min.
The column was transferred to a fresh tube.
50 µl of elution buffer was added carefully to the center of the column.
Incubate for 1 min
It was centrifuged at 13000rpm for 1 min by placing it in a fresh tube.

PCR Protocol with Taq Polymerase – IIT Madras – 2009
http://2010.igem.org/Team:IIT_Madras/Protocols
Mix contains:
0.4 µl Taq polymerase.
2 µl Bffer (10X).
0.8 µl dNTPs.
0.4 µl forward Primer.
0.4 µl backward Primer.
0.8 µl Template.
15.2 µl MilliQ water.
Program used:
96C for 2 min.
96C for 30sec.
(Tm-5)C for 30sec.
72C for 'x'min (1min per kb).
GOTO 2 30 times.
72C for 30min.
4C for storage.
Purification by Phenol/CHCl3 Method Kept for restrction digestion at 37 degC for 16hrs and purify by phenol/CHCl3 method
50uL mixture - make the volume to 100uL
add 100uL of phenol/CHCl3 – conc?
centrifuge 20,000 for 5 min
aqueous layer (approx. 100uL)
add 1/10 volume of 3M sodium Acetate (10uL) + 2.5 volumes of 95% ethanol (250uL)
incubate at -20/-40degC for 2 hrs
centrifuge 20,000xg for 20 min at 4 degC
pellet washed with 70% ethanol
air dry the pellet
dissolve pellet in 11uL MQ
V
V V
10uL 1uL load on gel and estimate its concentration

restriction digestion of vector as conc. Increases .... gel purification. 100uL [chk] kept for restiction
extract gel using gel extraction kit
quantify RE vector by running agarose gel 1:1 3:1 5:1</pre>

Vector Insert T4 DNA ligase Buffer MQ Total 10uL Length of insert [in bases] X ng of vector X (ratio)
lenght of vector [bases]
= ng of insert needed for [[ratio] Molar basis]</pre>

after 16 + 1 hr
Purification Of Ligation Mixture [using 2M potassium acetate pH=8] – IIT Madras – 2009
http://2010.igem.org/Team:IIT_Madras/Protocols
Both rxn mixture (1:3 & 1:5) was pooled (20uL) make up the ligation mixture volume to 50uL
add 6.25uL of 2M K-acetate (pH 8)
add 2.5 volume of 95% ethanol (125uL) – mix well
allow the ppt to form (-20degC, 2 hrs)

centrifuge at 14000 rpm for 10min in eppendorf
wash the pellet twice with 70% ethanol (125uL) 10 min , 14000 rpm (twice)
air dry to remove alcohol completely
resuspend the pellet in 5uL of deionozed water (sterile)
transfer the whole mixture for electroporation
transform the (40uL) cells with 5uL of ligation mix. (run a control also)
immediately add 1mL of media without antibiotic
incubate for 1.5 hr
centrifuge 5000rpm for 1 min
remove 800uL of supernatant
remaining 200uL, resuspend cells
plate in selection plate
Electroporation – IIT Madras – 2009 http://2010.igem.org/Team:IIT_Madras/Protocols

add 40uL cells in electroporation cuvette + 1uL DNA (or 5uL ligation mixture)
keep the cuvette on ice use biorad gene pulser 2000V, 25uF, 200ohms pulse approx. 4.5 to 5msec
add 1mL G-M17B +20mM $MgCl_2$ + 2mM $CaCl_2$ medium
keep the cuvette for 5min on ice
incubate 1 to 1.5 hr @ 30degC
centrifuge 1 min, 10,000rpm
remove 800 uL of media
resuspend in 200uL of media
spread plate on G-M17B + 1.5% agar + anti-biotic (10ug/ml) containing glucose 5g/l, 20 mM $MgCl_2$ & 2mM $CaCl_2$</pre>

Preparation of electro competent cells of L.lactis NZ9000 – IIT Madras – 2009
http://2010.igem.org/Team:IIT_Madras/Protocols
DAY 1: incubate 10mL culture in 10ml G-SGM17B media with -80degC stock grow at 30 degC
DAY 2: add 0.5ml culture in 10 ml G-SGM17B
grow at 30 degC
DAY 3: dilute the 10ml culture in 90ml of G-SGM17B media
grow till OD600 (0.2 to 0.3) approx. 3Hr[it should not go above 0.3]
centrifuge 10min at 5,000 rpm(4degC)
Wash cells with 40-45mL sucrose / 10% glycerol (4deg C)
Centrifuge (20-30mins, 5k rpm) keep cells on ice for 15 mins in 50ml of 0.5M sucrose 10% glycerol / 0.05M EDTA (4degC)
centrifuge 20-30 min , 5,000 rpm
wash cells with 25ml 0.5M sucrose /10% glycerol(4degC)
centrifuge 20min, 5,000rpm resuspend the cells in 0.8-1ml 0.5 sucrose /10% glycerol(4degC)
use 40uL per electroporation [keep on ice]
keep cells in aliquots -80degC let them defreeze on ice before each use

Reagents for electro competent cells prep

G-SGM17 (100mL) (M17 + 0.5M sucrose + 2.5% Glycine + 0.5% glucose)
2 X M17= 50ml
sucrose = 17.115g
glycine = 2.5g
20% glucose = 2.5 ml   {volume made to 100ml}
Note:add sucrose and glycine to M17 and sterilize by autoclaving
add sterile glucose when cooled

glucose stock = 20% - autoclave
1M sucrose
sucrose=34.2g
deionized water= make volume to 100ml
</pre>
Glycerol 50%
glycerol=50ml
deionized water= 50ml

0.5M sucrose - 10% Glycerol soln.
1M sucrose =100ml
50% glycerol= 40ml
deionozed water= 60ml
==200ml

0.5M sucrose - 10% Glycerol soln. -0.05M EDTA
1M sucrose =50ml
50% glycerol= 20ml
disodium EDTA = 1.86g
\/
heat to dissolve
\/
deionized water= make volume 100ml

0.5M CaCl2 (50ml)
CaCl2.2H2O= 3.676g
water = make vol 50ml

1M MgCl2 (50ml)
MgCl2.6H2O = 10.165g
deionized water = make up the vol to 50ml

revival medium - GM17CaMg
2 X M17= 5ml
0.5M CaCl2 = 0.04ml
1M MgCl2 = .2ml
20% Glucose = 0.25ml
deionized water = 4.5ml
==10ml

plate for selection
2 X M17 agar medium = 200ml

20% Glucose = 10ml
0.5M CaCl2 = 1.6ml
1M MgCl2 = 8ml
water = 180ml
chloramphenicol = 400ul
== 400ml
G5M17 -Cml plate
G5M17 -agar plate
G5M17 medium (5X 10ml)

also to be autoclaved MQ water, tubes, measuring cylinder.

Preparation of Competent cells (*E. coli* DH5a) – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Reagents
TB (Transformation Buffer)(at 4C, filtration)

		Final concentration
1 M $CaCl_2$ (at RT, autoclaved)	0.75 mL	15 mM
4 M KCl (at RT, autoclaved)	3.125 mL	250 mM
1 M $MnCl_2$ (at 4C, autoclaved)	2.75 mL	55 mM
1 M PIPES (pH 6.7 by NaOH, at 4C, filtration)	0.5 mL	10 mM
Total	50 mL	

Procedure
Single colony isolation on LB plate
Incubate the plate for 15-19 hrs at 37C
Lift a colony into 2 mL of LB
Culture cells at 37C for 12-16 hrs at 180-200 rpm
Transfer 30 uL, 100 uL, 300 uL of the culture into 100 mL SOB medium, respectively
Culture cells at 20C (for 24 hrs over) at 180-200 rpm (to ΔOD_{550nm} = 0.5~0.6)
Leave the 300 mL flask for 10 min on ice
Transfer the culture into two 50 mL Falcon tube
Centrifuge 7500 rpm at 4C for 20 min (TOMY TA-22 rotor), and discard sup
Suspend the pellet in ice-cold 15 mL of TB (Transformation Buffer)(7.5 mL/tube)
Centrifuge 7500 rpm at 4C for 2 min (TOMY TA-22 rotor), and discard sup
Suspend the pellet in ice-cold 3.2 mL of TB
Add 0.24 mL of DMSO (stirring, bit by bit)
Leave the 50 mL Falcon tube for 10 min on ice
Dispense 50 uL into 0.5 mL tube
Freeze the suspension in liquid nitrogen
Store at -80C

Bacterial Transformations – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29

Add DNA solution to thawed competent cells
Incubate the cells on ice for 30 min
Heat shock the cells by immersion in a pre-heated water bath at 42C for 60 sec
Incubate the cells on ice for 5 min
Add 200 uL of SOB broth
Incubate the cells at 37C for 2 hrs while the tubes are shaking
Plate 200 uL of the transformation onto the dish
Incubate the plate at 37C for 12-14 hrs

Mini-prep (Alkaline SDS Method) – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Reagents
Solution I (at RT, filtration 0.2 um, 50 mL)

		Final concentration
Glucose (at RT)	0.45 g	50 mM
1 M Tris-HCl (pH8.0, at RT, autoclaved)	1.25 mL	25 mM
0.5 M EDTA (pH8.0, at RT, autoclaved)	1 mL	10 mM
Total	50 mL	

Solution II (at RT, filtration 0.2 um, 20 mL)

		Final concentration
10 N NaOH (at RT)	0.4 mL	0.2 N
10% SDS (at RT, filtration)	2 mL	1%
Total	20 mL	

Solution III (at RT, filtration 0.2 um, 50 mL)

		Final concentration
5 M CH_3COOK	30 mL	3 M
CH_3COOH	5.75 mL	
H_2O	14.25 mL	
Total	50 mL	

Procedure
Lift colony *E. coli* into 2 mL LB contained antibiotics
Culture cells at 37C for 16-20 hrs at 180-200 rpm
Transfer 1.2-1.5 mL of culture into 1.5 mL tube
Centrifuge the culture at 15,000 rpm for 1 min at 4C and discard sup
Suspend the pellet in ice-cold 100 uL of Solution I
Add 200 uL of Solution II to the suspension
Mix by inverting the tube 10-20 times
Add ice-cold 150 uL of Solution III to the suspension
Mix by inverting the tube 10-20 times
Leave the tube for 5 min on ice
Add 10 uL of Chloroform

Mix by inverting the tube 5-10 times
Centrifuge the suspension at 15,000 rpm for 5 min at 4C
Transfer the supernatant into new 1.5 mL tube↓
Add equal volume of isopropanol and mix by voltexing
Leave the tube for 5 min at RT
Centrifuge the suspension at 15,000 rpm for 10 min at 4C and discard sup
Rinse the ppt by 70% EtOH and mix by voltexing
Centrifuge the suspension at 15,000 rpm for 2 min at 4C and discard sup
Dry up the ppt
Dissolve the ppt in 50 uL of TE (pH 8.0)
Add 1 uL of 10 mg/mL RNase A (4C and stock at –20C)
Incubate for 30 min at 37C
PCIAA and CIAA extraction
Ethanol precipitation
Dry up the ppt
Dissolve the ppt in 50 uL of TE (pH 8.0)

PCR – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Vector
Standard reaction setup

Component	Volume
10x PCR Buffer	5 uL
2mM dNTPs	5 uL
25mM $MgSO_4$	3 uL
Suffix-F primer	1 uL
Prefix-R primer	1 uL
Template DNA	1 uL
KOD -Plus- Neo	1 uL
DW	X uL
Total	50 uL

Cycling conditions (2-step cycle)

Predenature	94C 2 min
Denature	98C 10 sec
Extension	68C X sec (30 sec/kb)
Hold	4C

30-40 cycles
Insert
Standard reaction setup

Component	Volume
10x PCR Buffer	5 uL
2mM dNTPs	5 uL

25mM $MgSO_4$	3 uL
EX-F primer	1 uL
PS-R primer	1 uL
Template DNA	1 uL
KOD -Plus- Neo	1 uL
DW	X uL
Total	50 uL

Cycling conditions (2-step cycle)

Predenature	94C 2 min
Denature	98C 10 sec
Extension	68C X sec (30 sec/kb)
Hold	4C

30-40 cycles

Colony PCR – HokkaidoU Japan – 2010

http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29

resuspend a colony into 10 uL of DW (template suspension)

Standard reaction setup

Component	Volume
template suspension	4.8 uL
Quick Taq	5 uL
Forward primer	0.1 uL
Reverse primer	0.1 uL
Total	10 uL

Cycling conditions (2-step cycle)

Predenature	94C 2 min
Denature	94C 10 sec
Extension	68C X sec (60 sec/kb)
Hold	4C

30-40 cycles

Electroporation – HokkaidoU Japan – 2010

http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29

Preparation of electro-competent cells

Cell culture in 400 mL of SOB or LB and grow to ΔOD_{600} = 0.5~0.6

Dispense the medium into 8 Falcon 50 mL tube

Centrifuge at 3500 rpm for 5 min at 4C and discard sup

Add 5 mL of DW and suspend the ppt, mix 8 suspensions into single Falcon tube

Centrifuge at 3500 rpm for 5 min at 4C and discard sup

Add 40 mL of DW and suspend the ppt
Centrifuge at 3500 rpm for 5 min at 4C and discard sup
Add 10 mL of 10% Glycerol and suspend the ppt
Centrifuge at 3500 rpm for 5 min at 4C and discard sup
Add 10 mL of 10% Glycerol and suspend the ppt
Centrifuge at 3500 rpm for 5 min at 4C and discard sup
Add 5 mL of 10% Glycerol and suspend the ppt
Dispense 100 uL of the suspensions into 0.5 mL Eppendorf tube, respectively
Store at -80C freezer
Electroporation

PCIAA and CIAA extraction – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Reagent
PCIAA = Phenol : CIAA = 1 : 1
CIAA = Chloroform : IsoAmyl Alcohol = 24 : 1
Procedure
Add equal volume of PCIAA and vortex vigorously
Centrifuge at 15,000 rpm for 2 min at RT
Transfer the aqueous phase to a new tube, being careful not to transfer the phase interface
Add equal volume of CIAA and vortex vigorously
Transfer the aqueous phase to a new tube
Ethanol precipitation

Ethanol presipitation – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Add 1/10 volume of 3M CH_3COONa
Add 2.5 volume of 100% ethanol (EtOH)
Incubate on ice for few min
Centrifuge at 15,000 rpm for 10 min at 4C and discard sup
Wash precipitation with 100 uL of 70% EtOH (EtOH has to be cold)
Centrifuge at 15,000 rpm for 5 min at 4C and discard sup
Dry up the ppt (no EtOH should be left)
Resuspend ppt in wanted volume of TE

Mini-prep (QIAprep Spin Miniprep Kit) – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a micro-centrifuge tube
Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times
Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times
Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge
Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting
Centrifuge for 30–60 s. Discard the flow-through
Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through

Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s
Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer
Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min
see details (Official website)

Gel Extraction (Wizard® SV Gel and PCR Clean-Up System) – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5 mL microcentrifuge tube
Add 10 uL Membrane Binding Solution per 10 mg of gel slice
Vortex and incubate at 50–65C until gel slice is completely dissolved
Insert SV Minicolumn into Collection Tube
Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly
Incubate at room temperature for 1 min
Centrifuge at 16,000 g for 1 min
Discard flowthrough and reinsert Minicolumn into Collection Tube
Add 700 uL Membrane Wash Solution (ethanol added)
Centrifuge at 16,000 g for 1 min
Discard flowthrough and reinsert Minicolumn into Collection Tube
Repeat Step 4 with 500 uL Membrane Wash Solution
Centrifuge at 16,000 g for 5 min
Empty the Collection Tube and recentrifuge the column assembly for 1 min with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol
Carefully transfer Minicolumn to a clean 1.5 mL microcentrifuge tube
Add 50 uL of Nuclease-Free Water to the Minicolumn
Incubate at room temperature for 1 min
Centrifuge at 16,000 g for 1 min
Discard Minicolumn and store DNA at 4C or –20C
see details (Official website)

T3SS RK13 cell Injection Assay – HokkaidoU Japan – 2010
http://2010.igem.org/Team:HokkaidoU_Japan/Protocols#Preparation_of_Competent_cells_.28E._coli_DH5a.29
Seed RK13 cells
Remove the culture medium and wash 3 times with PBS follwed by trypsinization
Suspend RK13 cells with antibiotics free RPMI-10% FCS
Seed $2x10^5$/well RK13 cells on a 6 well plate or a glass bottom 3.5 cm dish 20 hrs before infection
Prepare *E. coli* culture
Grow E.coli-K12(SPI2/Signal-GFP/RFP), and *E. coli* K12(SPI2)in 4 mL of LB+0.4% arabinose with appropriate antibiotics at 37C overnight
10 hrs before injection
Centrifuge 4 mL of *E. coli* culture at 3,500 rpm for 10 min in the round tube.
Discard the sup and resuspend with 4 mL of MgM-MES(pH 7.2)+0.4% arabinose with appropriate antibiotics and grow for 4 hrs at 37C
4 hrs later(5.5 hrs before injection) centrifuge the culture 3,500 rpm for 10 min

Discard the sup and resuspend with 4 mL of MgM-MES(pH 5.0)+0.4% arabinose with appropriate antibiotics and grow for 4 hrs at 37C
4 hrs later(1 hr before injection) centrifuge the culture 3,500 rpm for 10 min, and discard the sup
Resuspend the pellet with 1 mL of antibiotics free RPMI-10% FCS+HCl(pH 5.0) and transfer the culture into micro tube
Centrifuge the culture 5,000 rpm for 2 min and discard the sup
Repeat step 6 and 7 three times
Measure and adjust the concentration of *E. coli* RPMI(pH 5.0) culture(ΔOD = 0.06)
Add 0.4% arabinose and appropriate antibiotics into this *E. coli* culture
Injection
Remove the RPMI on RK13
Add 1 mL of *E. coli* RPMI(pH 5.0) culture(ΔOD = 0.06)
Incubate the plate at 37C, 5%CO_2 and observe the cells by Cofocal Laser Scannning Microscope(OLYMPUS FV-1000D) under blue and green exciter light at every 1.5 hrs after first exposure

2009

Colony PCR – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

Pick a colony and streak a small patch on a plate (that way you can use one plate for all of your screening) and then pipette with the same tip up and down several times into one of the 10uL aliquots. Make sure you also run a positive and negative control.
Materials
12.5 µL Master Mix (Go Taq)
11 µL DNA in H20
0.5 µL Fwd Primer
0.5 µL Rev Primer

Thermocycler

Sequencing Reaction – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

Mixture
1 uL Big Dye
1.5 uL Big Dye Buffer
10 pmol Fwd Primer
10 pmol Rev Primer
0.1 ug DNA Template
The cycling protocol is in spencer's folder under SEQ - don't change the protocol! It is the same no matter what primer Tm you have.
Magnetic Bead Clean-up
Get graduate student in Brian's Lab to take reaction to center for processing.

Gel Loading Protocol – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

Make on Parafilm:
DNA Samples:
1 µL DNA
1 µL Dye
4 µL TAE Buffer
Ladder:
1 µL Ladder
1 µL Dye
4 µL TAE Bufer
Load all 6 µL (small well) or 12µL (same proportions)(big well)

Digestion – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

Materials
DNA to concentration of 1.5 µL/50 µL Rxn
5 µL Buffer 2
0.5 µL BSA
1 µL Enzyme #1
1 µL Enzyme #2
H20 up to 50 µL total Rxn

Ligation – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

Concentration
1 backbone : 5 insert
insert: [100 x (insert size bp/backbone size bp) x 5 / concentration(ng)] µL
Thermocycler
users: Dan A: Lig
2 hours 16 C or overnight
Materials
1µL T4 ligase
1 µL T4 ligase buffer
backbone + insert =< 8µL (remainder H20)

Polymerase Chain Reaction (PCR) – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

To amplify and to add BioBrick Restriction sites to your DNA your run a PCR of your gene.

You must first design Primers for you gene.

Tips:
EcoR1 (E)
5'- G A A T T G -3'
3'- C T T A A G -5'
Xba1 (X)
5'- T C T A G A -3'
3'- A G A T C T -5'
Spec1 (S)
5'- A C T A G T -3'
3'- T G A T C A -5'
Pst1 (P)
5'- C T G C A G -3'
3'- G A C G T C -5'

X and S have same sticky ends

Transformation of Plasmids into Competent Cells – UW Madison – 2009
http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

The process consists of growing cells to mid-log stage, harvesting, and performing multiple washes with sterile 10% glycerol to remove salts which interfere with electroporation.

General Considerations:
Keep everything cold, on ice
Glycerol pellets are not firm; try to remove as much supernate as possible, but be careful not to lose the pellet
All containers that come in contact with cells should be sterile
Keep centrifuge bottles dedicated for making Electrocompetent cells
Have 1 liter of 10% sterile glycerol chilled on ice, to less than 4C... or in a cold box overnight.
Keep manipulation of cells to a minimum, be gentle.
Resuspend pelleted cells using a sterile plastic pipette. Work quickly.
Harvest cells at 0.6 – 0.75 O.D. (A600nm)

A) Fermentation (Inoculum)
Streak for single colony from -70C glycerol stock
Start 50 ml, No Salt LB inoculum, 37C, overnight
Fermentation
Use 25 ml of the above Inoculum per liter of No Salt LB media (prewarm media to 37C)
Grow at 37C, shake at approximately 200 rpm
Grow to 0.6 – 0.75 O.D. (A600nm)......transfer to ice immediately to chill

B) Processing
1) Spin the chilled culture at 8,000 rpm, 10 minutes, 2 degrees C (use four 250 ml centrifuge bottles). Remove the supernate carefully. Save the pellets.
2) Resuspend all four pellets in a total volume of 200 ml cold 10% glycerol. Combine all resuspended pellets in one 250 ml centrifuge bottle.
3) Spin at 8,000 rpm, 10 minutes. Remove the supernate carefully.

4) Resuspend pellet in 150 ml cold 10% glycerol.
5) Spin at 8,000 rpm, 10 minutes. Remove the supernate carefully.
6) Resuspend pellet in 100 ml cold 10% glycerol.
7) Spin at 8,000 rpm, 10 minutes. Remove the supernate carefully.
8) To the pellet, add 2 ml 10% glycerol. Resuspend carefully with a 1 ml Pipetteman.
9) Transfer 110 ul of resuspended cells into cold***(-70C) 1.5 ml microcentrifuge tubes.
10) Transfer immediately to a -70C freezer (Do not use liquid nitrogen).
11) Freeze overnight before using cells.

The microcentrifuge tubes should be in a plastic tray, having been stored overnight at -70C freezer. Remove the tray and tubes from the -70C freezer immediately prior to aliquoting cells into the microcentrifuge tubes.

Transformation of Plasmids into Competent Cells – UW Madison – 2009
http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

1. Clean cuvettes with 95% Ethanol and UV in hood for about a minute, set on ice
2. Let competent cells and plasmids thaw on ice
3. Mix in cuvette:
40 μL of competent cells
1/3 – 1 μL plasmid
4. Let sit on ice for 5 min
5. Set Electroporator to ECORI and MS, align cuvette correctly, and shock
Should get milli second shock between 4 - 6 ms
ARC – too high of concentration of electrolights (plasmids)
6. Immediately after shock, add 950 μL of LB and mix well
7. Transfer to centrifuge tube
8. Let grow in water bath at 37 C (expressing antibiotic proteins)
Amp – 40 minutes
Cm – 1 hour
Kam - 1 hour
9. Plate 50 μL of cells, make 100x and 10,000x dilutions (3 plates)
Circle in center of plate, do not get sample in edges

Restriction Enzyme Mapping – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis

Use smallest micro cuvettes and mix (total volume 18 μL):
1.8 μL Buffer (10x)
0.18 μL BSA +3 (100x)
12 μL MiliQ H20
3 μL DNA
1 μL Enzyme (100x)
Incubate in water bath at 37 C for 2 hours:
3.6 μL Dye (6x)

Plasmid Preparation by Boiling Lysis – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis
Source
Hybrid of Maniatis and Qiagen
Materials
Qiagen PCR purification kit
Isopropanol
Sodium Acetate (2.5 M, pH 5.2)
STET
(10 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM EDTA (pH 8.0), 5% (v/v) Triton X-100)
Lysozyme (10mg/ml in 10 mM Tris-HCl, pH 8.0) Make Fresh each time
Protocol
1. Spin down cells (4-6 ml of overnight is fine)
2. Set up boiling water bath.
3. Prepare fresh lysozyme mix.
4. Aspirate cell pellet when centrifugation is complete.
5. Resuspend cells in 350 µl of STET, do so quickly by vortexing.
6. Make sure boiling bath is ready.
7. Add 25 ul of lysozyme to the resuspended pellet.
8. Set timer for 40 seconds.
9. Place cells in bath for exactly 40 seconds.
10. Centrifuge at max speed for 15 minutes at room temp.
11. Prepare fresh 2.0 ml tubes for each sample
12. Transfer the supernatant to a fresh tube.
13. Precipitate the nucleic acids by adding 40 µl of 2.5 M sodium acetate (pH 5.2), and 420 µl of isopropanol.
14. Mix by lightly vortexing, and let stand at room temp for 5 minutes.
15. Pellet the nucleic acids by centrifuging at maximum speed for 10 minutes at 4 oC.
16. Remove all isopropanol, by aspirating and then speed-vacing for 10 minutes.
17. Resuspend in 50-100 µl of Buffer P1 with RNase H to remove RNA.
18. Run PCR purification kit to clean up plasmid. Maniatis uses ethanol washes, and resuspension in RNase A containing TE.

Plasmid Preparation by Alkaline Lysis – UW Madison – 2009 http://2009.igem.org/Team:Wisconsin-Madison/Plasmid_Preparation_by_Alkaline_Lysis
Solution 1:
50 mM Glucose
25 mM Tris Cl (pH = 8)
10 mM EDTA (pH = 8)
Solution 2:
0.2 M NaOH (10 M)
1% SDS
Solution 3:
60 mL Potassium Acetate (5 M)
11.5 mL Glacial Acetic Acid
28.5 mL Water

Procedure:
1. Pellet the overnight culture(s) in a 1.5 ml or 2ml eppendorf tube.
2. Resuspend each pellet in 200 µl Alkaline Lysis Sol I, RnaseA added (final RNase A concentration should be 20 µg/mL)
3. Add 400 µl Alkaline Lysis Sol II. Invert 4-6 times to mix. Do not allow reaction to lyse for more than 5 min. Sample should clarify.
4. Add 300 µl Alkaline Lysis Sol III. Invert 4-6 times to mix. Sample should have a white precipitate.
5. Add 100 µl chloroform. Do this in a fume hood. Invert 4-6 times to mix.
6. Rest on ice for 5-10 minutes. This step is so that the chloroform does not get too hot in the centrifuges and leak out of the tubes. If you want to skip this step you might consider using less chloroform.
7. Centrifuge at max. speed (14,000 rpm) for 10 minutes.
8. Pipet 750µl of supernatant/aqueous layer into a fresh tube.
9. Add 1/10 volume (75µl) 3M NaOAc, pH 5.2. Vortex/flick to mix.
10. Add 0.7-1.0 Volume COLD isopropanol. Vortex/flick to mix. If in a hurry go straight to step 11, otherwise rest on ice for 10-30 minutes. I have even let it precipitate overnight at 4°C if convenient.
11. Centrifuge at max. speed for 25 min. Most miniprep protocols say to do this at 4°C, but I have not noticed decreased yield by centrifuging at room temp.
12. Remove and discard the supernatant. Don't disturb the pellet. Sometimes I can't see a pellet, and more often than not I still have DNA.
14. Add 1ml of 70% EtOH (at room temp.). Invert 4-6 times to rinse the tube.
15. Centrifuge at max speed for ~5 minutes. Room temp. is fine. Remove and discard the EtOH.
16. Repeat steps 14 and 15 to remove all traces of isopropanol. Pulse spin after removing bulk of final EtOH wash and pipet off remaining EtOH.
17. Air dry the pellet for ~15 minutes (pellet will change from white to clear as it dries). Resuspend in desired volume of H2O or T10E1, depending on downstream applications

Polymerase Chain Reaction (PCR) – Wash U – 2009
http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Polymerase Chain Reaction is used to amplify a small amount of purified DNA by several orders of magnitude. The key to PCR is thermal cycling which consists of three major steps which are repeated a number of times. The first is Denaturation which heats the DNA and splits it into two single stranded pieces. Next is the Annealing step where primers bind to each single stranded fragment. Finally Elongation takes place and Taq polymerase builds the entire strand of DNA from the primers. The process is exponential since each single strand of DNA becomes its own double strand creating a doubling effect. Only a few rounds of PCR are necessary to create large amounts of stock DNA. Note: This procedure assumes that you are starting with purified DNA.
Materials
5 uL AccuTaq LA 10X Buffer
2.5 uL dNTP mix
x uL Template DNA (about 40ng/uL)
1 uL DMSO
1 uL Forward primer
1 uL Reverse Primer
0.5 uL AccuTaq LA DNA Polymerase
dI H20 to 50 uL
Procedure
Begin by combining the top 8 ingredients in a microcentrifuge tube-add water until the volume reaches 50 uL.

Mix gently and briefly centrifuge to collect all components to the bottom of the tube. Add 50 uL of mineral oil to prevent evaporation.
Before thermocycling beings start with one period of 30 seconds at 98C
The following 3 step thermocycle should be repeated up to 30 times: 1) Denaturation 94C for 15 seconds 2) Annealing 65C for 20 seconds 3) Extension (Elongation) 68C for 20 minutes.
Finish cycling with 68C for 10 minutes.
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Transformation – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Transformation is the genetic alteration of a cell synthetically. In this case, plasmids are used to carry desired genes and coding sequences which are then taken up by the bacterial cells. Once in the cells, the plasmids are transcribed and translated into proteins in the same way as the host's genomic DNA. Transformations allow us to easily express things like antibiotic resistance and green florescent protein (GFP) in desired cells.
Materials
dI H20
Competent cells
Plasmid DNA (to be taken up by cells)
SOC media
LB agar
Antibiotics (vary depending on DNA used and are in LB plates)
Procedures
Start thawing the competent cells on crushed ice.
Add 50 µL of thawed competent cells and then 1 - 2 µL of the resuspended DNA to the labelled tubes. Make sure to keep the competent cells on ice.
Incubate the cells on ice for 30 minutes.
Heat shock the cells by immersion in a pre-heated water bath at 42ºC for 60 seconds. A water bath improves heat transfer to the cells.
Incubate the cells on ice for 5 minutes.
Add 200 µl of SOC broth (make sure that the broth does not contain antibiotics and is not contaminated)
Incubate the cells at 37ºC for 2 hours while the tubes are rotating or shaking. Important: 2 hour recovery time helps in transformation efficiency, especially for plasmids with antibiotic resistance other than ampicillin.
Label two petri dishes with LB agar and the appropriate antibiotic(s) with the part number, plasmid, and antibiotic resistance. Plate 20 µl and 200 µl of the transformation onto the dishes, and spread. This helps ensure that you will be able to pick out a single colony.
Incubate the plate at 37ºC for 12-14 hours, making sure the agar side of the plate is up. If incubated for too long the antibiotics start to break down and un-transformed cells will begin to grow. This is especially true for ampicillin because the resistance enzyme is excreted by the bacteria, and inactivate the antibiotic outside of the bacteria.
Note: Restriction sites E=EcoR1-HF; X=Xba1; S=Spe1; P=Pst1; M=Mixed Site
To view the full BioBrick Manual procedures, please click here.
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Miniprep – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
DNA Miniprep procedure is used to extract and isolate plasmid DNA from whole bacterial cells. This Miniprep kit was obtained from Sigma-Aldrich and exact solutions and formulas are unavailable (e.g. Wash Solution 1). To get exact ingredients please contact Sigma-Aldrich.
Materials
Wash Solution 1
Wash Solution 2

dI H20
Column Prep Solution
Lyse Solution
Elute solution
Resuspension Solution
RNase A solution
Lysis Buffer
Neutralization/Binding Buffer
GenElute HP Binding Columns
Procedures
Harvest Cells: Pellet 1-5mL of E. coli by centrifugation at 12,000RPM for 1 minute and discard supernatant.
Resuspend Cells: Resuspend cells with 200uL of the Resuspension Solution containing RNase A solution.
Cell Lysis: Lyse the resuspended cells by adding 200uL of Lysis buffer. Immediately mix by inverting the tube 6-8 times (Do Not Vortex). Do Not Allow Lysis to last longer than 5 minutes.
Neutralization: Precipitate the cell debris by adding 350uL of the Neutralization/Binding buffer and mix by inverting the tube several times. Centrifuge at 12,000RPM for ten minutes. Cell debris, proteins, lipids, SDS and chromosomal DNA should fall out of solution.
Prepare Column: Insert a GenElute Miniprep Binding Column into a microcentrifuge tube. Add 500uL of Column Preparation solution to each column and centrifuge at 12,000RPM for one minute and discard flow through liquid.
Load Cleared Lysate: Transfer the cleared lysate from step 4 to the column and centrifuge at 12,000RPM for 1 minute and discard flow through liquid.
Wash Column with Wash Solution 1: Add 500uL of Wash Solution 1 to the column and centrifuge at 12,000RPM for 1 minute and discard the flow through liquid.
Wash Column with Wash Solution 2: Add 750uL of Wash Solution 2 to the column and centrifuge at 12,000RPM for 1 minute and discard the flow through liquid
Centrifuge at 12,000RPM for 1 minute to remove excess ethanol.
Elute DNA: Transfer column to fresh collection tube. Add 50uL of Elution Solution to the column. centrifuge at 12,000RPM for 1 minute. DNA is now present in the eluate and ready for immediate use or storage at -20C.
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BioBrick Assembly – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
BioBrick Assembly is a standard protocol for combining two BioBrick parts and positioning them in a destination plasmid. Specific cut sites for standard restriction enzymes are utilized to simplify the protocol and include EcoR1-HF, Xba1, Spe1 and Pst1. The assembly consists of two major parts: digestion and ligation. Note that BioBrick assembly requires purified, isolated DNA for all parts involved (upstream, downstream and destination plasmid). To view the full Biobrick Assembly manual, please click here.
Materials
NEBuffer 2
BSA
Restriction Enzymes (EcoR1-HF, Xba1, Spe1, Pst1)
dI H20
10X T4 DNA Ligase Reaction Buffer
10X T4 DNA Ligase
purified upstream BioBrick part
purified downstream BioBrick part
purified destination plasmid
Procedures

Begin by thawing all DNA along with NEBuffer 2 and BSA.
Label three separate PCR tubes upstream, downstream, and destination plasmid. To each tube 500ng of respective DNA and dilute to 42.5uL. Add 5uL of NEBuffer 2 and 0.5uL of BSA to each tube.
Add 1uL of the first appropriate enzyme to each tube (see chart for correct enzyme). Then add 1uL of second appropriate enzyme to each tube.
Flick the tubes to mix contents and microcentrifuge for a few seconds to collect liquid in bottom of tube.
Incubate the three digests at 80C for 20 minutes to deactivate the restriction enzymes. The digestion portion of the procedure is now completed. You may wish to store the products at -20C or proceed directly to the ligation step.
Begin ligation by thawing 10X T4 DNA Ligase Reaction Buffer and enzyme. Agitate the buffer until all precipitate goes into solution.
Add 11uL of H20 to a 200uL PCr tube.
Add 2uL of each digest product to the new PCR tube.
Add 2uL of 10X T4 DNA Ligase Reaction Buffer to the PCR tube, then add 1uL of the T4 DNA Ligase to the tube.
Flick the tube to mix contents and spin the tube in a microcentrifuge for a few second to collect the liquid.
Incubate at room temperature for 10 minutes followed by 80C for 20 minutes. This will deactivate enzymes and improve transformation efficiency.
The ligation step is now completed. You may wish to store the products at -20C or begin a transformation immediately.
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Digestion – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Digestion cuts the DNA at very specific sites with restriction enzymes. This procedure is required for various DNA manipulation techniques including gel electrophoresis and BioBrick assembly. Note: The procedures from this point forward assume that DNA has been amplified and purified. If this is not the case, please read the Polymerase Chain Reaction procedure for amplification and the Mini-Prep procedure for purification.
Materials
NEBuffer 2
10x BSA
dI H20
Upstream, Downstream, and Destination Plasmid parts
Restriction Enzymes (varies depending on digestion)
Procedures
Begin by thawing the upstream, downstream, and destination plasmid parts along with the NEBuffer 2 and BSA.
In three separate PCR microcentrifuge tubes labeled upstream, downstream, and destination, add 750ng-1000ng of the respective dried DNA and dilute with dH20 to 38 uL.
Add 5 uL of NEBuffer 2 and 5 uL of 10x BSA to each tube.
Add 1 uL of the first appropriate enzyme to each tube. Then add 1 uL of the second appropriate enzyme.
Flick each tube to mix reagents and incubate at 37C for 1 hour.
Transfer the tubes to an incubator set at 80C for another 20 minutes. This step will deactivate the restriction enzymes.
Digestion is now finished and products should be stored at -20C or proceed to Ligation.
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Ligation – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Ligation formally joins two or more pieces of DNA together that are already annealed. Note: this procedure requires the products of a successful digestion.
Materials

DNA Ligase
10X T4 DNA Ligase Buffer
dI H20
Upstream, Downstream, and Destination Plasmid parts
Procedures
Thaw the 10X T4 DNA Ligase Reaction Buffer and mix to dissolve the precipitate.
Add 11 uL of dH2O to a 200 uL PCR tube. Then add 2 uL of each of the digestion products (upstream, downstream and destination) together into this new tube.
Add 2 uL of 10X T4 DNA Ligase Reaction Buffer to the 200 uL PCR tube.
Add 1 uL DNA Ligase to the PCR tube and flick to ensure the contents are mized.
Let the mix stand for 1 hour at room temperature before incubating at 80C for 20 minutes (deactivates enzymes).
Store the products at -20C until they are needed for a transformation.
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Gel Electrophoresis – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Gel Electrophoresis is a technique used to separate out DNA according to its length. DNA is loaded into a well, or hole in an agarose gel, and is then pulled through the gel via electric current (DNA has a negative charge). Smaller DNA fragments travel faster through the gel matrix while larger fragments travel slower. To better determine the length of DNA a ladder is often run parallel to an unknown piece of DNA. The ladder consists of several pieces of DNA of known length which serve as a reference point to the unknown fragment. Note that dyes are important in this procedure since DNA alone is invisible. Specific Dyes are needed to adhered to and travel with the DNA through the gel.
Materials
Agarose
1X TAE Buffer
Ethidium Bromide
DNA Ladder
DNA Samples
Gel Pouring Dock
Gel Tray
Gel Box
Power Supply
Procedures
Scale out agarose for gel (0.8% - 2.0%)
Add agarose powder to appropriately sized glassware
Add 1X TAE Buffer
Microwave until boiling
Allow solution to cool until glassware is comfortable in hand
Add 2ul ethidium bromide and swirl
Pour gel solution into level gel tray mounted on the gel pouring dock
Insert gel comb with appropriate number of wells
Allow gel to set up for approximately 20 minutes
Remove gel from pouring dock and submerge just below surface of 1X TAE buffer in gel box
Carefully remove gel comb
Load DNA ladder and samples
Add 3ul of ethidium bromide to the buffer near the positive electrode
Attach gel box electrodes to power supply and apply desired voltage
Add another 3ul of ethidium bromide to the buffer near the positive electrode when halfway to completion
Switch power supply off when gel has run to completion

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PCB Extraction – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Phycocyanobilin (PCB) is a blue phycobilin and is essential for the final testing of our project once implanted into *Rhodobacter sphaeroides*. It is most easily obtained from Spirulina, a cyanobacteria and popular dietary supplement.
Materials
Spirulina Powder
dI water
Methanol
HgCl2
Trifluoroacetic acid (TFA)
Trichloroacetic acid (TCA)
Acetonitrile
Procedures
Rehydrate Spirulina powder in dI water (30ml/g dry weight) for 10 min.
Centrifuge at 30,000xg for 20 min, decant and save supernatant.
Precipitate phycocyanin from supernatant with 1% (w/v) TCA by incubation for 1h at 4oC in the dark.
Collect by centrifugation at 30,000xg for 20 min.
Wash with methanol (2x 20ml/g Spirulina powder).
Resuspend blue pellet in methanol (2ml/g powder) containing 1mg/ml HgCl2.
Incubate for 20h at 42oC in the dark.
Remove protein by centrifugation at 10,000xg for 10 min.
Precipitate mercuric ion with 2-Mercaptoethanol (1ul/ml), remove by centrifugation at 30,000xg for 10 min.
Dilute bilin mixture 10-fold with 0.1% (v/v) TFA.
Apply to C18 Sep-Pak cartridge.
Wash cartridge with 0.1% (v/v)TFA (2x 3ml) and acetonitrile/0.1% TFA (20:80; 2x 2ml).
Elute bilin with 3ml acetonitriel/0.1% TFA (60:40) and dry in vacuo.
Note:Expected yields are 4 umol PCB per 6g Spirulina
Thanks to Dr. Clark Lagarias for providing this protocol. ref
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Glycerol Stock Solution – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Glycerol Stock Solutions are excellent for preserving and freezing cell cultures. It is very important that cells not be damaged or continue to grow too fast for various parts of our project.
Materials
Centrifuge/screw top tubes
50% glycerol sol
LB Cultures
Procedures
Label centrifuge/screw top tubes
Pipet 300 µl of 50% glycerol solution into each tube
Pipet 700 µl of LB culture into each tube
Close & vortex to mix.
Put in -80 C.
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Designing Primers – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
We found the Registry's protocol extremely helpful for designing primers and constructed most of our primers from this template. To visit the website, please clickhere.

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Colony PCR – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Colony PCR is similar to regular PCR in that it is used to amplify DNA. We have used colony PCR to verify successful transformations by adding grown up cells into the PCR mix, lysing the cells, and then amplifying the DNA. The amplified DNA can then be run through a gel and the number of base pairs can then be compared to an expected result.
Materials
0.5uL AccuTaq LA DNA Polymerase
5uL 10x Buffer
2.5uL dNTP mix
1 uL DMSO
0.5 uL BioBrick forward Primer
0.5 uL BioBrick reverse Primer
0.5 uL Enzyme
40 uL dI H20
LB agar plate
E. coli colony to amplify.
Procedures
Create the PCR mix by combining the first 8 ingredients.
Touch the colony of E. coli with a sterile plastic loop and do a small streak across a new plate. Swirl this tip in the PCR mix.
Begin process of thermocycling with a heat shock of 98C for 30.
Next begin the cycling steps with 94C for 15 seconds, then 71C for 20 seconds, then 68C for 5 minutes. Repeat these three steps thirty times.
Move solution to 68C for 20 minutes.
PCR is now complete and should be held at 4C.
Note: any further streaking should be done with a sterile tip.
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Genomic DNA Purification – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Characterization and modeling of individuals parts of our system will require the isolation and purification of genomic DNA from *Rhodobacter sphaeroides*. One of the main goals of the project is to make BioBricks out of ompR, ompF, pucB/A and the puc promoter coding regions. This DNA however is in chromosomal, not plasmid, form and will require a separate protocol to obtain. Note: This protocol and materials were obtained in a kit called DNeasy from Qiagen. To obtain exact recipes and formulas please contact them.
Materials
Overnight cultures
Buffered ATL
Proteinase K
Buffered AL
Ethanol
DNeasy mini column
Buffer AW1
Buffer AW2
Buffer AE
Procedures
Harvest cells by taking 1.5mL from overnight cultures and spinning them down to a pellet.
Resuspend pellets in Buffered ATL
Add 20uL Proteinase K, mix by vortexing and incubate at 55C for 3 hours shaking every 20-30 minutes

Vortex 15 seconds and then add 20uL Buffer AL to the samples mixing thoroughly and incubate at 70C for 10 minutes
Add 200uL ethanol to the sample and mix by vortexing
Pipet mixture from step 4 into DNeasy mini column sitting in a collection tube. Centrifuge at 12,000RPM for 1 minute and discard the flow through in the collection tube.
Place the mini column in a new collection tube and add 500 uL of Buffer AW1. Centrifuge at 12,000RPM for 1 minute and discard the flow through and collection tube.
Place the mini column in a new collection tube and add 500 uL of Buffer AW2. Centrifuge at 12,000RPM for 3 minutes and discard the flow through and collection tube.
Place the mini column in a clean collection tube and pipet 200 uL Buffer AE on the DNeasy membrane. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at 12,000RPM.
Repeat elution as described in step 9 with same collection tube to combine both elutes.
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Gel Extraction – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
Gel Extraction is intended to recover and reuse DNA that has been purified by gel electrophoresis. The process re-isolates DNA and removes all primers, dyes, and ethidium bromide that may still by present in the band of DNA in the gel, leaving up to 95% of the original DNA. We will be using the GenElute Gel Extraction Kit from Sigma-Aldrich so individual formulas and solutions may be unattainable. Please contact them for more details.
Materials
gel with desired DNA band
Column preparation solution
Gel Solubilization Solution
Wash Solution Concentrate G
Elution Solution
GenElute Binding Column G
spin columns
Ethanol
Isopropanol
3M Sodium Acetate Buffer (pH 5.2)
Procedures
Dilute the Wash Solution Concentrate G with 48mL of ethanol.
Cut out the desired band of DNA from the gel leaving as little excess gel as possible.
Weight the excised gel and place into a microcentrifuge tube. Add 3 gel volumes of Gel Solubilization Solution to the tube (for every 100mg of gel, add 300uL of solution). Incubate tube at 60C for 10 minutes or until gel is completely dissolved. Vortex occasionally to ensure gel is evenly dissolved.
Prepare the binding column by adding 500uL of Column Preparation Solution to the column and centrifuging for 1 minute. Discard the flow through.
Check the color of the tube containing the dissolved gel. If the solution is red, add 10uL of 3M Sodium Acetate to the mix until the solution turns yellow.
Add 1 gel volume of 100% isopropanol and mix (may need slightly more isopropanol for gels greater than 2%).
Load the gel solution into the binding column and centrifuge for 1 minute and discard the flow through.
Add 700uL of Wash Solution to the column and cetrifuge for 1 minute and discard the flow through.
Centrifuge the column again for 1 minute to remove excess ethanol.
Transfer the column to a new collection tube. Add 50uL of preheated Elution solution (65C) to the column and centrifuge for 1 minute. The desired DNA is now in the collection tube.
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Making Competent Cells – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures

Competent cells are necessary for performing transformations. This protocol prepares cells to take up new, foreign plasmids. Note that different cells may require slightly different preparatory protocols.
Materials
SOB media
SOC media
CCMB80
Glycerol
Procedures Preparing Seed Stocks
Streak cells on an SOB plate and grow for single colonies at 23C. Pick single colonies into 2mL of liquid SOB media and shake overnight at 23C. Add to 15% glycerol. Aliquot in 1mL samples and place in a -80C freezer to store indefinitely. Note competent cells lose efficiently every time they are frozen and then refrozen.
Preparing Competent Cells
Innoculate 250mL of SOB media with 1mL vial of seed stock and grow at 20C to an Optical Density (OD) of 0.3 for approximately 16 hours.
Centrifuge at 3,000g at 4C for 10 minutes and discard liquid. Resuspend the cells in 80mL of CCMB80 buffer and incubate on ice for 20 minutes.
Centrifuge again at 4C at 3,000g for 10 minutes. Discard the solution and resuspend the cells in 10mL of CCMB80 buffer. Test OD of a mixture of 200uL SOC and 50uL of the resuspended cells. Add chilled CCMB80 to yield a final OD of 1.0-1.5.
Incubate on ice for 20 minutes and then aliquot to 2mL vials and store indefinitely at -80C.
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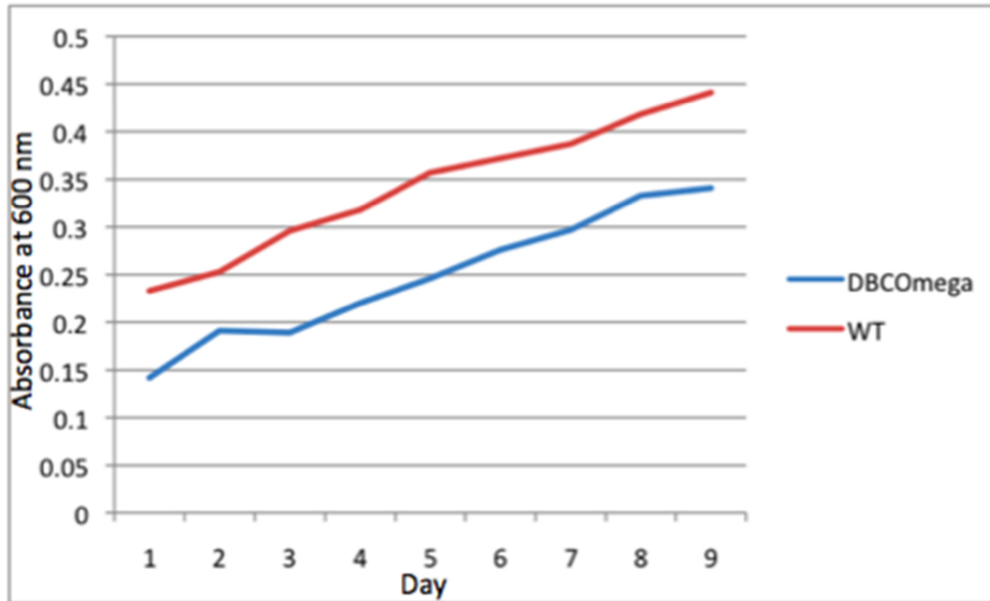
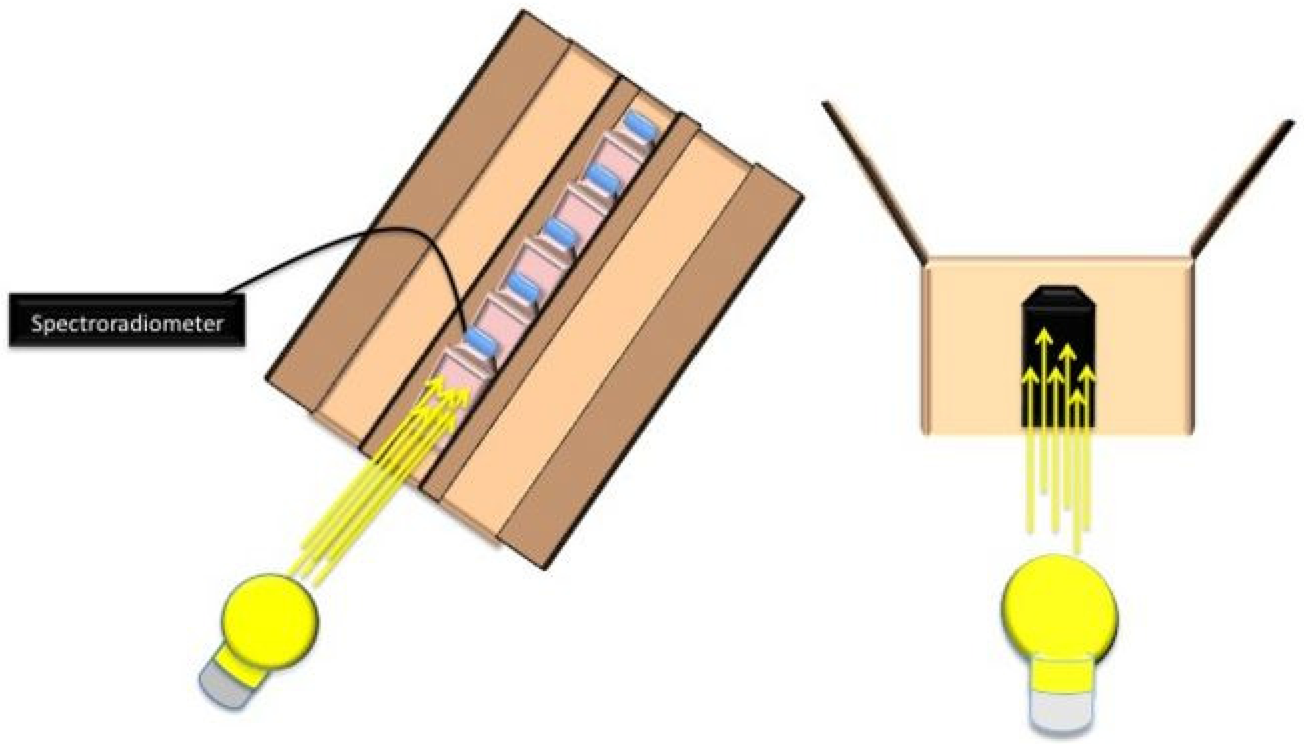
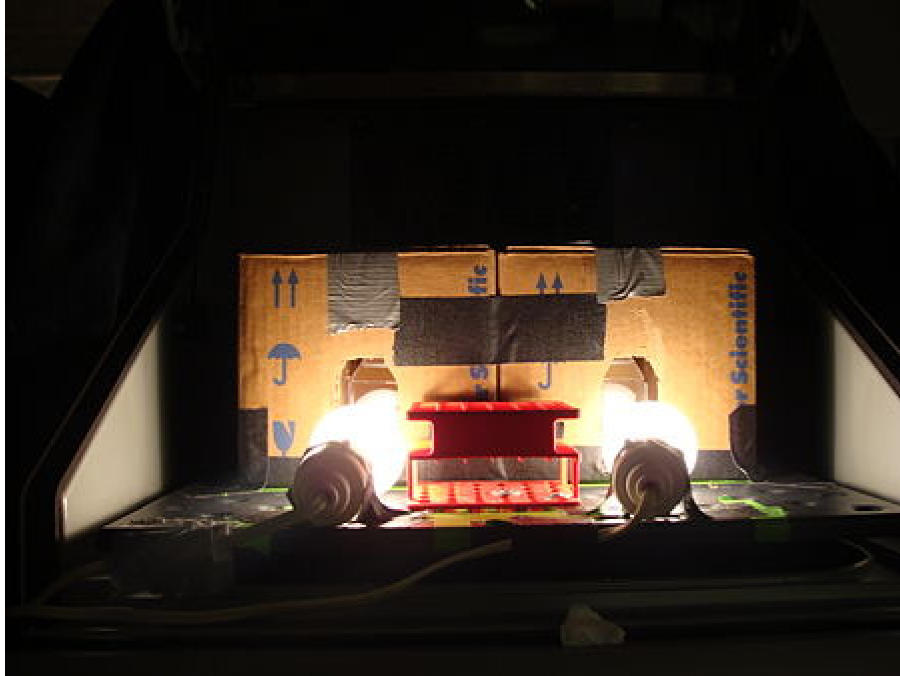
Tissue Flask Experiment – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
This experiment to monitor the growth and spectra of Rhodobacter shaeroides under anaerobic, photosynthetic conditions was performed as follows:
-To ensure optimal growth conditions, an Excella E24 incubator shaker was used to maintain a temperature of 34° C and shaking at 160 rpm and the lid of the incubator was propped open 0.5 cm to prevent overheating. The incubator was covered by a black, opaque cloth to prevent external light from influencing culture growth. Tissue flasks were enclosed by 2 growth boxes inside the incubator, which allowed light to enter only through a tissue flask sized opening on the end nearest the light source. These boxes had the dimensions: 34.8 cm x 18.3cm x 16.8cm.
-5 BD Falcon ™ 50 ml 25 cm² Cell Culture Flasks with blue plug-seal screw caps were used to grow cultures within each box. Cell culture flasks were arranged in series with the first flask recessed 2.5 cm from of box opening and with 2.5 cm separations between each flask. The cell culture flasks were flanked on each side by an opaque barrier so that light incident on each flask was either from the light source, in the case of the 1st flask, or from the light transmittance from the preceding flask in the series
-A constant light source was centered in front of each of the growth boxes The Light source was a 40 watt incandescent light bulb located 10.8 cm from the growth box opening, as measured from the closest point to the box.
-Tissue culture flasks were inoculated with R. sphaeroides cultures in the exponential growth phase from a 50 ml inoculation culture grown at 34° C in the dark in M22 liquid media.
-The test tissue culture flasks were inoculated with Volume = 0.478ml / OD600nm of inoculation culture



-M22 liquid media was added to flasks for a final volume of 65ml and just after inoculation each flask had OD600nm = 0.011
-Cell culture growth was monitored by measuring OD600nm daily at a standard time for each tissue culture flask. To conduct this measurement,1ml of culture was extracted from each flask for measurement and 1ml M22 liquid media was added to each flask to replace the extracted volume and to ensure minimal oxygen exposure to the culture by reducing headroom.

-Spectroradiometer readings were taken with an Ocean Optics QP-600-1-UV-Vis EOS25354-2 optic cable transmitting data to an Ocean Optics HR2000CG-UV-NIR High-Resolution Spectrometer. Absolute irradiance and transmittance data were recorded in front of the first flask, then behind this flask and each subsequent flask. The absolute irradiance data were calibrated to a Mikropack HL-2000 Halogen Light Source. The transmittance data were recorded relative to the reading at the front of the first flask.


Spectroradiometer



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Recipes
Luria Broth (LB) Media – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
10g Tryptone
5g Yeast Extract
10g NaCl
15g Agar
up to 1 Liter H20
adjust pH to 7.5
ATCC medium: Van Niel's yeast agar – Wash U – 2009
http://2009.igem.org/Team:Wash_U/Protocol#Procedures

1.0 g K_2HPO_4
0.5 g $MgSO_4$
10.0 g yeast extract
20.0 g agar
1.0 L tap water
Adjust pH to 7.0-7.2
Super Optimal Broth (SOB) – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
2% w/v bacto-tryptone (20g)
0.5% w/v bacto-yeast extract (5g)
8.56mM NaCl (0.5g) or 10mM NaCl (0.584g)
2.5mM KCl (0.186g)
ddH_2O to 1L
Super Optimal Broth with Catabolite repression (SOC) – Wash U – 2009
http://2009.igem.org/Team:Wash_U/Protocol#Procedures
In addition to all of the SOB components,
10mM $MgCl_2$ (0.952g)
20mM glucose (3.603g)
50X TAE Buffer – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
242 g Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) (= 2 mole)
57.1 ml glacial acetic acid (= 100% acetic acid) (57.19 ml = 1 mole)
100 ml 0.5 M Na_2 EDTA (pH 8.0)
ddH_2O to 1L
5X TBE Buffer – Wash U – 2009 http://2009.igem.org/Team:Wash_U/Protocol#Procedures
53 g of Tris base (CAS# 37186)
27.5 g of boric acid (CAS# 11280)
20 ml of 0.5 M EDTA (CAS# 60004) (pH 8.0)
ddH_2O to 1L
Tris EDTA (TE) buffer 5X 1L – Wash U – 2009
http://2009.igem.org/Team:Wash_U/Protocol#Procedures
750 mL d-H20
242 g Tris base
57.1 mL glacial acetic acid
100 mL 0.5M EDTA (93.05 g EDTA in 500 mL d-H20, pH ~8.0)
fill to 1 L
adjust pH to 8.5
Agarose Gel (for electrophoresis of DNA >100bp) – Wash U – 2009
http://2009.igem.org/Team:Wash_U/Protocol#Procedures
50 mL 1X TAE buffer
0.8 g agarose
2.5 microliters EtBr (Caution: EtBr is a known carcinogen)
Note: Jacob suggested adding 1.0 microliter EtBr to gel and 3.0 microliters to TAE buffer in rig.
CCMB80 Buffer
10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
80 mM $CaCl_2 \cdot 2H_2O$ (11.8 g/L)
20 mM $MnCl_2 \cdot 4H_2O$ (4.0 g/L)
10 mM $MgCl_2 \cdot 6H_2O$ (2.0 g/L)
10% glycerol (100 ml/L)
adjust pH DOWN to 6.4 with 0.1N HCl if necessary
Note: adjusting pH up will precipitate manganese dioxide from Mn containing solutions.
sterile filter and store at 4°C
slight dark precipitate appears not to affect its function

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Bacterial Transformation – Utah State – 2009
http://2009.igem.org/Team:Utah_State/Notebook#protocols
Once the target DNA has been successfully ligated into the plasmid vector, the plasmid must be transferred into the host cell for replication and cloning. In order to do this, the bacterial cells must first be made "competent." The term "competent" is to describe a cell state in which there exist gaps or openings in the cell wall which will allow the plasmid containing the target genes to enter into the cell. Several methods to make bacterial cells competent exist, such as the calcium chloride method and electroporation. Competent cells may also be purchased commercially. The team at USU has purchased competent ce lls for all experiments. The following is the method used by the USU team to insert the plasmids containing various biobricks into the cells.
Method

Ensure the necessary antibiotic agar plates have been prepared or begin their preparation now. Four plates per transformation will be necessary (two today, then two tomorrow for streaking). Also ensure that 10 ml liquid media is made up per transformation (also for tomorrow).
If using Biobrick parts from iGEM distribution, use registry to identify appropriate will containing plasmid of interest and proceed to step 3, if using other DNA proceed to step 5.
Add 10ul of sterile water to distribution well to dissolve DNA. Remove 10ul and place in 0.5ml bullet tube. Label tube with part number, use 2ul to transform and save the other 8ul in the BioBrick part box.
Take competent cells from the -80˚C freezer and place on an ice bath.
Add 2 µl of the DNA solution (or 4ul of ligation reaction) to the competent cells. Ensure the pipetting is done directly into the cell solution. Let cells incubate on ice for 30 minutes. Heat water bath to 42˚C.
Heat shock cells in the 42˚C water bath for 30 seconds. Remove and place back in the ice bath for 2 minutes.
In the hood, add 250 µl SOC media to each tube, bringing the total cell solution to 300 µl. Incubate at 37˚C for 1 hour.
Add 200 µl of each transformed cell solution to the appropriate antibiotic plate. Use the Bunsen burner to create a "hockey stick" out of a glass pipette tip by holding over the flame until it bends. Allow to cool.
Spread cell solution uniformly over the agar plate using the "hockey stick," then before discarding, spread residual solution on the "stick" over a second plate to get more a more sparse colony distribution.
Parafilm all plates and place in 37˚C incubator 12-14 hours, or overnight if that is not possible.

Streak Plates and Liquid Cultures from Transformed Colonies – Utah State – 2009
http://2009.igem.org/Team:Utah_State/Notebook#protocols
After bacterial cells have been transformed, successfully transformed cells must be selected. Because 100% of the cells do not receive the desired plasmid and target gene, it is essential to select for cells that do have the target genes. The USU team uses antibiotic resistance to select for successful transformations. To do this, an antibiotic resistance gene is also added to the plasmid vector that contains the target genes. By doing so, it is possible to know that a cell was successfully transformed based on its ability to grow on an agar plate with antibiotics added. Because the cell is able to grow, the antibiotic resistance gene must be present as well as the target gene. From the agar plates containing the antibiotics, a colony is picked and transferred into a liquid culture for further analysis. The following is the method used by USU to clone the DNA and select for the successful transformation of various BioBricks in E.coli.
Method

Prepare two 15 ml tubes per transformation, each with 5 ml media containing the appropriate antibiotic. Use a pipette tip to extract half of each colony and inoculate one agar plate per colony. Using a pipette with a tip, extract the other half of each colony and inoculate one liquid media tube per colony. Label all tubes and plates and place in the 37˚C incubator until the next morning.

Plasmid DNA Isolation – Utah State – 2009 http://2009.igem.org/Team:Utah_State/Notebook#protocols
Following successful bacterial cloning and isolation, it is important to verify that the target gene is in the cell and that the resultant plasmid is correct. To do this, it is a common practice to sequence the plasmid DNA. To obtain enough DNA for sequencing, the bacterial clones are grown in a liquid culture. The cells are harvested by centrifugation and then prepared for DNA plasmid extraction. DNA plasmid extraction can be done several ways, and the overall purpose is to lyse the cells and separate the plasmid DNA from all other cellular proteins, DNA, and debris. The following is the method used by the USU team to isolate plasmid DNA containing the various biobricks.
Method

Prepare two water baths, one boiling and the other 68C.
Centrifuge bactrerial cultures (3 to 5 ml) at 3K RPM for 20 min. Discard supernatant.
Resuspend cell pellet in 200 µl of STET buffer. Transfer to 1.5 ml tubes.
Add 10 µl of lysozyme (50 mg/ml) and incubate at room temperature for 5 min.
Boil for 45 sec and centrifuge for 20 min at 13K RPM (or until pellet gets tight).
Use a pipette tip or toothpick to remove the pellet.
Add 5 µl RNase A (10 mg/ml) to supernatant and incubate at 68C for 10 minutes.
Add 10 µl of 5% CTAB and incubate at room temperature for 3 min.
Centrifuge for 5 min at 13K RPM, discard supernatant, and resuspend in 300 µl of 1.2 M NaCl by vortexing.
Add 750 µl of ethanol and centrifuge for 5 min at 13K RPM.
Discard supernatant, rinse pellet (which cannot be seen) in 80% ethanol, and let tubes dry upside down with caps open.
Resuspend pellet in either sterile water or TE buffer.

Restriction Enzyme Digestion and Electrophoresis – Utah State – 2009
http://2009.igem.org/Team:Utah_State/Notebook#protocols
Restriction enzyme digestion is the process by which an insert DNA sequence is separated from the rest of the DNA molecule. Specific knowledge of the DNA insert is needed to determine which enzyme and conditions to use during the digestion reaction. Once the DNA sequence is known and the correct enzymes have been selected, the DNA may be digested. Listed below is the procedure used by USU to digest the plasmid DNA. After enzyme digestion, electrophoresis is used to separate the plasmid from the insert. A gel is prepared and the respective reaction mixes are loaded into the gel. Using a DNA ladder, and knowing the size of the insert, the corresponding band can be seen and cut out of the gel. The insert may then be removed and isolated from the gel, thus yielding the desired DNA. The DNA from this may then be used in PCR reactions, sequencing, ligations for further experimentation, etc. Listed below are example protocols used by the USU team for a restriction enzyme digestion and subsequent agarose gel electrophoresis.
Method

Resuspend DNA in 20 to 40 µl water, vortex, and do a brief centrifuge to get solution to the bottom of the tube.
Add components to the digestion solution in the following order: DNA (23 µl), 10X restriction enzyme buffer (3 µl), Xba1 (2 µl), and Pst1 (2 µl). The volume and restriction enzymes can be varied, but it

should be ensured that the total volume is 10X the amount of RE buffer. Tap tubes periodically and allow to digest at appropriate temperature while preparing electrophoresis gel.
Prepare electrophoresis gel by adding 2 g agarose to 200 ml TAE (1% solution). This is best done in an Erlenmeyer flask of adequate volume as swirling will need to be done. Place in the microwave and microwave on high for 20 seconds at a time, pulling it out and swirling until solution is homogeneous again, then repeating (BE CAREFUL to watch the solution closely when swirling – it superheats and can boil over and cause severe burns). Continue until solution is seen boiling in the microwave then gently swirl again.
Add 20 µl ethidium bromide to solution and swirl until dissolved evenly.
Add 6 µl of 6X loading dye to each tube of digested DNA solution.
Prepare the electrophoresis unit by orienting the basin sideways with rubber gaskets firmly against the side. Place desired well template in the basin.
When the agarose solution is cool enough to comfortably touch the flask, pour into the basin until the solution is about ¾ of the way to the top of the well template.
When the gel is solidified (should look somewhat cloudy), remove the well template and change basin orientation to have the wells closest to the negative pole (as the DNA will flow towards the positive pole).
Pour 1X TAE buffer into both sides of the electrophoresis unit until it just covers the gel and fills the wells.
By inserting the pipette tip below the TAE liquid and into the well, add 10 µl of DNA ladder solution to first (and last if desired) well, skip one well, then begin adding the digested DNA solutions to the wells by adding about 2 µl less than the total volume in the tubes to prevent air bubbles in the wells.
Place the cover on the electrophoresis unit, plug into the power source, and turn on voltage to 70 V (this can be as high as 100 V if time is an issue), and press the start button. Separation should take two to three hours. The yellow dye shows the location of the smaller nucleotide lengths and the blue dye shows the location of the larger nucleotide lengths. DNA separation can be observed as time goes on by turning off the power supply then gently removing the basin from the electrophoresis unit (be careful not to let the gel slip out of the basin) and placing on the UV transilluminator to see DNA bands. The basin can then be placed back in the electrophoresis unit for further separation if desired. Take care to not have the power supply on without the lid to the unit in place.
When the desired level of separation is obtained, the basin can be placed on the transilluminator for picture taking. Place the cone-shaped cover over the transilluminator and place the digital camera in the top hole for pictures.

Media Preparation – Utah State – 2009 http://2009.igem.org/Team:Utah_State/Notebook#protocols
For all experimentation involving the need for bacterial biomass and experimentation, proper media is needed to grow the cells. The following is the media composition and methods used by USU to prepare the media.
Add 5 g yeast extract, 10 g NaCl, 10 g Bacto Tryptone, and 15 g agar (if desired) to a 2 L Erlenmeyer flask and bring the volume up to 1 L with ddH20. Mix by swirling. Cover top with foil.
Autoclave for 45 minutes (liquid setting, 0 minutes drying time). It will take an additional half hour for the autoclave to finish cooling then an additional 20 to 30 minutes until the media is cool enough to pour.

Polymerase Chain Reaction (PCR) – Utah State – 2009
http://2009.igem.org/Team:Utah_State/Notebook#protocols
PCR is used to amplify a desired DNA sequence. The reaction is first set up by designing primers that will bind only to the desired regions of the DNA sequence. Once the primer and polymerase have been selected, the reaction parameters of time and temperature must be optimized. When the reaction works properly only the target DNA will be amplified into large quantities that may then be isolated and used for further experimentation. The following is the procedure used by USU for PCR reactions to amplify various biological parts. A useful set of primers are the universal BioBrick primers VF2 and VR that can be used to amplify almost any BioBrick part.

Method

Obtain the following reagents from the freezer: DNA template (cells or DNA), 10X Taq buffer (+KCl, -Mg/Cl2), MgCl2, 10 mM dNTP Mix, Taq polymerase (take out of freezer only immediately when needed and put back), and sterile distilled H2O. Place all reagents on ice. Also obtain PCR (either 0.2 or 0.5 ml) tubes.
Add the following reagents to a tube (50 µl reaction) in the following volumes and order:
32 µl sterile H2O,
5 µl 10X buffer,
2 µl dNTP Mix,
6 µl MgCl2
3 µl cells/DNA,
0.25 µl Taq Polymerase
1 µl primer 1
1 µl primer 2
MgCl2 volume can be varied (lower to increase specificity – just ensure total volume is 50 ul with H2O).
If many reactions are to be constructed, a master mix can be made up to cut down on time and pipette tip usage (if this is done, ensure primers are added to the appropriate reaction, i.e. perhaps not to the master mix). Tap or vortex tubes and take to the thermocyler. Place all reagents back in the -20˚C freezer.
Choose thermocycler temperatures. The Eppendorf Mastercycler will cycle between three temperatures: typical temperatures are 94˚C for denaturing, 50-60˚C for primer annealing, and 72˚C for polymerase extending. Lowering the annealing temperature decreases DNA specificity; 55˚C is a good temperature to begin if no trials have been made with the sample.
Turn on thermocycler with the switch in the back of the unit and open the lid. The placement of the tubes depends on the size of the tube (0.2 or 0.5 ml) and whether or not a temperature gradient is to be used.
If no temperature gradient will be used, tubes can be placed anywhere on the unit in the appropriately-sized hole. Select "Files" and press enter. Select "Load" and then "Standard." If cells will be used in the reaction, include a 1-minute lysing step at the beginning (step 1); this will be followed by a 1-minute DNA denaturing step (step 2). If purified DNA will be used, set step 1 to 1 second. Set an annealing temperature for step 3. Ensure the lid temperature is 105˚C and the extending temperature is 72˚C. Press exit. If prompted to save, save by pressing enter three times. Press exit to return to the main menu. Choose "Start" on the main menu and select "Standard." The program should begin.
If a temperature gradient is to be used, temperature will vary according to column. A 20˚C range is the maximum range that can be used (+/- 10˚C). The range is made by setting a temperature for the middle column and then setting a +/- range. To see what the temperatures will be if a gradient is used, select "OPTIONS" on the main menu, then select "Gradient." Select the size tube that is being used by pressing "Sel," then press enter. Choose a temperature for the center column, press enter, then select a +/- range and press enter. The column number along with the corresponding temperature is shown. Decide tube placement based on this information. Press exit twice to return on the main menu. Select "Files" then "Load," then "Gradient." If cells are being used, set the cell lysing step (step 1) to 1 minute (1:00); if purified DNA is being used, set this time to 1 second (0:01). Step 2 should be 94C, Step 4 should be 72˚C, and the lid temperature should be 105˚C. Go to step 3 and set an annealing temperature for the center column. Leave the next two lines as they are, and change the gradient setting ("G") to the +/- the center temperature amount. Press exit. If prompted to save, press enter three times; if not prompted to save, press enter once. Press exit to get back to the main menu. To begin cycle, select "Start," then select "Gradient." The program should begin.
The thermocycler is set to store the completed reaction tubes at 4˚C when finished.

Ligation – Utah State – 2009 http://2009.igem.org/Team:Utah_State/Notebook#protocols

Ligation is the process by which the insert (target DNA gene) is inserted into a plasmid. Both the plasmid and insert have been digested and have the proper “sticky” or blunt ends which are compatible for joining the two DNA pieces together into one molecule. These two DNA pieces are placed in a reaction tube and the proper DNA ligase, buffer, and cofactors are added for the reaction to take place. When done properly, the ligation will result in a successful combination of the insert and plasmid into one plasmid. This newly formed plasmid may then be isolated using gel electrophoresis and then used for bacterial transformation or other experimentation. The following is the procedure used by USU to ligate together various biobrick parts.
Method

1. Obtain the following reagents, some of which are in the -20˚C freezer: DNA vector, DNA insert, 10X ligation buffer, T4 DNA ligase (take out only when needed, then return immediately to freezer), and sterile distilled water.
2. Ideally, it is desirable to have the concentration of insert ends (or moles of insert) be two to three times the concentration of vector ends (or moles of vector), with a total DNA concentration of 50-400 ng/µl in the reaction. If determining the DNA concentration is not possible, place two to three times the volume of vector as the volume of insert in the reaction. As this is often the case, place the following reagents in a thin-walled PCR tube in the following volumes: • 10 µl insert DNA • 3 µl vector DNA • 2 µl 10X ligation buffer • 4 µl $H2O$ • 1 µl T4 DNA ligase = 20 µl total This could also be done in different volumes depending on DNA concentration/total volume desired.
3. Gently mix the tube, and place the tube in the PCR thermocyler, turn on the machine, select “Start,” from the main menu, select “22” and press “Start.” The thermocycler will keep the reaction at 22˚C.
4. Incubate for 60 minutes. Heat-inactivate by placing tubes in 68C water bath for 10 minutes. Place in the freezer if storing for later use.

Western Blot – Utah State – 2009 http://2009.igem.org/Team:Utah_State/Notebook#protocols
Western blotting is a procedure that allows for the identification of proteins using a specific antibody after protein separation on an SDS polyacrylamide gel.
Method

Collect bacterial cells by centrifugation and lyse the cells using any of a variety of procedures.
Spin at 14,000 rpm (16,000 g) in a microfuge for 10 min at 4°C.
Transfer the supernatant to a new tube and discard the pellet.
Determine the protein concentration (Bradford assay, A280, or BCA)
Mix 20 µl of sample (20ug) with 10ul of 3x sample buffer.
Boil for 1 min, ool at RT for 5 min.
Flash spin to bring down condensation prior to loading gel.
Assemble pre-prepared polyacrylamide gel into gel running rig.
Load protein samples into individual wells.
Use 10 µl of Kaleidoscope standard.
Run at 35 mA (constant current) for approximately 2hrs.
Disassemble gel when done running.
Cut a piece of PVDF membrane (Millipore Immobion-P #IPVH 000 10).
Wet for about 10 min in methanol on a rocker at room temp.
Remove methanol and add 1x Blotting buffer until ready to use.
Assemble "sandwich" for Bio-Rad's Transblot.
Sponge - filter paper - gel - membrane - filter paper - sponge
Transfer for 1 hr at 100V at 4°C on a stir plate. Bigger proteins might take longer to transfer.
When finished, immerse membrane in blocking buffer (5% nonfat dry milk)and block overnight.

Incubate with primary antibody diluted in 0.5% blocking buffer for 60 min at room temp.
Wash 3 x 10 min with 0.05% Tween 20 in PBS.
Incubate with secondary antibody diluted in 0.5% blocking buffer for 45 min at room temp.
Wash 3 x 10 min with 0.05% Tween 20 in PBS.
Detect with TMB stabilized substrate for HRP.

Site-Directed Mutagenesis – Utah State – 2009
http://2009.igem.org/Team:Utah_State/Notebook#protocols

QuikChange II Site-Directed Mutagenesis Kit (Stratagene)
Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence.
Prepare the control reaction as indicated below:
5 µl of 10× reaction buffer (see Preparation of Media and Reagents)
2 µl (10 ng) of pWhitescript 4.5-kb control plasmid (5 ng/µl)
1.25 µl (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/µl)]
1.25 µl (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/µl)]
1 µl of dNTP mix
39.5 µl of double-distilled water (ddH2O) to a final volume of 50 µl
Then add
1 µl of PfuTurbo DNA polymerase (2.5 U/µl)
Prepare the sample reaction(s) as indicated below:
Note: Set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping the primer concentration constant.
5 µl of 10× reaction buffer
X µl (5–50 ng) of dsDNA template
X µl (125 ng) of oligonucleotide primer #1
X µl (125 ng) of oligonucleotide primer #2
1 µl of dNTP mix
ddH2O to a final volume of 50 µl
Then add
1 µl of PfuTurbo DNA polymerase (2.5 U/µl)
If the thermal cycler to be used does not have a hot-top assembly, overlay each reaction with ~30 µl of mineral oil.
Cycle each reaction using the cycling parameters as outlined in Table I of the Stratagene QuikChange II Site-Directed Mutagenesis Kit manual. We used an annealing temperature of 55C for 1 min and an extension temperature of 68C for 5 min and 18 cycles.
Following temperature cycling, place the reaction on ice for 2 minutes to cool the reaction to ≤37°C. If desired, amplification may be checked by electrophoresis of 10 µl of the product on a 1% agarose gel. A band may or may not be visualized at this stage. In either case proceed with Dpn I digestion and transformation.

Dpn I Digestion of the Amplification Products – Utah State – 2009
http://2009.igem.org/Team:Utah_State/Notebook#protocols
Add 1 µl of the Dpn I restriction enzyme (10 U/µl) directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip.Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA.
Transform into XL1-Blue Supercompetent Cells and proceed as previously described.

X Gal/Beta Galactosidase Assay – Harvard – 2009 http://2009.igem.org/Team:Harvard/Protocols
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X-Gal/Beta-galactosidase assays
Solutions
Z Buffer
0.75 g KCl
16.1 g Na2HPO4 ω 7H2O
0.246 g MgSO4 ω 7H2O
5.5 g NaH2PO4 ω H2O
Bring the final volume to 1 liter using ddH2O, adjust pH to 7 and store at 4deg C.
Alternatively, for 500mls, using these reagents:
0.375 g KCl
4.27 g Na2HPO4 ω H2O
0.061 g MgSO4 ω H2O
2.75 g NaH2PO4 ω H2O
Bring the final volume to 500 mL using ddH2O, adjust pH to 7 and store at 4deg C.
Z Buffer with 2-Mercaptoethanol:
Add 270 µl of 2-Mercaptoethanol per 100 ml of Z Buffer, just prior to use.
X-gal, 100X stock solution:
100mg/ml in Dimethyl formamide
Add X-gal stock solution to Z Buffer with 2ME, to a final concentration of 1mg/ml, prior to use.

Procedure
1. For colony lifts from solid agar, nitrocellulose filters were used. After cutting an appropriately sized filter to fit the surface of a petri plate, nitrocellulose was laid on the agar surface and then removed using tweezers.
2. The filters were then placed onto an aluminum sheet that was floated on liquid nitrogen, for approx. 20 seconds. The filters were then immersed in liquid nitrogen for 2 seconds, thawed at room temperature.
3. Filter lifts were then placed cell side up onto pads of Whatman #1 filter paper, in empty petri-plates, that had been soaked with Z-buffer solution containing 2-mercaptoethanol and X-gal.
4. The filter lifts were then incubated at 30 C for 30min to overnight, until the development of a blue precipitate was clearly visible.
5. A similar approach was used in the semi-quantitative assays. Alternatively, yeast cells from liquid culture were immobilized onto nitrocellulose filter sheets using a Bio-Rad "Bio-Dot" apparatus connected to a vacuum manifold. The immobilized cells were lysed using liquid nitrogen and incubated with x-gal containing buffer as outlined above.
6. For the analysis of beta-galactosidase expression in cells grown in 96 well micro-titer dishes, cotton applicators were used to remove cells from each well and then immersed directly into liquid nitrogen. After thawing at room temperature, they were then incubated for ~30-overnight in wells containing 150 µL of X-gal buffer.

References:
Cell, Vol. 74, 205-214, July 16, 1993
Mammalian Ras interacts directly with the serine/threonine kinase raf.
Anne B. Vojtek, Stanley M. Hollenberg and Jonathan A. Cooper.

A very good overview of various beta-galactosidase activity assays performed in yeast:
http://www.foxchase.org/research/labs/golemis/betagal/beta_gal_yeast.htm

Colony PCR – Harvard – 2009 http://2009.igem.org/Team:Harvard/Protocols
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Same as 2nd link for normal PCR except:
a) Colony is streaked with a toothpick and put in tube in place of DNA
b)Use of Magnesium Chloride

Restriction Digest – Harvard – 2009 http://2009.igem.org/Team:Harvard/Protocols
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1. Find restriction enzymes of choice
2. Use double digest finder to find conditions
3.Add enzymes, DNA, buffers and amount of water necessary
4.Place digestions in 37 degree for correct amount of time and prepare a diagnostic gel

Bacteria-Yeast Communication – Harvard – 2009 http://2009.igem.org/Team:Harvard/Protocols
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Bacteria-Yeast Communication Protocol
Yeast
1. Grow cells overnight, 20 hours in 6 mL media with 120 uL of PCB (50x stock) (!!need to grow total of 18 mL cells, 360 uL of PCB!!), in leu-/trp- media supplemented with extra histidine. Use a little scraping of cells from the aliquot. (Take stuff from aliquot B, has a mL of frozen culture, use end of a bacterial spreader)
2. At 16-20 hours of growth, want cell concentration at 1x10^6 cells/mL. On the hemocytometer, if you count two diagonal blocks there should be 200 cells total. If you take the OD, the OD of 2E7 cells is 0.8.
3. Spin down the 6 mL of cells (at top speed, 8000rpm, 1 minute should pellet all of the yeast). Resuspend in 200 uL of fresh medium with PCB (4 uL 50x stock).

Bacteria
1. Grow luciferase bacteria in LB amp overnight , 10 mL.
2. Grow bacteria overnight, induce 5 hours ahead of time with IPTG, 10 mMolar, 0.238 g per 100 mL, dump powder in directly into media, should dissolve well. Use 2x YT, we have 300 mL, use 100 mL.
3. Centrifuge the bacteria down at max (4400 rpm for 10 minutes), and resuspend in 5 mL sodium citrate buffer, with 10 mMol luciferin, so 0.032 g in 10 mL sodium citrate.
4. ONLY SPIN DOWN AND RESUSPEND IMMEDIATELY BEFORE EXPOSING THE YEAST

Filter Lifts
1. Get the liquid nitrogen

2. Prepare the Z buffer
3. Lay filter paper down on the yeast plate, rough side down, and lift up. Place filter on foil boat on liquid nitrogen, then submerge in liquid nitrogen for 2-5 seconds.
4. Place in small dish with Z buffer
3 plates: Experimental, negative control in bioreactor (no light), laser control. All PCB treated, grown overnight.
3.5 mm radius for a well in the 96 well plate, 27 mm radius for miniplate, so we need 6 mL cells for whole plate as opposed to 100 uL per well for same cell density.

CPEC Cloning – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols
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Materials
Phusion™ High-Fidelity PCR Kit (FINNZYMES, Cat. No. F-553)
Thermocycler
Preparation
5x Phusion HF Buffer 4 ul
10 mM dNTPs 0.4 ul
Vector 50 ng/1kb
Insert x ng*
Phusion DNA Polymerase 0.2 ul

H2O to 20 ul
*The amount of insert is determined so that the molar ratio for vector and insert is 1 to 2.
Procedures
98°C 30sec
10X
98°C 10 sec
Annealing** 30 sec
72°C x sec***
72°C 5min
4°C hold
** Anneal at Tm + 3°C. The Tm should be calculated with the nearest-neighbor method.
***The extension time is usually calculated according to the shortest piece with 15 sec /kb if the cloning is not complicated. For example, if there is only one insert and is shorter than the vector, say, 600 bp, then I will use 15 sec for extension. Refer to the published paper for detailed information.
DNA Purification – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols
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Materials :
E.Z.N.A Gel Purification Kit (Omega Bio-Tek, Cat No. D2500-02)
Water bath equilibrated to 55-65C
Microcentrifuge capable of at least 10,000 x g
Nuclease-free 1.5 ml centrifuge bottles
Absolute (95%-100%) ethanol
Protective eye-wear
Isopropanol (for fragments < 500 bp only)
Protocol:
Perform agarose gel electrophoresis to fractionate DNA fragments. Any type or grade of agarose may be used. It is strongly recommended, however, that fresh TAE buffer or TBE buffer be used as running buffer. Do not re-use running buffer as its pH will increase and reduce yields.

When adequate separation of bands has occurred, carefully excise the DNA fragment of interest using a wide, clean scalpel.
Determine the approximate volume of the gel slice by weighing it in a clean 1.5 ml microfuge tube. Assuming a density of 1 g/ml of gel, the volume of gel is derived as follows: A gel slice of mass 0.3 g will have a volume of 0.3 ml. Add equal volume of Binding Buffer (XP2). Incubate the mixture at 55C-60C for 7 min or until the gel has completely melted. Mix by shaking or inverting the tube every 2-3 minutes. Centrifuge the tube briefly to collect all the liquid to the bottom of the tube.
Note: For DNA fragment less than 500bp, add 1 sample volume of isopropanol after the addition of Binding Buffer (XP2).
Apply up to 700 ul of the DNA/agarose solution to a HiBind® DNA spin column assembled in a clean 2 ml collection tube (provided) and centrifuge in a microcentrifuge at 8,000-10,000 x g for 1 min at room temperature. Discard the liquid. Re-use the collection tube in Steps 5-8. For volumes greater than 700 ul, load the column and centrifuge successively, 700 ul at a time. Each HiBind® spin-column has a total capacity of 25-30 ug DNA.
Discard liquid and add 300ul Binding Buffer. Centrifuge at 10,000 x g for 1 minutes.
Add 700 ul of SPW Buffer diluted with absolute ethanol into the column and wait 2-3 minutes. Centrifuge at 10,000 x g for 1 min at room temperature to wash the sample.
Discard liquid and repeat step 6 with another 700 ul SPW Buffer.
Discard liquid and, re-using the collection tube, centrifuge the empty column for 1 min at maxi speed (>13,000 x g) to dry the column matrix. This drying step is critical for good DNA yields.
Place column into a clean 1.5 ml microcentrifuge tube (not provided). Add 30-50 ul depending on desired concentration of final product) Elution Buffer (or sterile deionized water) directly to the center of the column matrix, then incubate for 1 minute. Centrifuge 1 min at maxi speed (>13,000 x g) to elute DNA. This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lo

PCA (Polymerase Cycle Assembly) – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols

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Materials

Phusion™ High-Fidelity PCR Kit (FINNZYMES, Cat. No. F-553)
Thermocycler

Preparation

5x Phusion HF Buffer	5 ul
10 mM dNTPs	0.5 ul
Oligo mixture	125 ng /250 ng /500 ng /
Phusion DNA Polymerase	0.25 ul
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H2O	to 25 ul

Procedures

98°C 30sec
40X
98°C 7 sec
70-50°C slow ramp, 0.1°C/sec
50°C 30 sec
72°C 15 sec /kb
72°C 5 min
4°C hold

PCR Product Clean-up for DNA Sequencing – Duke – 2009
http://2009.igem.org/Team:Duke#Protocols

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Materials:
ExoSAP-IT® (usb, Cat. No. 78200)
Thermocycler
Protocol:
Remove ExoSAP-IT from -20°C freezer and keep on ice throughout this procedure.
Mix 5 µl of a post-PCR reaction product with 2 µl of ExoSAP-IT for a combined 7 µl reaction volume.
Incubate at 37°C for 15 min to degrade remaining primers and nucleotides.
Incubate at 80°C for 15 min to inactivate ExoSAP-IT.
The PCR product is now ready for use in DNA sequencing etc.
PCR – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols
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Materials
Phusion™ High-Fidelity PCR Kit (FINNZYMES, Cat. No. F-553)
Thermocycler
Preperation

5x Phusion HF Buffer	10 ul
10 mM dNTPs	1 ul
DNA template	1 pg – 10 ng
Forward primer (10 uM)	2.5 ul
Reverse primer (10 uM)	2.5 ul
Phusion DNA Polymerase	0.5 ul
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H2O	to 50 ul

Procedure
98°C 30sec
30X
98°C 10 sec
Annealing* 30 sec
72°C 15 sec per 1 kb
72°C 5min
4°C hold

* Anneal at Tm + 3°C. The Tm should be calculated with the nearest-neighbor method.
Single Colony PCR – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols
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Materials
Taq DNA Polymerase with Standard Taq Buffer (NEB, Cat. No. M0273)
10 mM dNTP Mix (NEB, Cat. No. N0447)
Thermocycler
Preperation

10x Standard Taq Buffer	2 ul
10 mM dNTPs	0.4 ul
Bacteria culture*	1 ul
Forward primer (10 uM)	1 ul
Reverse primer (10 uM)	1 ul

Taq DNA Polymerase	0.2 ul
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H2O	to 20 ul

*Bacteria culture refers to E. coli cultured in LB solution overnight.

Procedures

94°C 5 min
25X
94°C 15 sec
Annealing** 30 sec
72°C 1 min per kb
72°C 5min
4°C hold

**Anneal at Tm which is calculated with salt-adjusted method.

Transformation – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols

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Materials:

GC5 Chemical Competent Cells (Genesee Scientific, Cat. No. 42-653)
SOC Medium (Sigma, Cat. No. S1797)
LB Agar (Sigma, Cat. No. L3027)
Petri Dishes (VWR, Cat. No. SC25373-187)
Cell Spreader (VWR, Cat. No. 89042-018)
37°C incubator
37°C shaker
water bath

Protocol:

1. Thaw 1 tube of competent cells on ice;
2. Add 3 ul of cloning product or 1-50 ng of plasmid into competent cells while stirring gently;
3. Keep the tube covered by ice for 30min;
4. Heat-shock the competent cells in water bath for 45 sec at 42°C;
5. Put the tube on ice for 2 minutes;
6. Add 450 ul of SOC medium and then put it in a 37°C shaker for 1 hour;
7. Dilute and spread an appropriate amount on an LB agar plate with the appropriate antibiotics;
8. Place the plate up-side-down in 37°C incubator for 16-18 hours (overnight).

NMR – Duke – 2009 http://2009.igem.org/Team:Duke#Protocols

Login and start NMR program.
Click Acqi. Load sample. Make sure spin is on and lock is off.
Go to lock. Decrease number of sine waves to 1 to obtain step function. Make sure Zo is 1100, lockpower is below 30, lockgain is 36, lockphase is 352, and spin is 20.
Go to shim. Increase lock level.
Click main menu. Click set up. Click H1CDCl3. Type 'nt=64' (number of scans), 'ss=2' (dummy scans), 'go', 'lb=0.2' (line broadening).
Viewing: Type 'wft', 'dscale', 'dfp' to display peak frequency.
Zooming in: Click display. Go to interactive. Type 'cr=8p, delta=8p' to set width of zoom. Use cursor to set boundaries. Click to cut.
Printing: Type "pL pscale(0) pltext ppf page' to print spectra with text and peak frequencies.

Media Prep: - CityColSanFrancisco – 2009
http://2009.igem.org/Team:CityColSanFrancisco/Notebook/Protocols

LB (Lauria Broth) 1 Liter:

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
For agar plates add:	
Bacto-agar	15g

YP (Yeast Peptone) 1 Liter:

Yeast extract	10g
Bacto-peptone	20g
Glucose	20g
For agar plates add:	
Bacto-agar	15g

FEM (anaerobic media) 1 Liter:

*PLEASE use all appropriate PPE for the preparation of this media. Aerosols are harmful!

All proponents for media are weighed on a digital balance. Approximately 75-80% of the distilled water is added to the beaker on a stir plate. Once all ingredients have been added to the dH_2O and homogenized for a few minutes (no solid is seen) the contents are added to a graduated cylinder and brought to volume with dH_2O. The media is then transferred back into the beaker and put back on the stir plate for final homogenization. After thorough mixing, the media is either transferred into a large autoclavable bottle or aliquoted for liquid culture in Morton closure glass tubes. It is important to leave capped closures partially opened, as the increase of heat and pressure creates gas that will expand beyond the volume of the container. Also, it is important to remove the liquid from the autoclave once the containers have cooled to a reasonable temperature. The caps are then tightened to avoid contamination and evaporation. Leaving the liquid in the autoclave promotes unnecessary evaporation and concentration of the liquid media.

When making media to pour plates, the agar is added last. The stir bar should remain in the media for the autoclave cycle and the media should be "homogenized" after the container is at an appropriate temperature. The agar will not incorporate into the media until after autoclaving as its melting point is quite high; trying to incorporate it into the media pre-autoclave is futile. Label all plates before pouring them!! Plates must be sterilely poured before the agar begins to congeal.

Any antibiotics that are to be added to media (either liquid or for plates) must be added after autoclaving. They do not survive this process, and are rendered useless. If antibiotics were added before, they must be added again afterwards, and only when the liquid is cooled significantly (50-55°C).

Cell Culture: - CityColSanFrancisco – 2009
http://2009.igem.org/Team:CityColSanFrancisco/Notebook/Protocols

P. aeurigonsa grown in LB - 37°C
R. palistrus (both strains TIE-1, TIE-3) grown in YP - 30°C
E. coli grown in LB - 37°C
R. ferrireducins grown aerobically in both LB and YP, but anaerobically in FEM -
S. oneidensis (all three strains: WT, MTRB, OMCB) grown in LB - 37°C

After the media is prepared and autoclaved in liquid cycle, liquid cultures are inoculated from the culture slants received from various labs.

This begins the cycle of creating plates from liquid cultures, then selecting a single colony from a plate to inoculate another liquid culture. Every 48 hours, plates were created from liquid cultures, then after another 48 hours, liquid cultures were inoculated again.

Liquid cultures were also used to create a frozen cell bank. Glycerol (50-90% - depending on what was available, and that was sterile) was used for preserving the culture by preventing crystallization of the cell walls. The liquid culture was added to the Cryo-tube first, and then glycerol added on top. After mixing, they were placed in a -80°C freezer.

Plasmid Digestion: - CityColSanFrancisco – 2009
http://2009.igem.org/Team:CityColSanFrancisco/Notebook/Protocols

Two plasmids were digested: pSB1A3 (high copy plasmid) – 2156bp (small fragment – 22bp, large fragment – 2134bp)
Bba_J63009 (low copy plasmid) – 2098bp (sm fragment – 21bp, lg fragment – 2077bp)
Plasmids were purified and eluted in optimized buffer for a final concentration of 150ng/ µL.
Protocols from iGEM were followed. The plasmids were cut with EcoRI and SpeI. Measurements are as follows:

1µL EcoRI
1µL SepI
5µL Buffer 4
0.5µL BSA
5µL of plasmid DNA
37.5µL dH_2O

Because 4 reactions were done for each plasmid type, this recipe should be multiplied by 4, then evenly aliquoted into 4 tubes for digestion. (TV: 45µL per reaction)

The DNA is added last to the mix. Each tube was incubated at 37°C for 45 minutes. This should give complete digestion. The tubes are then put into an 80°C heat block for about 10 minutes to deactivate the enzymes, and stop the digestion process. Portions of the product are run through gel electrophoresis to determine if digestion has happened, and is complete.

PCR Reactions: - CityColSanFrancisco – 2009
http://2009.igem.org/Team:CityColSanFrancisco/Notebook/Protocols
Several reactions have been done. Traditional and Colony PCR were the most useful to this project. Primers were designed based on the OprB gene (1340bp) found in *P. aeurginosa*. The amount needed for each reaction is based on final concentration, and total volume. A spreadsheet was created to help with calculations, depending on the total volume for each reaction done. Reaction volume was also amended based on the amount of genomic DNA available. Colony PCR uses a single colony from a plate, that is added individually to each of the reaction tubes just before going into the thermocycler. Parameters for cycling were changed depending on the reaction, primers used, and as a troubleshooting tool to optimize the reactions.

How to calculate CFU's – Brown – 2009 http://2009.igem.org/Team:Brown/Notebook_Protocols

(pg plasmid DNA/total reaction volume) x plated volume = pg DNA plated

CFU value: (avg # of colonies/pg DNA plated) x (10^6 pg / µg) = (# of transformed cells/ µg)

Start by taking a tube of competent cells that you have prepared and add 100pg of plasmid DNA from any one of your miniprep samples.
Go through the transformation the same way that you would any other time.
I usually take the volume of transformed cells (100µL) and add the same volume of LB.
I then plate out a number of dilutions of the mix, 0.5 (100µL), 0.05 (10µL), and 0.005 (1µL) onto the appropriate selection media (you obviously can't spread 10 or 1µL so add more LB on the plate to spread the cells effectively).
Let them grow up overnight and count the plate that has between 20 and 200 colonies and go through the calculations.

Compiled by Adrian Reich, July 2009

Immunoblotting (Western Blotting) – Brown – 2009
http://2009.igem.org/Team:Brown/Notebook_Protocols
Adapted for Spot-Blotting

Reagents needed:
1) Blotto (per liter, 10.8 gms NaCl; 20 mM Trip, pH 8.0; 30 gms not-fat dry milk; 0.01% Tween-20)
2) Antibody (anti-6-his, in blotto, diluted 1/10,000)
3) Alkaline phosphatase buffer, (per liter, 50 mM Tris pH 9.6, NaCl 10.8gms, 5mM MgCl2)
4) Staining components: NBT (4-nitro blue tetrazolium chloride; 0.5 gms in 10mls 70% N,N-dimethylformamide) BCIP(5-bromo-4-chloro-3-indolyl-phosphate; 0.5 gms in 100% N,N-dimethylformamide)
Notes: BCIP (colorless) oxidation → forms blue precipitate. BCIP-NBT naturally forms this bluish purple precipitate over time; however, alkaline phosphatase speeds up the process 1000 fold. BCIP binds very tightly in the alkaline phosphatase active site, but when NBT reacts with BCIP, it is released from the enzyme and the colored precipitate forms.

Procedure:
1) Following blotting/spotting, immediately immerse the blot in excess blotto. Agitate for 5-10 minutes.
2) Pour off blotto and add limiting amounts – 'cause it is expensive (!) (alkaline-phosphatase conjugated, anti-6X His) antibody at appropriate dilution. Agitate for 20 minutes.
3) Wash the blot 3 times, for 5 minutes each with blotto, and then with alkaline phosphatase buffer for 5 minutes.
4) Make up (5 mls) staining solution: to 5mls of alkaline phosphatase buffer, add 33 ul of NBT, and 17 ul of BCIP.
5) Pour off wash alkaline phosphatase buffer and add staining solution. Agitate and monitor for several minutes. When ready to stop the reaction, wash several times with water.
6) To record, photograph immediately, or store dry (though it will lose some color intensity).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) – Brown – 2009
http://2009.igem.org/Team:Brown/Notebook_Protocols
The standard method to separate proteins in a complex mixture according to mass (formally, according to their ability to migrate through the gel). The proteins are denatured by SDS and boiling, and must be stained with dyes in order to see, yet the gels can also be blotted to identify specific proteins by antibodies, or lectins. "Bands" of individual proteins can be excised and analyzed by mass spectrometry or, eluted and used to make antibodies.
Components:
1) Gel box, electrode buffer, power supply, pre-pored gels.
2) Protein molecular weight marker (not 1kb ladder), 2x sample buffer, samples
The protein MW marker is in sample buffer and ready to load. Simply thaw, and load 10 µl.
Electrode buffer: per 3 liters – 43.2 grams of Glycine, 9.09 grams of TRIS base, and 15 mls of 20% SDS, pH8.0
2x Sample Buffer: 5mM TRIS, pH 6.8; 2% SDS; 5mM EDTA; 20% sucrose; and a pinch of Bromphenol blue
Sample preparation:
1) For protein in aqueous solution, add equal volume of 2x sample buffer and treat at 95oC for 5 minutes. Spin for 1 minute at high speed in the microfuge and load supernatant (being careful not to disturb pellet).
2) For bacteria: pellet (spin at high speed in the microfuge for 30 seconds) appropriate amount of cells (e.g. 100ul of liquid culture). Resuspend cells in dH2O (e.g. 20 ul) by vortexing vigorously. Add equal volume of 2x sample buffer and incubate at 95oC for 5 minutes with intermittent vigorous vortexing to lyse the cells. Spin for 1 minute at high speed in the microfuge and load supernatant (being careful not to disturb pellet).
Running gels: Assemble the gels in the gel box. For one gel use a blank to form a reservoir. Remove gel comb (and perhaps label wells for easier loading), add buffer to the two chambers, wash wells with pipette, and load samples (about 10-20 µl, being careful to not bump the gel box). Run 125V for 90 minutes. Turn off power supply before disassembling the gel box.
Staining gels: Carefully ('cause it will stain you, your clothes, shoes, teeth, fingers etc) place the gel in Coomassie Blue (or similar choice) for >4 hours on a rotary shaker (not a rocker). Destain to taste.

DNA digestion protocol & hints – Brown – 2009
http://2009.igem.org/Team:Brown/Notebook_Protocols

Overview: Although it is pretty standard to digest DNA with restriction enzymes, here is a standardized protocol and some hints…

Materials:
• DNA sample in water or TE buffer
• 10x digestion buffer
• restriction enzyme
• DNA loading buffer
• Agarose gel 0.8% (or different depending on expected band sizes)

Procedure:
1. Pipet the following into a microfuge tube:
20 µl reaction 50 µl reaction DNA 0.1 to 4 µg 0.1 to 4 µg 10x Digestion buffer 2 µl 5 µl Enzyme (as appropriate) Water (Rest of volume)
2. Add the enzyme (1-5u/µg DNA)

3. Incubate at recommended temperature for an hour.
4. Take 2 to 5 µl of the digested sample, add loading buffer, and run on agarose gel to check the result.

Tips:
1. DNA:
• For checking DNA, use 0.1 µg; (or 4-8 µl from a good DNA mini prep)
• For cloning, 4 µg DNA is enough
2. Buffer: besides the buffer that comes with the enzyme, buffers from other company can be used, too (as long as the contents are the same)
3. Enzyme: the maximum volume that an enzyme can be used is 1/10 of the total reaction volume (example: 2 µl for 10 µl reaction)
4. Incubation time: can be longer than 1 hr, but don't do overnight digestion if you don't have to (genomic DNA requires overnight digestion)
5. Gel: make sure to run the uncut DNA along with the digested DNA. And, always run a DNA marker!

Reference: *Current protocols in molecular biology* (3.1.1 - 3.1.2)

Glycerol Stocks – Brown – 2009 http://2009.igem.org/Team:Brown/Notebook_Protocols

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Materials and Stocks needed:
1. Sterile screw cap microcentrifuge tubes.
2. Appropriate media, liquid & solid.
3. 80% Glycerol (80 ml)
4. dH20 (20 ml)
5. Autoclave
Notes: The reason for using 80% glycerol is that it is simply much easier to handle than is 100%)

A. To create a glycerol stock:
(1) Pick a SINGLE colony of the clone off of a plate and grow an overnight in the appropriate selectable liquid medium (e.g. LB amp).
(2) Make a label (clone ID # and date) for the construct (use the time tape). Place this label onto a sterile screw cap microcentrifuge tube.
(3) Add 0.5ml of the o/n culture to 0.5ml of 80% sterile glycerol in the sterile screw cap microcentrifuge tube. (Certain antibiotics in the medium should be removed first as they are supposedly toxic over time. Tetracycline is one such antibiotic. To do this: spin the culture down and resuspend in straight LB with no antibiotic).
(4) Screw a lid onto the tube and write the clone ID # on the lid of the tube.
(5) Vortex.
(6) Freeze the glycerol stock at –80 degrees C.
(7) Enter any and all pertinent information (host strain, vector, cloning site(s), selection criteria, date prepared, origin/source and/or reference, and any other important information) regarding this accession into the lab stock collection book. Also include a map or sequence if possible.
(8) If this is a plasmid construct, perform a mini prep on a portion of the same culture medium that was used to prepare the glycerol construct in order to verify that it is what you think it is.
(9) Store the mini prep DNA away in the lab DNA stock box in the –20oC.

B. To streak out from a glycerol stock:

(1) Take the tube to the place that you intend to streak the clone out (e.g., your bench or the laminar flow hood, etc).
(2) Flame a metal inoculating loop until it is red hot.
(3) Scrape off a portion from the top of the frozen glycerol stock and streak it onto your plate.
(4) Return the construct to the –80oC. DO NOT LET THE GLYCEROL STOCK THAW! FREEZE THAW CYCLES WILL KILL THE CLONE! If you need to streak out multiple constructs take out only one or two from the freezer at a time.
(5) Flame the metal inoculating loop a second time, cool it by inserting it into the agar of the plate and finish streaking out the clone so that it will be possible to isolate single colonies.

Protocol compiled by Stockinger Lab; 01/31/01

Protocol: Polymerase Chain Reaction – Brown – 2009
http://2009.igem.org/Team:Brown/Notebook_Protocols

Background/Purpose
Polymerase Chain Reaction (PCR) is used to amplify DNA segments by way of template strands, primers, and DNA polymerase. PCR is initiated by a short sequence of primers (one forward and one reverse). Primers are short, complementary sequences of roughly 18-28 nucleotides that anneal to the template strands to begin the synthesis of the new DNA strands. Following the primers annealing to the separate template strands respectively, the DNA polymerase begins to add complementary nucleotides to the template strands thereafter to complete the polymerase chain reaction.
DNA is synthesized in the 5’→3’ direction. Since DNA is antiparallel, the DNA polymerases add nucleotides in opposite direction. That is, each primer anneals at opposite ends of the desired DNA of interest, prompting the DNA polymerases to build towards each other. The region in between the two primers is replicated. See Figure 1 for the PCR Mechanism.

Methods
Primer Design
1. Identify gene of interest
2. Go to NCBI and navigate to the “All Databases” drop-down menu in the upper left part of the webpage and select the “Protein” tab.
3. Now type in the text box “gene of interest organism” Example: Search “Protein” for “Groucho sea urchin”
4. Click on “Go”
5. Click on one of the relevant results
6. Scroll down to “Origin.” Copy and paste this one-letter code amino acid sequence into a Word document. Format this by deleting the numbers dispersed in the overall sequence. Spaces in between the sequence will suffice. Example: Pristionchus pacificus
mdkragsrgg ggggnpflda leklkddynh mqaqlssqra eldkmnaeke qlqrhymmyy
emscglnmem qkqsevakrm tallqsmlqy aphdaqasti qamerakqis lqelqqltaa
sqaqqmlgmt gpmaamgglg gmagalggpg glnmaaiaaa mgaglrppap pgggggddrp
apsssrqsss rqrsgspagg ekkpkleted gdddeidvqn ddpagpaang ktggrdsvhs
gisssgastp aaaaaknfaa qlgqqrlpla qldpatrmmm qgmmapngka pysyrvdstg
nlaptmfppd altdpgvpks vkavhdlphg evvcavaisk daqrvftggk gcvkiwdlaa
ntsaararle clednyirsc klfaegthlv vggeasnill fdietqkeva kldttaqacy
alalnqeskl lyaccadgav vifdlasmqe varlpghtdg ascvdlsgdg lrlwtggldh

tlrswdirer relsnidfas qifslgcspt edwvavgldt nqievvntap gvkeryqlhr
hdscvlslrf ahsgkwfctt gkdnllnvwr spygalsvra sesssvlscd ishddsvivt
gsgekkatvy qvqyesss

7. Identify another relevant organism with the same gene of interest and repeat the aforementioned process.

8. Alignment: Go to Clustal W. Paste amino acid sequences in the following format (the title ">speciesname1" should not contain any spaces):

>speciesname1
mdkragsrgg ggggnpflda leklkddynh mqaqlssqra eldkmnaeke qlqrhymmyy
emscglnmem qkqsevakrm tallqsmlqy aphdaqasti qamerakqis lqelqqltaa
sqaqqmlgmt gpmaamgglg gmagalggpg glnmaaiaaa mgaglrppap pgggggddrp
apsssrqsss rqrsgspagg ekkpkleted gdddeidvqn ddpagpaang ktggrdsvhs
gisssgastp aaaaaknfaa qlgqqrlpla qldpatrmmm qgmmapngka pysyrvdstg
nlaptmfppd altdpgvpks vkavhdlphg evvcavaisk daqrvftggk gcvkiwdlaa
ntsaararle clednyirsc klfaegthlv vggeasnill fdietqkeva kldttaqacy
alalnqeskl lyaccadgav vifdlasmqe varlpghtdg ascvdlsgdg lrlwtggldh
tlrswdirer relsnidfas qifslgcspt edwvavgldt nqievvntap gvkeryqlhr
hdscvlslrf ahsgkwfctt gkdnllnvwr spygalsvra sesssvlscd ishddsvivt
gsgekkatvy qvqyesss
>speciesname2
mypspvrhpa aggpppqgpi kftiadtler ikeefnflqa qyhsiklece klsnektemq
rhyvmyyems yglnvemhkq teiakrlntl inqllpflqa dhqqqvlqav erakqvtmqe
lnliigqqih aqqvpggppq pmgalnpfga lgatmglphg pqgllnkppe hhrpdikptg
legpaaaeer lrnsvspadr ekyrtrspld iendskrrkd eklqedegek sdqdlvvdva
nemeshsprp ngehvsmevr dreslngerl ekpsssgikq erppsrsgss ssrstpslkt
kdmekpgtpg akartptpna aapapgvnpk qmmpqgpppa gypgapyqrp adpyqrppsd
paygrpppmp ydphahvrtn giphpsaltg gkpaysfhmn gegslqpvpf ppdalvgvgi
prharqintl shgevvcavt isnptkyvyt ggkgcvkvwd isqpgnknpv sqldclqrdn
yirsvkllpd grtlivggea snlsiwdlas ptprikaelt saapacyala ispdskvcfs
ccsdgniavw dlhneilvrq fqghtdgasc idispdgsrl wtggldntvr swdlregrql
qqhdfssqif slgycptgdw lavgmenshv evlhaskpdk yqlhlhescv lslrfaacgk
wfvstgkdnl lnawrtpyga sifqsketss vlscdistdd kyivtgsgdk katvyeviy

9. Click on "ClustalW" under "Multiple Alignment"

10. Copy and paste the aligned sequence into a Word document. If the sequences do not align, change the font to Courier and/or minimize the font.

The sequences are presented in the direction of 5'→3'.The stars designate conserved regions between the species. Take note of the conserved regions as they will be crucial for primer design.

11. Two primers must be designed, one forward and one reverse.

Selection of Primers: Select a conserved region of ~7 one-letter amino acid codes. Explicitly write out the nucleotide sequences that correspond to the one-letter amino acid code. The U must be replaced by a T in the three-letter codon.

Example:
Forward Primer 5' MMFECKW 3' (amino acid sequence)
5' ATG ATG TTc/t GAa/g TGc/t AAa/g TGG 3' (nucleotide sequence)
In this case, the template nucleotide sequence is also the forward primer sequence. As indicated in Figure 1, the forward primer is replicating DNA in the 5'→ 3' direction, meaning that the primer is annealing to the bottom 3'→ 5' template strand, which is just the complementary sequence of the nucleotide sequence obtained from ClustalW. The forward primer's sequence is exactly the same as the parental strand sequence.

FINAL FORWARD PRIMER SEQUENCE:

5’ ATG-ATG-TTc/t-GAa/g-TGc/t-AAa/g-TGG 3’

Reverse Primer:
5’ PCYTHNMC 3’ (amino acid sequence)
5' CCa/c/g/t TGc/t TAc/t TGG CAc/t AAc/t ATG TGc/t 3' (nucleotide sequence)
Because this is the reverse primer, the nucleotide sequence is the reverse complement of the DNA strand.
5’ GGa/g/c/t ACg/a ATg/a ACC GTg/a TTg/a TAC ACg/a 3'
Ideally, it is best to avoid a wobble base at either end of the primer. This problem can be solved by simply eliminating the last nucleotide if need be. In this case, the g/a nucleotide can be eliminated on the 5’ end.
FINAL REVERSE PRIMER SEQUENCE:
5’ CA-CAT-g/aTT-a/gTG-CCA-a/gTA-a/gCA-a/g/c/tGG 3’

12. Some amino acids have degeneracy, the condition in which more than one codon corresponds to one amino acid. During primer design, it is best to minimize degeneracy.
13. Other factors to consider during Primer Design:
Each primer should be ~18-28 nucleotides long (~7 amino acids).
The 3’ end of primers should end with a G or C or GC, but not all three should be G/C. If the 3’ end does not end with G/C, eliminate the final nucleotide.
No wobble base should be located on either end of the primer.
Distance between the two primers should be ~ 500-1000 nucleotides, which is ~ 160-330 one-letter amino acid codes.
Melting temperature should be between 55-70* Celsius. Calculate this at Oligo Calc.
GC content should be roughly 50-60%. Calculated this at Oligo Calc.
14. After having determined the best possible primer sequences that satisfy all of the aforementioned criteria, order the primers. Polymerase Chain Reaction
PCR employs the method of thermal cycling: Denaturation: At 94°C, hydrogen bonds between complementary bases of the DNA strands are disrupted, separating the two template strands. Annealing: At 58 °C, the primers anneal to their respective single-stranded DNA templates. Extension/Elongation: At 72 °C, DNA polymerase synthesizes the new DNA strands by adding dNTPs that are complementary to the template in 5' to 3' direction.
The key ingredients are listed as follows:
• dH2O (Adjusted to the total 50 µL volume. In this case, ~28.5 µL)
• Buffer .10 µL
• Primers .4 µL/ primer
• dNTPs (building blocks from which the DNA polymerases synthesizes a new DNA strand) .2 µL
• template DNA (DNA region to be amplified) .1 µL
• Taq Polymerase (enzyme originally isolated from the bacterium Thermus aquaticus; heat-stable) 0.5µL
MgCl2 is necessary for the activation of active sites. It may or may not be included in the buffer. Adjust the volumes accordingly.
Notes: When working with the 200 µL PCR tubes, be careful not to touch the cap since human DNA will contaminate the sample. Place the PCR tubes in the PCR machine. If necessary, program the machine for the 35 cycles. Press “Start.” Collect the PCR product after 2-3 hours.

Primer resuspension – Brown – 2009 http://2009.igem.org/Team:Brown/Notebook_Protocols

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Goal: 5pM/ul. This is a 1x stock solution.

Protocol:

1) Identify the nM amount on the tube and resuspend the primers in 10x (in ul) water, e.g. 23.5 nM would be resuspended in 235 uL water. This is a 100 pM (or 0.1 nM) and is the 20x that you protect!
2) Dilute a portion of this 20x into a fresh tube, appropriately labeled, to make a 1X stock solution, or 5pm/uL. This is your working solution. Use 2uL per reaction.
3) Store at -20oC.

Moving DNA (subcloning) – Brown – 2009 http://2009.igem.org/Team:Brown/Notebook_Protocols

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Often pieces of DNA (biobricks, PCR fragments, promoters, etc) need to be moved into a different vector, combined with other pieces, or modified in various ways. This is usually accomplished as follows:
1) **Restriction enzyme digest** of plasmid and DNA pieces, making them compatible for recombination
2) **Gel purification** of each appropriate piece (agarose gel, excising appropriate DNA bands using Qiagen gel extraction kit – see manual fordetails)
3) **Ligation** of DNA fragment to vector
4) **Transformation** of new cells with ligation mixture.

Details of procedures:
1) **Restriction enzyme (RE) digest**: Each restriction enzyme works best in a particular buffer of pH, salt, etc etc. The REs are often compatible between different buffers, and the buffers are provided with the enzymes. Many websites give plenty of such information e.g. neb.com.
A normal digest is in 20µl as follows: 10x buffer - 2 µl Restriction Enzyme 1 µl (about 10 units) DNA (about a µg) Water to 20 µl (Remember the best order of addition)
Incubate the digest reaction 1-2 hours at 37 degrees C, and run on a gel along with the plasmid digested similarly. A good stopping point is to freeze the digest after 1-2 hours at 37 degrees C, but do not let the incubation go longer. It is usually best if you can double digest with two different REs that will not only force the orientation of the cloning, but increase its efficiency. Compatible REs are best, but even if not possible, you simply isolate the DNA (like in the gel extraction procedure), or change the buffer after the first digest (depending on buffer components this may mean simply adding a salt, or changing the pH). If you know the sequence of your insert, make sure you check that the REs do not digest internally.

2) **Gel purification procedure**: Run a 1% agarose gel in TAE using gel loading buffer in your digest (so it stays in the well), and loading a ladder for reference. (See Qiagen kit manual for DNA purification)

3) **Ligation of DNA fragment to vector:** Following purification of DNA, mix isolated DNA such that copy number insert to vector is about 2:1 (remember staining intensity and size and mass considerations?) 5x Ligation buffer 2 µl (T4 DNA) Ligase 1 µl DNA mixture XX µl Water to 10 µl Mix all components on ice (make sure ligase buffer was mixed thoroughly) with the pipette. Keep at 4 degrees C overnight. Before adding to transformation – dilute in water to 100 µl.

4) **Bacterial cell transformation:** This procedure is used to get DNA into bacteria. It requires fresh cells for optimal transformation – but it is quite reliable. Supercoiled DNA works much better than ligated DNA in transformations. the important part of this protocol is to plan ahead, and once cells are in CaCl2, to keep them ice-cold.
1. Grow a 5ml overnight culture of XL1-Blue in LB broth at 37 oC with vigorous shaking.
2. The next morning, add 50ul of this culture to a fresh tube of 5ml LB. Use the plastic 15ml snap top tubes. Again incubate at 37 o C with vigorous shaking, and grow to A600 = 0.6-0.7. This takes a couple of hours, and the suspension will begin to take on a silky appearance. Do not let this culture overgrow -

the health of the cells declines with confluence. An alternative procedure is to start a culture of XL1-Blue cells from a recently streaked plate, (streaked on LB/tetracycline) and to grow for several hours.
3. Cool cells on ice for 10 min., then take the tops off the tubes and while using the green tube adapters, spin in Sorvall SS-34 rotor for 5 minutes, 4 o C, at 3,000 rpm.
4. Discard the supernatant and quickly put cells back on ice. [From here on, keep cells on ice as best you can, even when resuspending them in fresh buffers].
5. Resuspend the cells in 2mls of ice cold (sterile) CaCl2 (50mM) using your P-1000 Pipettman. Leave on ice for 15 minutes. (For 50 mM CaCl2 use 0.73 gms of CaCl2 - 2H2O per 100 mls of water, and autoclave)
6. Centrifuge the cells as in #3 above. Discard the supernatant and resuspend cells in 500 ul CaCl2. Use immediately for transformation or store at -70 oC. If cells used for transformation are frozen, remove tube from freezer and immediately put on ice. Allow these cells to thaw on ice, which takes about 30 minutes.
7. To transform competent cells, put 100-200ul of the suspension in a precooled 13 x 100mm disposable glass culture tube (microfuge tube works fine). Add DNA ligation mixture to the cells, mixing gently with your pipette on ice. [For a positive control of transformation, use 1 ul of supercoiled plasmid pBS DNA from any miniprep]. Cover the tube with parafilm and incubate for 30-40 minutes on ice. Prepare a beaker of water (tap water is fine) at 42 degrees C. Heat shock the cells by placing the glass culture tube at 42 degrees C for 2 minutes, and then back on ice. Add 0.5ml LB media and then incubate cells in the 37 degrees C water bath for 1 hour, making sure the tube is capped with Parafilm (or snapped for a microfuge tube).
8. Spread the cells over one or two LB/Amp plates, invert in 37 degrees C incubator overnight.

Bacterial Basics – Brown – 2009 http://2009.igem.org/Team:Brown/Notebook_Protocols
Bacteria Structure

Bacteria are prokaryotic organisms. They are surrounded by a firm cell wall that helps maintain their structure. Bacteria have no nucleus; their genomic DNA is contained in the nucleoid. Bacteria also contain extrachromosomal DNA, called a plasmid. Bacteria reproduce asexually.

Different Forms of DNA Transfer:
- Transformation
- Conjugation
- Transduction

Bacterial Transformation

Bacterial Transformation is the process by which bacteria uptakes DNA from the media (liquid) surrounding it. The DNA can be from cells from the same species or from different species. Once taken up by bacteria, the DNA will integrate into the bacteria's chromosome. As seen in the diagram at the right, the DNA binding complex that the free DNA passes through is contained within the bacteria's cell wall. This process is commonly used in genetic engineering.

Bacterial Conjugation

During conjugation, DNA from one bacteria cell (donor) is transferred to another bacteria cell (recipient). The two bacteria cells must be in physical contact; the donor bacterium extends its pili to transfer its DNA to the recipient. Plating bacteria on different media is a common way to see whether any mutants (bacteria that have received new DNA) survived. Auxotrophic mutants will not grow if there are no supplements in the media, whereas prototrophic mutants can grow without supplements.

Bacterial Transduction

A phage (virus) attaches to a donor bacterium and injects its DNA. It uses the bacterium as a "factory" to make more phages. The newly made phages then break out of the cell and attach to recipient bacterium. They insert their DNA, which then recombines into the bacterium's DNA. The recipient bacterium is considered transduced and contains the genetic information to synthesize the phage.

More Details of Chemical Bacterial Transformation

Although the mechanism by which calcium chloride-mediated bacterial transformation is not completely understood, some basics are known.
During early logarithmic growth, the cell membrane of E. Coli bacteria develops many pores called adhesion zones. These zones differ from the surrounding lipid bilayer membrane by the existence of lipopolysaccharide (LPS) molecules, which can bind foreign DNA. The LPS molecules alone, however, are not enough to bring the foreign DNA into the cell due to the electrostatic repulsion between the DNA's sugar phosphate backbone and the polar lipids of the lipid membrane. Calcium cations from a calcium chloride solution should theoretically be able to create an electrostatically neutral situation. Cooling the bacteria while in this solution congeals the lipid membrane and shields the ionic charges effectively. Heat-shocking the bacteria to 42ºC creates a heat gradient, through which the foreign DNA along with outside water can enter the cell (this causes the cells to swell).
Cells that can take up foreign DNA from a nutrient-rich external solution are termed chemically competent. The foreign DNA is incorporated into the bacterial genome in one of the three aforementioned ways. If these cells recognize the origin of the foreign DNA, they will replicate it along with the rest of their genome during cell division.
If the exogenous DNA is tagged with an antibiotic resistance gene, only cells that incorporated the correct foreign DNA can be selected by the application of the antibiotic.

Chemical Transformation Procedure
Based on Invitrogen's One Shot® TOP10 Competent Cells
1. Choose ligation DNA and make sure that it is tagged with an antibiotic resistance gene (usually ampicillin)
2. Centrifuge the vial(s) containing the ligation reaction(s) [foreign DNA] briefly and place on ice.
3. Thaw, on ice, one 50 µl vial of One Shot® cells [chemically competent cells] for each ligation/transformation.
4. Pipette 1 to 5 µl of each ligation reaction directly into the vial of competent cells and mix by tapping gently. Do not mix by pipetting up and down. The remaining ligation mixture(s) can be stored at -20°C.
5. Incubate the vial(s) on ice for 30 minutes.
6. Incubate for exactly 30 seconds in the 42°C water bath. Do not mix or shake.
7. Remove vial(s) from the 42°C bath and place them on ice.
8. Add 250 µl of pre-warmed S.O.C medium to each vial. S.O.C is a rich medium; sterile technique must be practiced to avoid contamination.
9. Place the vial(s) in a microcentrifuge rack on its side and secure with tape to avoid loss of the vial(s). Shake the vial(s) at 37°C for exactly 1 hour at 225 rpm in a shaking incubator.
10. Spread 20 µl to 200 µl from each transformation vial on separate, labeled LB agar plates (+ antibiotic – usually ampicillin). The remaining transformation mix may be stored at +4°C and plated out the next day, if desired.
11. Invert the plate(s) and incubate at 37°C overnight.
12. Select colonies and analyze by plasmid isolation, PCR, or sequencing.

Sources:

http://www.dnai.org/text/mediashowcase/index2.html?id=1009
http://www.genome.ou.edu/protocol_book/protocol_adxF.html
Griffiths, Anthony J. F. Introduction to Genetic Analysis, 9th Edition. W.H. Freeman and Company: New York, 2008.
Panja et al. "Plasmid DNA Binds to the Core Oligosaccharide Domain of LPS Molecules of E. coli Cell Surface in the CaCl2-Mediated Transformation Process." Biomacromolecules 9(2008): 2501–2509.

Restriction Reaction – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
- 1X Reaction Buffer
- Total DNA - 0.1-4 μg
- Endonuclease - 1 unit per μg of DNA
- Autoclaved distilled water to 20 uL

Temperature - 37°C - or the suggested by the manufacturer
Time - 3 h or overnight
Protocol according to Current Protocols in Molecular Biology

Prepare of eletrocompeten *S. cerevisiae* – Unicamp-Mexico – 2009
http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
1- Grow yest strain in 50ml YEPD medium overnight, 250 RPM, 30ºC.
2- Inoculate 100 ml YEPD in a 500ml flask in a OD=0,1, 250 RPM, 30ºC util OD= 1,3
3- Place the culture on ice for 15 minutes to stop the growth
4- Centrifuge for 4 min at 4000g, 4ºC
5- Ressuspend cells in 20ml of cold water in 50ml tubes, complete volum to 50ml.
6- Centrifuge for 4 min at 4000g, 4ºC
7- Repeat steps 5 and 6.
8- Ressuspend cells in 10ml Sorbitol 1M
9- Centrifuge for 4 min at 4000g, 4ºC
10- Ressuspend cells in 200ul

S. cerevisiae Transformation – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
1- In a 1,5ml tube, pipete 40ul of fresh eletrocompetent cells, and addthe DNA (5-100ng in 5ul)
2- mix gently and place on ice for 5 minutes
3- transfer the contents of the tube to a cold electroporation bucket.
4- Proceed electroporation
5- Add immediately 1ml of cold sorbitol (1M)
6- Plate in seletive media (YNB Ura-)

Culture Media – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
YEPD 10g yeast extract, 20g peptone, 20g glucose, Final volume: 1l

YNB Ura- 6,7g YNB, 30g glucose, 30g agar, 10ml tryptophan (100x), 10ml histidine (100x), 30ml leucine (100x), 30ml Drop out
Drop out:
0,5g/l Adenine
1,2g/l L- aspartic acid
1,2g/l L- glutamic acid
0,24g/l L- arginine

0,36g/l L- lysine
0,24g/l L- methionine
0,6g/l   L- phenylalanine
4,5g/l   L- serine
2,4g/l   L- treotonine
0,18g/l L- tyrosine
1,8g/l   L- valine

Electroelution – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
Protocol according to Stratagene Quick-Pik electroelution capsule protocol
Electroelution Protocol

T4 DNA Ligase – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
- 5X Ligase Reaction Buffer - 4 µl
- Insert: Vector Molar Ratio - 3:1
- Vector Ends - 3-30 fmol
- Insert Ends - 9-90 fmol
- Total DNA - 0.01-0.1 µg
- T4 DNA Ligase - 0.1 unit
- Autoclaved distilled water to 20 uL
Temperature - 14°C
Time - 1 h
Protocol according to Invitrogen

CIAP Dephosphorylation – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
Protocol for Dephosphorylation of 5'-ends of DNA:
1. Dilute sufficient CIAP for immediate use in CIAP 1X Reaction Buffer to a final concentration of 0.01u/µl. Each picomole of DNA ends will require 0.01u CIAP. (1µg of 1,000bp DNA = 1.52pmol DNA = 3.03pmol of ends.)
2. Purify the DNA to be dephosphorylated by ethanol precipitation, and resuspend the pellet in 40µl of 10mM Tris-HCl (pH 8.0). Set up the following reaction:
- DNA (up to 10 pmol of 5´-ends) - up to 40µl
- CIAP 10X Reaction Buffer - 5µl
- Diluted CIAP (0.01u/µl) - up to 5µl
- MiliQ Water - to 50µl
3. Incubate at 37°C for 30 minutes.
4. Add another aliquot of diluted CIAP (equivalent to the amount used in Step 2), and continue incubation at 37°C for an additional 30 minutes.
5. Add 300µl of CIAP stop buffer. Phenol:chloroform extract and ethanol precipitate by adding 0.5 volume 7.5M ammonium acetate (pH 5.5) and 2 volumes of 100% ethanol to the final aqueous phase.
Protocol according to Promega

SAP Dephosphorylation – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols

Protocol for Dephosphorylation of 5'-ends of DNA:

1. Resuspend 1 pmol of DNA ends (about 1 µg of a 3 kb plasmid) in nuclease-free water.
2. Prepare reaction mix in a 20 µl volume according to the following:

- DNA - 1 µl
- 10X SAP Reaction Buffer - 2 µl
- Water, Nuclease-Free - up to 19 µl
- Shrimp Alkaline Phosphatase (1 unit/µl) - 1 µl

3. Incubate at 37°C for 30-60 min.
4. Stop reaction by heating at 65°C for 15 min. This completely inactivates SAP.

Protocol according to USB www.usbweb.com

Isolation of RNA using Trizol Reagent (Invitrogen) – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols

1. Homogenization

a. Tissues Homogenize tissue samples in 1 ml of TRIzol® Reagent per 50-100 mg of tissue using a glass-Teflon® or power homogenizer (Polytron, or Tekmar's TISSUMIZER® or equivalent). The sample volume should not exceed 10% of the volume of TRIzol Reagent used for homogenization.

b. Cells Grown in Suspension Pellet cells by centrifugation. Lyse cells in TRIzol® Reagent by repetitive pipetting. Use 1 ml of the reagent per 5-10 × 106 of animal, plant or yeast cells, or per 1 × 107 bacterial cells. Washing cells before addition of TRIzol® Reagent should be avoided as this increases the possibility of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer.

OPTIONAL: An additional isolation step may be required for samples with high content of proteins, fat, polysaccharides or extracellular material such as muscles, fat tissue, and tuberous parts of plants. Following homogenization, remove insoluble material from the homogenate by centrifugation at 12,000 × g for 10 minutes at 2 to 8°C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. In samples from fat tissue, an excess of fat collects as a top layer which should be removed. In each case, transfer the cleared homogenate solution to a fresh tube and proceed with chloroform addition and phase separation as described.

2. Phase Separation Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIzol® Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 × g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIzol® Reagent used for homogenization.

3. RNA Precipitation Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIzol® Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 × g for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

4. RNA Wash Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol® Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 × g for 5 minutes at 2 to 8°C.

5. Redissolving the RNA At the end of the procedure, briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C. (Avoid SDS when RNA will be used in subsequent enzymatic reactions.) RNA can also be redissolved in 100% formamide (deionized) and stored at -70°C (5).
RNA Isolation Notes:
1. Isolation of RNA from small quantities of tissue (1 to 10 mg) or Cell (102 to 104) Samples: Add 800 µl of TRIzol® to the tissue or cells. Following sample lysis, add chloroform and proceed with the phase separation as described in step 2. Prior to precipitating the RNA with isopropyl alcohol, add 5-10 µg RNase-free glycogen (Cat. No 10814) as carrier to the aqueous phase. To reduce viscosity, shear the genomic DNA with 2 passes through a 26 gauge needle prior to chloroform addition. The glycogen remains in the aqueous phase and is co-precipitated with the RNA. It does not inhibit first-strand synthesis at concentrations up to 4 mg/ml and does not inhibit PCR.
2. After homogenization and before addition of chloroform, samples can be stored at -60 to -70°C for at least one month. The RNA precipitate (step 4, RNA WASH) can be stored in 75% ethanol at 2 to 8°C for at least one week, or at least one year at –5 to -20°C.
3. Table-top centrifuges that can attain a maximum of 2,600 × g are suitable for use in these protocols if the centrifugation time is increased to 30-60 minutes in steps 2 and 3.

Troubleshooting

Expected yields of RNA per mg of tissue or 1 x 106 cultured cells
* Liver and spleen, 6-10 µg
* Kidney, 3-4 µg
* Skeletal muscles and brain, 1-1.5 µg
* Placenta, 1-4 µg
* Epithelial cells (1 x 106 cultured cells), 8-15 µg
* Fibroblasts, (1 x106 cultured cells) 5-7 µg

Low yield
* Incomplete homogenization or lysis of samples
* Final RNA pellet incompletely redissolved

A260/A280 ratio < 1.65 RNA sample was diluted in water instead of TE prior to spectrophotometric analysis. Low ionic strength and low pH solutions increase absorbance at 280 nm (6,7).
* Sample homogenized in too small a reagent volume.
* Following homogenization, samples were not stored at room temperature
for 5 minutes.
* The aqueous phase was contaminated with the phenol phase.
* Incomplete dissolution of the final RNA pellet.

RNA degradation
* Tissues were not immediately processed or frozen after removal
from the animal.
* Samples used for isolation, or the isolated RNA preparations were
stored at -5 to-20°C, instead of -60 to -70°C.
* Cells were dispersed by trypsin digestion.
* Aqueous solutions or tubes were not RNase-free.
* Formaldehyde used for agarose-gel electrophoresis had a pH below 3.5.

DNA contamination
* Sample homogenized in too small a reagent volume.
* Samples used for the isolation contained organic solvents
(e.g., ethanol, DMSO), strong buffers, or alkaline solution.

Proteoglycan and polysaccharide contamination

The following modification of the RNA precipitation (step 3) removes these contaminating compounds from the isolated RNA. Add to the aqueous phase 0.25 ml of isopropanol followed by 0.25 ml of a high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) per 1 ml of TRIzol® Reagent used for the homogenization. Mix the resulting solution, centrifuge and proceed with the isolation as described in the protocol. The modified precipitation effectively precipitates RNA while maintaining polysaccharides and proteoglycans in a soluble form. A combination of the modified precipitation with an additional centrifugation of the initial homogenate (note 2, RNA isolation protocol) is required to isolate pure RNA from plant material containing a very high level of polysaccharides.

PureLink™ Quick Gel Extraction Kit - Invitrogen – Unicamp-Mexico – 2009
http://2009.igem.org/Team:UNICAMP-Brazil/Protocols

1. Equilibrate a water bath or heat block to 50°C.
2. Excise the area of the gel containing your desired DNA fragment and minimize the amount of agarose surrounding the DNA fragment.
3. Weigh the gel slice containing the DNA fragment using a scale sensitive to 0.001 g, then place the gel into a polypropylene microcentrifuge tube and add Gel Solubilization Buffer (GS1) as directed below.
4. For ≤2% agarose gels: place up to 400 mg of the excised gel containing the DNA fragment into a 1.5 ml polypropylene tube. Add 30microL Gel Solubilization Buffer (GS1) for every 10microL volume of gel.
5. Place the tube containing the gel slice and GS1 into a 50°C water bath or heat block.
6. Incubate at 50°C for 15 minutes. Invert the tube by hand every 3 minutes to mix and ensure gel dissolution.
7. After the gel slice appears dissolved, incubate for an additional 5 minutes.
8. Place a Quick Gel Extraction Column inside a Wash Tube and load the dissolved gel mixture with DNA onto the center of the Column.
9. Centrifuge at >12,000 × g for 1 minute.
10. Add 500–700 µl Wash Buffer (W1) containing ethanol.
11. Centrifuge at >12,000 × g for 1 minute, then discard the flow-through.
12. Centrifuge again at maximum speed for 2–3 minutes to remove residual Wash Buffer and ethanol. Discard the Wash Tube, and place the Column into a Recovery Tube.
13. Add 50 µl Elution Buffer (E5) to the center of the Column.
14. Incubate for 1 minute at room temperature.
15. Centrifuge at >12,000 × g for 1 minute to elute the purified DNA into the Recovery Tube.
16. Store the purified DNA at -20ºC.

Yeast RNA extraction – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols

1-Transfer 1.5 ml of liquid culture of yeast grown for 20 - 24 h at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose) into a microcentrifuge tube. Pellet cells by centrifugation at 20,000 × g for 1-5 minutes
2- Ressuspend cells with 200 µl of TES, Add 400 µl of acid phenol and Incubate 1 hour 65C, vortexing every 10 minutes.
3- Incubate on Ice 10 min
4- Spin 5 min at 4 deg and transfer top layer to a new tube.
5- Add 400 µl acid phenol
6- Repeat step 4
7- add 400 µl chloroform, vortex and repeat step 4

8- Add 40 µl 3M Na Acetate, pH 5.3, 2.5 volumes of ethanol, repeat step 4, discart supernadant.
9- Wash pellet with 500 µl cold 80% ETOH, and air dry
10- Resuspend in 50 microliters H2O. Store RNA at –70 deg

TES:
0,5% SDS
10 mM Tris pH 7.5
10 mM EDTA
RNase-free water

Yeast DNA extraction – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
1. Transfer 1.5 ml of liquid culture of yeast grown for 20 - 24 h at 30°C in YPD (1% yeast extract, 2% peptone, 2% dextrose) into a microcentrifuge tube. Pellet cells by centrifugation at 20,000 × g for 1-5 minutes.
2. Add 200 µl of Harju- buffer
3. Add ~300 µl glass beads. Add 200 µl of phenol /chloroform/ IAA (25:24:1).
4. Vortex 2 minutes.
5. Centrifuge 3 minutes at room temperature, 20,000 × g.
6. Transfer the upper aqueous phase to a microcentrifuge tube containing 400 µl ice-cold 100% ethanol. Mix by inversion or gentle vortexing.
7. Incubate on ice temperature, 1 hour.
8. Centrifuge 5 minutes at room temperature, 20,000 × g.
9. Remove the supernatant
10. Wash the pellet with 0.5 ml 70% ethanol
11. Centrifuge 5 minutes at room temperature, 20,000 × g.
12. Remove supernatant.
13. Air-dry the pellets at room temperature or for 5 minutes at 60°C in a vacuum dryer.
14. Resuspend in 25- 50 µl water.

Harju- Buffer
– 2% Triton X-100
– 1% SDS,
– 100 mM NaCl
– 10 mM Tris-HCl, pH 8.0,
– 1 mM EDTA

Preparation of electrocompetent *E. coli* – Unicamp-Mexico – 2009
http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
1. Preferably, select single colony of E. coli from fresh LB plate to inoculate a 100 ml LB overnight (O/N) starter culture. Grow the starter culture O/N in 37°C shaker (250rpm).
2. Inoculate 1L of LB media and place culture in 37° shaker. Grow cells and measure OD600 every 30min. When the OD600 equals 0.5-0.8 (log phase growth), remove the cells from the shaker and place on ice.
NOTE: It very important to keep the cells at 4°C (or on ice) for the remainder of the procedure.
3. Split the 1L culture into four equal parts by pouring ~250ml of culture into each chilled 250ml Corning pointed bottle.

4. Spin in GPR centrifuge at 4000rpm, 15min at 4°C.
5. Place bottles on ice. Remove supernate immediately as cell pellet begins to lift off quickly. Gently resuspend each pellet in 250ml ice-cold 10% glycerol.
6. Spin in GPR centrifuge at 4000rpm, 15min at 4°C.
7. Place bottles on ice. Remove supernate. Gently resuspend **each** pellet in 250 ml of ice-cold 10% glycerol.
8. Spin in GPR centrifuge at 4000rpm, 15min at 4°C.
9. Place bottles on ice. Remove supernate. Gently resuspend **each** pellet in 10ml ice-cold 10% glycerol.
10. Spin in GPR centrifuge at 4000rpm, 15min at 4°C.
11. Place tubes on ice. Remove supernate. Gently resuspend **each** cell pellet in 1ml of ice-cold 10% glycerol.
12. With cell suspensions on ice, prepare aliquots of 40 ul of cells in pre-chilled 1.5ml eppendorf tubes. Snap freeze tubes containing cells in liquid N2. Store frozen cells at -80°C.

Electroporation – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
1. Set the electroporation apparatus to 2.5 kV.
2. Add 2 µl plasmid DNA to tubes containing 40 µl fresh or thawed cells on ice. Mix by swirling with pipette tip.
3. Transfer the DNA and cells to a pre-chilled electroporation cuvette (0.2 cm electrode gap) using a narrow pipette tip. Wipe any ice or water from sides of cuvette using a Kimwipe. Place the cuvette into the sample chamber.
4. Energize the electroporation apparatus and deliver the pulse by pushing in both charging buttons simultaneously and holding until a short beep is heard. Note the time constant of the pulse and the actual voltage delivered.
5. Remove the cuvette from the sample chamber. Immediately add 1 ml LB medium and transfer the cells to a sterile polypropylene culture tube using a Pasture pipette. (Failure to immediately add SOC to the electroporated cells can significantly reduce cell viability and decrease transformation efficiency.)
6. Incubate cultures for 60 to 180 minutes at 37°C on a roller or with moderate shaking to allow for plasmid expression.
7. Plate aliquots of the electroporation mixture on L-agar plates supplemented with the appropriate antibiotics. Incubate plates at 37°C.
Protocol adapted from: http://wheat.pw.usda.gov/~lazo/methods/goldberg/electro.html

Thermal transformation – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
1. Keep the E. coli on ice for 30 minutes
2. Add 10µl ligation reaction
3. Keep the E. coli on ice for more 30 minutes
4. 42ºC for 1`30``
5. Put in the ice immediately for 5 minutes.
6. Add 500µl LB media
7. 37ºC for 1 hour
8. Plate aliquots of the mixture on LB plates supplemented with the appropriate antibiotics. Incubate plates at 37°C.

Miniprep – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols

This procedure is used to extract plasmid DNA from bacterial cell suspensions and is based on the alkaline lysis procedure developed by Birnboim and Doly (Nucleic Acids Research 7:1513, 1979).
Procedure
1. Gently swirl the contents of the culture tube to resuspend the cells.
2. Label 1.5 mL tubes and pipet 1500 uL of the cell suspension into each tube.
3. Close the caps and place the tubes in a centrifuge and spin at maximum speed for 2 minutes.
4. Withdraw and discard the supernatant in a waste container.
5. Add 300 uL of Buffer 1 (50 mM Tris-HCl, 10 mM EDTA, 100 ug/mL RNase A, pH 8.0) to each tube and resuspend the cells by vortexing. It's very important that the cell suspension is homogenous and no clumps are visible.
6. Add 300 uL of Buffer 2 (1% SDS, 0.2 M NaOH) to each tube. Close the caps and mix the solutions by rapidly inverting them a few times. DO NOT VORTEX since the chromosomal DNA released from the broken cells could be sheared into small fragments and contaminate your plasmid prep.
7. Let tubes stand on ambient temperature for 5 minutes.
8. Add 300 uL of ice-cold Buffer 3 (3.0 M Potassium Acetate, pH 5.5) to each tube. Close the caps and mix the solutions by rapidly inverting them a few times. A white precipitate will form.
9. Let tubes stand on ambient temperature for 15 minutes.
10. Place the tubes in a centrifuge (balanced) and spin at maximum speed for 15 minutes. The precipitate will pellet along the side of the tube.
11. Transfer the supernatants into clean 1.5 mL tubes, being careful not to pick up any of the precipitate. Discard the tubes with the precipitate and KEEP the tubes with the supernatant.
12. To each tube of supernatant add 400 uL of isopropanol to precipitate the nucleic acids. Close the caps and mix vigorously, place them in a centrifuge (balanced) and spin at maximum speed for 10 minutes. This step pellets the nucleic acids but if you leave it around too long, proteins remaining in solution will begin to precipitate as well.
13. Plasmid DNA pellet may be difficult to see. Carefully remove and discard the supernatant. The pellet is usually visible at this point. If not, do not despair. It may be too small to see but there is probably enough DNA there.
14. Add 600 uL of absolute ethanol to each tube and mix by inversion several times.
15. Spin the tubes at maximum speed in a centrifuge for 5 minutes.
16. Carefully remove and discard the supernatant. Try to get as much out as possible without dislodging the pellet of plasmid DNA.
17. Place the tubes in the fume hood with the caps open for 15-20 minutes to dry off the last traces of ethanol.
18. When the ethanol is gone (you can check this by smelling the tube) add 20 uL of MiliQ water to dissolve the pellet. Pipet the 20 uL in and out, up the side of the tube to ensure that all of the plasmid DNA comes into contact with the water.
19. Pool the 20 uL solutions into one labeled tube and store it in the freezer

DNA extraction – Unicamp-Mexico – 2009 http://2009.igem.org/Team:UNICAMP-Brazil/Protocols
Aubusel 1998 protocol with modifications
1- Grow bacterial strain in 4ml of LB medium overnight.
2- Centrifuge 2ml of the medium for 5 minutes at 6000rpm to pellet the cells
3- Resuspend the pellet in 400µl TE buffer by repeated pipetting. Add 30µl SDS 10% and 2,5µl 20mg/ml proteinase K. Mix thoroughly and incubate for 30 minutes at 37°C.
4- Add 100µl NaCL 5M. Mix thoroughly.
5- Add 100µl CTAB/NaCL solution (0,8G/l CTAB, 0,4g/l NaCl). Incubate 10 min at 65°C.

6- Add 750µl chloroform/isoamyl alcohol (24:1). Mix thoroughly and centrifuge for 10 min at 1200rpm. The original protocol uses phenol in this phase, but we are avoiding using this substance due its risks to our health and the environment.
7- Recover aqueous supernatant to a new tube, leave the interface behind. Add 600µl isopropanol at -20°C to precipitate the nucleic acids. Incubate at room temperature for 30min.
8- Centrifuge for 30 min at 12000rpm, discard the liquid maintaining the pellet in the tube. Wash the pellet 2x with ethanol 70% at -20°C.
9- Let the pellet dry at room temperature and then resuspend with 50µl H2O or TE.

MIDIPREP – IPN-UNAM-Mexico – 2009 http://2009.igem.org/Team:IPN-UNAM-Mexico/Protocols
Modified from "QIagen Plasmid Purification Midi".
Inoculate a colony in 25 - 50 ml of LB Medium with antibiotic. For low copy plasmids, inoculate 100 - 200 ml of medium.
Centrifuge 20 minutes at 4000 rpm and get the pellet.
Suspend the pellet in 4 ml of P1 QIagen Buffer (Be sure to add RNAse to the buffer).
Add 4 ml of P2 QIagen and mix on inversion 6 times.
Add 4 ml of P3 QIagen and mix on inversion, add 10 ml of chloroform and mix on inversion 6 times.
Incubate on ice 10 minutes and centrifugate 20 minutes at 4000 rpm.
Recover the aqueous phase and add 5ml of isopropanol, incubate 30 minutes on ice.
Centrifugate 15 minutes at 13000 rpm.
Wash with EtOH at 70% and centrifugate 2 min at 13000 rpm, let the pellet dry and suspend on 500 ml of esterile deionized water (Water for pcr).
Competent cells with RbCl – IPN-UNAM-Mexico – 2009 http://2009.igem.org/Team:IPN-UNAM-Mexico/Protocols
Take 5 ml of liquid SOB and incubate at 37 °C overnight
Take 1 ml of cell culture and innoculate into 500 ml of YENB media and incubate at 37 °C and 200 rpm until O.D.=0.5-0.55
Transfer the cells to 250 ml bottles (must be cold)
Incubate for 30 minutes on ice
Centrifuge at 2500 rpm for 15 min at 4°C.
Resuspend cells in 20 ml of ice-cold RF1 solution
Incubate for 15 min on ice
Centrifuge at 2500 rpm for 15 min at 4°C.
Resuspend cells in 2 ml of ice-cold RF2 solution.
Incubate for 15 min on ice and divide into 200 µl aliquots and store at -70°C
RF1 solution
Rubidium chloride...................... 100 mM
Manganese chloride tetrahydrate......... 50 mM
Potassium Acetate...................... 30 mM
Calcium chloride dihydrate............. 10 mM
Gycerol................................ 15 %
Adjust pH to 5.8 with 0.2 M of glacial acetic acid and sterilize by filtration using a 0.22 µm filter

RF2 solution
Rubidium chloride...................... 100 mM
MOPS................................... 10 mM
Calcium cloride dihydrate............... 75 mM
Gycerol................................ 15 %
Adjust pH to 6.8 with 0.2 M of NaOH and sterilize by filtration using a 0.22 µm filter

CTAB miniprep – IPN-UNAM-Mexico – 2009 http://2009.igem.org/Team:IPN-UNAM-Mexico/Protocols
Take 1.5 ml of cell culture and centrifuge at 15000 rpm for 3 minutes.
Discard liquid phase
Repeat 1 and 2
Resuspend cells with 1 ml of NaCl 1.2 M with vortex
Centrifuge at 15000 rpm for 3 minutes and discard supernatant.
Add 100 µl of sterile water and resuspend with vortex
Add 200 µl of STET and mix with vortex.
Add 4 µl of lysozyme and mix with vortex.
Incubate for reaction to occur for 5 min at 37°C then boil for 45 seconds.
Boil 45 seconds inside a glass with water
Centrifuge at 15000 rpm for 10 min.
Discard pellet.
Add 8 µl of CTAB and centrifuge at 15000 rpm for 5 min.
Discard supernatant
Resuspend with 300 µl of NaCl 1.2 M using vortex.
Add 1 ml of ethanol and incubate at -20°C for 20 min.
Centrifuge at 15000 rpm for 10 min.
Discard supernatant.
Wash with 1 ml of 70% ethanol and centrifuge for 3 min.
Drop out ethanol by decantation.
To dry remaining ethanol use a thermoblock at 65°C.
Resuspend with 100 µl of sterile water and add 1 µl of RNase.
Incubate at 37°C for 30 min.
DNA purification from agarose gel – IPN-UNAM-Mexico – 2009 http://2009.igem.org/Team:IPN-UNAM-Mexico/Protocols
In a UV room, cut the gel with a knife and place DNA bands inside falcon tubes
Place tubes on a tared balance to get the gel weight.
Multiply the gel weight by three to obtain the volume of QG buffer to be added
Add the volume of QG buffer obtained in the previous step.
Place tubes on thermoblock for 10 min at 55°C.
Prepare one column for every tube.
Take 800 µl of the tubes and place the volume in the column.
Centrifuge the columns at 13000 rpm for 1 minute with the lid open.
Add 800 µl of PE to every column and wait 6 minutes.
Centrifuge at 13000 rpm for 1 minute.
Discard supernatant and centrifuge again at 13000 rpm for 30 seconds.
Place column inside a Eppendorf tube
Add 25 µl of deionized water to the column and wait for 6 minutes.
Centrifuge at 15000 rpm for 2 minutes.

Making cells competent – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
Back to top
Most of the time, we used Top10 chemically competent cells. We did make a stock of chemically competent DB3.1 cells with the following protocol (found on OpenWetWare). We found that these cells were indeed very competent.
You will need TSS Buffer.
Preparing the cells:

Grow a 5ml overnight culture of cells in LB media.
In the morning, dilute this culture back into 25-50ml of fresh LB media in a 200ml conical flask. You should aim to dilute the overnight culture by at least 1/100.
Grow the diluted culture to an OD600 of 0.2 - 0.5. (You will get a very small pellet if you grow 25ml to OD600 0.2)
Put eppendorf tubes on ice now so that they are cold when cells are aliquoted into them later. If your culture is X ml, you will need X tubes. At this point you should also make sure that your TSS is being chilled (it should be stored at 4oC but if you have just made it fresh then put it in an ice bath).
Split the culture into two 50ml falcon tubes and incubate on ice for 10 min.
All subsequent steps should be carried out at 4oC and the cells should be kept on ice wherever possible
Centrifuge for 10 minutes at 3000 rpm and 4oC.
Remove supernatant. The cell pellets should be sufficiently solid that you can just pour off the supernatant if you are careful. Pipette out any remaining media.
Resuspend in chilled TSS buffer. The volume of TSS to use is 10% of the culture volume that you spun down. You may need to vortex gently to fully resuspend the culture, keep an eye out for small cell aggregates even after the pellet is completely off the wall.
Add 100 µl aliquots to your chilled eppendorfs and store at – 80oC.
Transformations – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
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Standard transformation procedure
Remove competent cells from -80, let thaw for 10 min on ice and aliquot in 50 ul amounts.
add 2-5 ul of vector, usually in H2O, to 50 ul cells, no mixing by pipet due to shear induction.
keep on ice for 20 minutes (vector spreading through volume)
heat shock (42°C) for 45 seconds
keep on ice for 2 minutes
add 200 ul SOC, put on 37°C for 1 hour or longer with agitation.
plate out 250 ul on appropriate antibiotics.
Prepering chemically competant cells - TMF Buffer – TUDelft – 2009
http://2009.igem.org/Team:TUDelft/Protocols
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Materials
Plate of cells to be made competent
TMF buffer
LB media
Ice
Glassware & Equipment
Falcon tubes
500µl Eppendorf tubes, on ice
200ml conical flask
200µl pipetman or repeating pipettor
5ml pipette
Preparation
Grow a 5ml overnight culture of cells in LB media. In the morning, dilute this culture back into 40ml of fresh LB media with 0.8 ml of Mg-mix (0.5M Magnesium chloride + 0.5M Magnesium sulfate) in a 100ml conical flask. You should aim to dilute the overnight culture by at least 1/100.
Grow the diluted culture to an OD_{600} of 0.5 - 0.8.
Put eppendorf tubes on ice now so that they are cold when cells are aliquoted into them later. If your culture is X ml, you will need X tubes. At this point you should also make sure that your TMF is being chilled (it should be stored at 4°C but if you have just made it fresh then put it in an ice bath).
Split the culture into two 50ml falcon tubes and incubate on ice for 10 min.

All subsequent steps should be carried out at 4°C and the cells should be kept on ice wherever possible

Centrifuge for 15 minutes at 4000 rpm and 4°C.

Remove supernatant. The cell pellets should be sufficiently solid that you can just pour off the supernatant if you are careful. Pipette out any remaining media.

Resuspend in 4ml chilled TMF buffer and add 1 ml of 40% glycerol. You may need to vortex gently to fully resuspend the culture, keep an eye out for small cell aggregates even after the pellet is completely off the wall.

Add 100 µl aliquots to your chilled eppendorfs.

Flash freeze the eppendorfs containing the cells with liquid nitrogen.

Store the cells at -80°C.

It is a good idea to run a positive control on the cells.

Preparing electro-competent cells – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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For making the electro-competent cells we used this protocol from openwetware.org

Electroporation – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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For doing the electroporation on the electro-competent cells we used this protocol from openwetware.org

Restrictions and Ligations – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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We used the New England Biolabs' Assembly Kit for this purpose and exactly followed the protocol specified by them. It works well.

Purifying small DNA parts – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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We used standard Qiagen PCR purification kit.

DNA precipitation – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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Standard Qiagen Minprep Kit was used for plasmid isolation.

Colony PCR – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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Make biobrick mastermix, containing per sample:

12.5 ul *Taq* mastermix

2.5 ul 10x forward biobrick primer

2.5 ul 10x reverse biobrick primer

7.5 ul H2O

Put 25 ul in the PCR tubes.

With a toothpick or pipet point, touch a colony and stir it through the fluid

Run the iGEM colpcr program *(to be added later)*

PCR using *Taq* Mastermix – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols

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Contents of the PCR mix is the for a large part the same as mentioned above for the Colony PCR. Differences will be noted here. First, instead of biobrick primer, any primer of choice can be added, also 2.5ul if standard solution has a concentration of 10 pmol/ul. Also x ul template DNA from a sample is added, where x depends on the total concentration of DNA in the sample. Typically 50 to 100 ng of total DNA is added. 7.5 - x ul of H_2O is added to the mix.

PCR program is:

1. 5' @ 95ºC
2. 1' @ 95ºC
3. 1' @ annealing temperature of the primer
4. 1' @ 72ºC (1' is long enough for 1kb, longer times can be used if larger products are formed)
5. repeat steps 2-4 29x (total of 30 cycles, more can be added if necessary)

6. 5' @ 72ºC
7. ∞ @ 4ºC (PCR can be stopped and stored in the fridge at any time from this point on)
PCR using *Pfx* polymerase – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
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Mastermix does not exist for the *Pfx* polymerase. This means the components have to be added seperately. The mix consists of:
x ul template DNA (again 50 - 100 ng total)
5.0 ul 10x buffer
2.5 ul forward primer (10 pmol/ul)
2.5 ul reverse primer (10 pmol/ul)
0.2 ul *Pfx*
1.5 ul dNTP's (10 mM)
1.0 ul $MgSO_4$ (50 mM)
37.3-x ul H_2O
The PCR program looks the same as mentioned above for Taq polymerase, only difference is the elongation temperature in step 4. This is 68ºC for *Pfx*.
DNA gels – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
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Take a flask of 0.8% up to 1.5% molten agarose from the 70oC stove.
Pour a it in a taped gel tray.
Add ca. 5 ul of SYBRSafe (depending on size gel)
Add a comb and let the gel harden for ca. 15 minutes.
Remove the comb and the tape and put the gel tray in an electrophoresis tray.
Add enough 1x TBE to completely cover the gel.
Add DNA loading buffer to your samples and load them.
Let the gel run at a voltage between 60V and 120V, depending on desired resolution/time available.
Visualize the DNA by putting it in the imager for taking a picture, or if you want to cut out your DNA, put it on the blue light emitter.
Fluorescence Measurements – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
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The samples to be tested are cultured from plates in 2ml of the Basal Minimal Medium with appropriate antibiotics and incubated overnight at 37°C at 175 rpm.
The culture is next day checked for OD600 and then diluted to 100 times in a 96 well plate by the same medium with antibiotics.
The plate is then first read at OD600 and is then incubated again at 37°C with medium shaking for around 3 hours.
The plate is then taken out and read at OD600 based on which the cultures are diluted to 10 times which must be around (0.1) with calculated samples induced with 0.1mM IPTG and 0.2mM IPTG.
The plate reader is then read by the automatically repeating protocol with shaking at medium speed created by BioTek Synergy.
The program does the following:
Set Temperature to 37°C
In a kinetic loop of fixed time (We used 2 hour 30 mins or 16 hour 30 mins) following measurements are taken in a time interval of 10 minutes or 20 minutes with shaking: Absorbance (600 nm filter) and Fluorescence (485nm and 520nm for GFP).
Then a delay of 100 seconds is made.
If the protocol is programmed to generate the results in excel sheet then it is easy to get the results of the well data and interpret them.
Antibiotics (1000x stock solutions) – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
Ampicillin: 100 mg/ml in H_2O
Chloroamphenicol: 34 mg/ml in etOH

Kanamycin: 10 mg/ml in H_2O
Tetracycline: 5 mg/ml etOH
SOB (Super Optimal Broth) – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
For 1 liter dissolve in H_2O
20 g Bacto tryptone
5 g Bacto-Yeast extract
0.5 g NaCl
10 ml 250 mM KCl
adjust pH to 7.0
before use add 5 ml of 2mM $MgCl_2$
SOC (Super Optimal broth with Catabolite repression) – TUDelft – 2009
http://2009.igem.org/Team:TUDelft/Protocols
add 20 mM glucose to 1L SOB.
You can also order small bottles from Invitrogen (which is what we did)
LB medium (Lysogeny Broth[1], but better known as Luria-Bertani Medium) – TUDelft – 2009
http://2009.igem.org/Team:TUDelft/Protocols
In 950 mL H_2O
10 g Bacto Tryptone
5 g Bacto-Yeast extract
10 g NaCl
adjust pH to 7.0
TSS buffer – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
For 50 mL:
5g PEG 8000
1.5 mL 1M MgCl2 (or 0.30g MgCl2*6H20)
2.5 mL DMSO
Add LB to 50 mL
Filter sterilize (0.22 µm filter) TSS buffer and store at 4ºC or -20ºC
TMF buffer – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
For 50 mL:
100mM CaCl2
50mM RbCl
40mM MnCl2
Add ddH2O to 50 mL
Filter sterilize (0.22 µm filter) TMF buffer and store at 4ºC or -20ºC
Basal Minimal Medium – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
For 1 litre:
K2HPO4 - 9 g
KH2PO4 - 3 g
(NH4)SO4 - 2 g
NaCitrate - 0.5 g
MgSO4 10x (10gr/L) (1%)
Glucose 10x 20%
vitamin B1 (thiamine) 200x 2 mg/mL
Amino Acids 20x 10 mg/mL
Add all except glucose solution and mix well in ddH2O. Autoclave and then add filter sterilized glucose solution.
10x TBE (Tris, Boric Acid, EDTA) – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
To make 1L, dissolve in 950 ml H_2O
54 g Tris
27.5 g Boric Acid

4.65 g EDTA or 20 ml 0.5M EDTA pH 8.0
6x DNA Gel loading buffer – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
Dissolve in H_2O
0.25% Bromophenolblue
0.25% Xylene Cyanol FF
40% (w/v) Sucrose
10x PBS (Phosphate Buffered Saline) – TUDelft – 2009 http://2009.igem.org/Team:TUDelft/Protocols
In 950 mL H_2O dissolve:
11.5g Na_2HPO_4
2g KH_2PO_4
80g NaCl
2g KCl
Adjust volume to 1L
The pH of 1x PBS should be 7.4

Protocol for Primer PCR – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
dNTP mix (10mM)
10ul 100mM dGTP
10ul 100mM dATP
10ul 100mM dTTP
10ul 100mM dCTP
60ul H2O
(Total V=100ul)

PCR reaction
39ul H2O
5.0ul Pfx-buffer (-MgSO4)
1.0ul 50mM MgSO4
1.5ul 10mM dNTP mix
1.5ul forward primer
1.5ul reverse primer
0.5ul Pfx enzyme (add just before PCR run)
PCR program
For PCR of BioBricks:

1.	Start	94˚C	2min
2.	Denaturing	94˚C	1min
3.	Annealing	52˚C	1min
4.	Elongation	68˚C	2min
5.	GOTO 2		rep. 29x
6.	End	68˚C	3min

7.	Hold	4˚C	

Protocol for colony PCR – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols

- A little colony is transfered to each tube (afterwards the same tip is used for plating out on a LA+Amp plate)
- The tubes are placed in the microwave at full power for 2 min with an open lid.
- 25 µl Pre-mix (with taq-pol) is added to the PCR tubes. Mix well by pipetting up/down.

Pre-mix (to 1 colony)
2,5 µl 10 x TAQ Buffer + $MgCl_2$
1,25 µl 10 pmol/µl forward primer
1,25 µl 10 pmol/µl reverse primer
0,50 µl dNTP
19,25 µl H_2O
0,25 µl Taq. Pol → Pre-mix is made without taq pol. which is added after the colonies have been zapped in the microwave,
=25 µl taq . pol is added to the pre-mix → PCR-tube
Make enough premix for your number of colonies + 3
PCR program

1.	Start	94˚C	2min
2.	Denaturing	94˚C	1min
3.	Annealing	52˚C	1min
4.	Elongation	72˚C	2min
5.	GOTO 2		rep. 29x
6.	End	72˚C	3min
7.	Hold	4˚C	

Protocol for purification of DNA from TAE and TBE agarose gel bands – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
Kit from GFX
Sample capture
Weigh a DNase-free 1,5 ml microcentrifuge tube
Excise band of interest from the gel and place in microcentrifuge tube
Weigh microcentrifuge tube plus agarose gel band
Calculate weight of agarose gel slice
Add 10 ul Capture buffer type 2 for each 10 mg agarose gel slice
Mix by inversion
Place at 60 degrees until agarose is completely dissolved
Sample binding
Add up to 600 ul Capture buffer-sample mix to assembled GFX MicroSpin columns and Collection tubes.
Leave at room temperature for 60 sec.
Centrifuge for 30 sec at 16000 g.

Discard the flow through in the Collection tube and place the MicroSpin column in the Collection tube again.
Repeat sample binding step until all sample is loaded onto the MicroSpin column.
Wash & dry
Add 500 ul Wash buffer type 1
Centrifuge for 30 sec at 16000 g.
Discard flow through and keep Collection tube as above.
Centrifuge again for 30 sec at 16000 g. More flow through will appear in the Collection tube. It is important to centrifuge this second time to get the sample completely dry. This step is not provided in the original protocol.
Discard Collection tube and transfer MicroSpin column to a clean 1,5 ml DNase-free microcentrifuge tube.
Elution
Add 10 – 50 ul Elution buffer type 4 or 6. We eluted with 10 ul in order to obtain a small volume and a high concentration of purified DNA. Only very big amounts of sample require higher elution volumes.
Leave at room temperature for 60 sec.
Centrifuge for 60 sec at 16000 g.
Retain flow through and discard MicroSpin columns
Store purified sample DNA at -20 degrees or proceed to cutting DNA or ligation.
Protocol for purification of DNA from PCR mixtures or an enzymatic reaction – SDU Denmark – 2009
http://2009.igem.org/Team:SDU-Denmark/Protocols
Kit from GFX
Sample capture
Add 500 ul Capture buffer type 2 to up to 100 ul sample
Mix thoroughly
Sample binding
Add Capture buffer-sample mix to assembled GFX MicroSpin columns and Collection tubes
Centrifuge for 30 sec at 16000 g.
Discard the flow through in the Collection tube and place the MicroSpin column in the Collection tube again.
Wash & dry
Add 500 ul Wash buffer type 1
Centrifuge for 30 sec at 16000 g.
Discard flow through and keep Collection tube as above.
Centrifuge again for 30 sec at 16000 g. More flow through will appear in the Collection tube. It is important to centrifuge this second time to get the sample completely dry. This step is not provided in the original protocol.
Discard Collection tube and transfer MicroSpin column to a clean 1,5 ml DNase-free microcentrifuge tube.
Elution
Add 10 – 50 ul Elution buffer type 4 or 6. We eluted with 10 ul in order to obtain a small volume and a high concentration of purified DNA. Only very big amounts of sample require higher elution volumes.
Leave at room temperature for 60 sec.
Centrifuge for 60 sec at 16000 g.
Retain flow through and discard MicroSpin columns
Store purified sample DNA at -20 degrees or proceed to cutting DNA or ligation.
Protocol for making cells (E.coli) competent for transformation – SDU Denmark – 2009
http://2009.igem.org/Team:SDU-Denmark/Protocols
(Cells are kept on ice at all times!! If the cells temperature rises above ~5º C they'll lose their competency!)
600 µl overnight (ON) Top10 E. coli culture is added to 60 ml Luria-Bertani (LB) medium.

Grows at 37º C while being shaken until the optic density (OD550) is 0,2.
Cool cells on ice.
Harvest the cells in screwcap tubes (4 × 10 ml).
Pour away the supernatant and keep the pellet on ice.
Wash the cells with 10 ml cold 50mM CaCl2.
Distribute 200 µL cells to each of many eppendorf tubes.
Add 41.7 µl 87% glycerol and mix well.
Store at -80º C.
Protocol for making cells (E. coli) competent for electroporation – SDU Denmark – 2009
http://2009.igem.org/Team:SDU-Denmark/Protocols
(Cells are kept on ice at all times!! If the temperature rises above ~5º C they'll lose their competency!)
2ml over-night cell culture is transferred to 200ml Luria Bertani (LB)-medium.
Incubate at 37˚C on shaker until OD450=0.5-0.7
Keep on ice for 15-30min and harvest by 4000 x g for 15 min at 4˚C.
Remove all supernatant and resuspend carefully in 200ml ice cold dH2O. Harvest as in 3.
Resuspend in 100ml ice cold dH2O. Harvest as in 3.
Resuspend in 20ml ice cold 10% glycerol. Harvest as in 3.
Resuspend in ice cold 10% glycerol until a total volume of 1ml. Cell concentration should be 1-3x1010 cells/ml.
Cells are distributed in tubes with 40ul in each and kept at -80˚C.
Protocol for transforming (E.coli) – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
Take 2 and 5 µl of the plasmid from the distribution plates and add it to 2 different tubes with 50 µl competent cells in each. As controls use 1 tube with competent cells only (control of the cells) and 1 tube with puc plasmid which is supercoiled and known to work (control of our plasmid).
Store on ice for 40 min.
Keep at 42º C for 2 min.
Keep on ice for 5 min.
Add 1 ml LB to each tube.
The tubes are stored at 37 º C for 2 hours while being shaken.
Centrifuge the tubes at 3500 rpm for 5 min.
Suck up and throw out 850 µl and resuspend the remaining by pipetting up and down.
Spread out 75 µl on 2 LA (LB + agarose) plates with antibiotics (100 µg/ml ampicillin). The competent cells of the control are spread out on 1 LA plate with antibiotics and 1 without, since they are not resistant.
Store at 37 º C over night.
Electroporation – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
This protocol is very useful compared to standard-transformation, because it uses the power of ZAP! Requires less raw material than a normal transformation and is faster.
Thaw competent cells at room temperature and then keep on ice. 0.2cm cuvettes for electroporation are cooled on ice.
For each transformation, transfer 40ul cells to a 1.5ml Eppendorf tube and add 1.5ul plasmid from distribution plates. Mix well and transfer to the bottom of a cuvette without making air bubbles. Keep on ice for 5min.
Place the cuvette in the electroporator and pulse once. Setup: Gene pulser: 25uF 2.5kV Pulse controller: 200ohm
Immediately add 1ml SOC medium to the cuvette, mix well by pipetting, and transfer to a 1.5ml Eppendorf tube and incubate immediately at 37˚C for 1h on shaker (500 rpm).
Plate the transformed cells onto LA plates containing antibiotics (100 ug/ml ampicillin). Untransformed cells (negative control) should be plated with and without antibiotics.
Miniprep protocol – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols

We used BIO-RAD Quantum Prep, Plasmid Miniprep Kit.
All centrifugation steps are performed at maximum speed (12.000-14.000 g = 14.000 rpm).
Transfer an ON culture (2 mL) of plasmid-containing cells to a microcentifuge tube. Pellet the cells by centrifugation for 30 sec. Remove all of the supernatant by aspirating or pipetting.
Add 200 µl of the Cell Resuspension Solution and vortex or pipet up and down until the cell pellet is completely resuspended.
Add 250 µl of the Cell Lysis Solution and mix by gently inverting the capped tube about 10 times (do not vortex). The solution should become viscous and slightly clear if cell lysis has occurred.
Add 250 µl of the Neutralization Solution and mix by gently inverting the capped tube about 10 times (do not vortex). A visible precipitate should form.
Pellet the cell debris for 5 min in a microcentrifuge. A compact white debris pellet will form along the side or at the bottom of the tube. The supernatant (cleared lysate) at this step contains the plasmid DNA.
While waiting for the centrifugation step at step 5, insert a Spin Filter into one of the 2 ml wash tubes supplied with the kit. Mix the Quantum Prep matrix by repeated shaking and inversion of the bottle to insure that it is completely suspended (no tubes are supplied with the sample kit, however, most 2 and 1,5 ml tubes will accommodate the Spin Filters).
Transfer the cleared lysate (supernatant) from step 5 to a Spin Filter, add 200 µl of thoroughly suspended matrix, then pipet up and down to mix. If you have multiple samples, transfer the lysates first, then add matrix and mix. When matrix has been added to all samples and mixed, centrifuge for 30 sec.
Remove the Spin Filter from the 2ml tube, discard the filtrate at the bottom of the tube and replace the filter in the same tube. Add 500µL of Wash Buffer and wash the matrix by centrifugation for 30 seconds.
Remove the Spin Filter from 2 ml tube, discard the filtrate at the bottom of the tube and replace the filter in the same tube. Add 500 µL of Wash buffer and wash the matrix by centrifugation for a full 2 minutes to remove residual traces of ethanol.
Remove the Spin Filter and discard the microcentrifuge tube. Place the filter in one of the 1.5 mL collection tubes supplied with the kit or any other standard 1.5 mL microcetrifuge tube which will accommodate the Spin Filter. Add 50 µL of deionized H2O. Elute the DNA by centrifugation for 1 minute at top speed.
Discard the Spin Filter and store DNA at -20 degress Celsius.
Restriction digest - Protocol 1 – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
Pool plasmid and dry on vacuum centrifuge down to about 50uL
Mix the following into one tube:
4uL Plasmid and RIP
5uL 10x Buffer
0,5uL BSA
Fill with water to 47uL (37,5uL)
1,5uL Enzyme 1
1,5uL Enzyme 2
Incubate for 2 hours on 37 degress C.
Inactivate the enzymes at 80 degress C for 20 minutes.
Place product on -20 degress C or continue immediately to ligation.
Restriction digest - Protocol 2
Fast digest restriction enzymes. Fast digest restriction enzymes have proved more efficient for cutting DNA, and is less time-consuming to work with. Fast Digest enzymes can be bought at Fermentas.
24 ul water
2 ul enzyme
4 ul Fast Digest buffer
10 ul PCR product
Leave for 15 min at 37 degrees. Afterwards, inactivate the enzyme for 20 min at 80 degrees.
Be aware, cut only with ONE enzyme at a time.

After cutting with enzyme 1, isolate and purify the DNA fragments on a gel before applying enzyme 2. The final volume when cutting is 40 ul.
Add 4 ul loading buffer to the eppendorf tube and load directly onto gel.
Run gel and purify from gel.
Eluate with 10 ul when purifying from gel.
Eventually, place your sample in the vacuum centrifuge in order to get a smaller volume and greater concentration before ligation is applied. Final volume prior to ligation should be 5 ul.
Restriction digest - Protocol 3

Fav
Restriction protocol á la Anna.
Check that you have all the enzymes and prepare two gels.
2 ul PCR product
2 ul buffer (Green buffer)
1 ul Enzyme 1
1 ul Enzyme 2
14 ul H20
Mix the products gently together. Quick spin down. Incubate 5 min. at 37 degrees, mildly shaken.
After 5 min. quickly load 15 ul in one gel (purification gel), and the remaining 5 ul in the other gel (test-gel). After 15-20 min. check the test-gel, print a picture, and cut out the correct band of the purification gel and purify using the TAE/TBE protocol.
Ligation - Protocol 1 – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols

Fav
Mix the following:
2uL 10x Ligase buffer
1uL T4 DNA ligase
2 or 4 uL cut backbone
5 or 10 uL cut PCR product
Leave at 17 degress C overnight.
Use for electroporation.
Ligation - Protocol 2
Takes place in eppendorf tube.
2 ul 10x T4 ligase buffer
1 ul T4 ligase
5 ul PCR product (cut) of each brick which is to be ligated together - or 1 part plasmid and 5 part bricks
Leave at 17 degrees over-night.
Test ligation using PCR and run a test gel afterwards in order to check the PCR product has the right size.
Ligation - Protocol 3

Fav
Use our plasmid ligation helper to calculate the volume of Plasmid and Insert to be mixed.
Mix cut Plasmid and cut Insert with the following:
2uL 10x Ligase buffer
1uL T4 DNA ligase
Leave at 17 degress C overnight.
Use for electroporation.

Protocol to Hot Phenol RNA Isolation – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
Solution 1: 0,3M sucrose; 0,01M NaAc, pH 4,5
Solution 2: 2% SDS; 0,01M NaAc, pH 4,5
Phenol saturated in 0,01-0,03M NaAc pH 4,5 containing 0,1% 8-hudroxyquinolin

I. 200 ml phenol + H_2O (2cm water on top)
II. Add 0,2g 8-hudroxyquinolin
III. Add 6,66ml 3M NaAc pH4,5
IV. Shake, leave over night and in the end check pH of the water phase.

1. Resuspend frozen cell pellet in 300 µl ice cold solution 1 and transfer into a 1,5 ml microcentrifuge tube containing 300 µl solution 2. Proceed immediately with 2.
2. Add 400 µl Phenol, mix and incubate for more than 3 min. at 65˚C. When handling multiple samples these steps are repeated until all tubes are collected in the heating block. Snap Freeze in liquid nitrogen, 15s and spin at max speed for 5 min.
3. Transfer the water layer to a fresh 1,5 ml microcentifuge tube containing 600 µl hot phenol (it is important to avoid the organic phase in all steps). Mix and incubate for 3 min. at 65˚C. Snap Freeze in liquid nitrogen, 15s and spin at max speed for 5 min.
4. Transfer the water layer to a fresh 1,5 ml micorcentrifuge tube containing 300 µl phenol and 300 µl chloroform. Mix and spin.
5. Transfer the water layer to a fresh 1.5 ml microcentrifuge tube containing 40 µl 3M NaAc pH 4,5 + 900 µl 96% EtOH. Incubate at -20˚C for 15 min (or over night if required).
6. Centrifuge at 20.000g (14681 rpm, Eppendorf 5417R) for 20 min. At 4˚C.
7. Carefully remove the supernatant and wash the pellet with 200 µl ice cold 70% EtOH. Spin at 20.000 g for 5 min. at 4˚C.
8. Carefully remove the supernatant and dry the pellet for max. 10 min. in speed-vac.
9. Resuspend the pellet in 50 µl RNase-free H_2O. spin again and transfer to a new tube.

Test of RNA
1. Mix. 2 µl RNA, 2 µl H_2O and 2 µl formaid loading buffer.
2. Run on agarose gel.
3. Test concentration on nanodrop.

Protocol for Northern Blotting – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
Gel mix (6%):
- 50 ml 10×TBE
- 75 ml 40% Acrylamide
- 30ml 2% Bis-acrylamide
- 240g $CH(NH_2)_2$
- Ajust with, H_2O to 500 ml

Preparation of samples
1. After determining the concentration, delute the samples to a concentration of 2 µg/µl. Take 5 µl of the diluted sample. This gives you 10 µg/lane.
2. Run prepared samples on agarose gel to check for decomposition.
3. Add the calculated amount to an eppendorf tube and adjust with loading buffer to 15 µl. (minimum 10 µl). Make sure the ratio between loading buffer and sample is 3:1
4. Preheat the gel for 30 min.
5. Denature the prepared samples at 95˚C for 3 min. Put samples on ice immediately after to avoid renaturation. The samples are now ready to be loaded on the gel.

6. Run the gel at 300 V for 2-3 hours until the BPB colour (the lowest) is 3-4cm form the bottom of the gel.
7. The gel is now ready for blotting

Semi dry northern electroblotting – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
1. Cut 6 pieces Whatman 3MM paper and 1 piece of Zeta-probe membrane (14,5 ×16 cm for small gel, 24×17 cm for large gel-or whatever area of the needs to be blotted)
2. Wet 2 pieces of Whatman paper in 1×TBE and place them on the top electrode (the cathode).
3. Place a pieces of paper on the gel and cut away the gel not covered by the paper. Remove the gel and paper from the glass plate and wet it slowly in 1×TBE. Place it on top of the sandwich with the gel facing up.
4. Wet the membrane in 1×TBE and place it on top of the gel.
5. Wet the remaining 3 pieces of paper in 1×TBE and place them on top of the membrane. Roll carefully with a glass or plastic pipette to remove air bubbles.
6. Assemble the blotting device and turn it around. Run at 400 mA for 1 hour.
Protocol for 5S rRNA hybridization – SDU Denmark – 2009 http://2009.igem.org/Team:SDU-Denmark/Protocols
Probe to 5S rRNA:
• 2 µl ss RNA 1pmol/ µl
• 1 µl PNK buffer (10x)
• 4 µl H2O
• 2 µl 32P-ATP
• 1 µl PNK (polynucleotide kinase)
• Incubate for 1 hour at 37˚C
• Add 1 µl DNAse
• Incubate for 15 min. at 75˚C.
• Store in LED tubes at -20˚C until use.
• Prior to use denature probe for 2 min. at 90˚C.

Hybridization
1. Preincubate the membrane in 7 ml hybridization buffer for 30 min. at 42˚C.
2. Add 10 µl probe
3. Incubate over night at 42˚C.

Wash of membrane
1. Add Low stringency buffer (fill up the tube half-way) at room temp. and wash for 5 min.
2. Add High stringency buffer (fill up the tube half-way) and wash for 10min at 42˚C.
3. Let the membrane air-dry and wrap it in vita-wrap.
Buffer-solutions:
Low stringency buffer (2X SSC, 0,1% SDS) To 500ml milliQ water add 100ml 20X SSC and 10 ml 10% SDS. Adjust with milliQ water to 1l.
High stringency buffer (0,5% SSC, 0,1%SDS) To 500ml milliQ water add 25ml 20X SSC and 10ml 10% SDS. Adjust with milliQ water to 12l.

Recovery of cwlD spores – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/MoreProtocols
Sheffield graciously gave us some non-germinating spores. There are two methods which can be used to recover the cwlD spores, however, neither give complete restoration of germination.

Method A
Method A (fast method resulting in partial germination ~ approx. 0.1% recovery)
Sekiguchi, J., Akeo, K., Yamamoto, H., Khasanov, F., Alonso, J. & Kuroda, A. (1995). Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, cwlD, which affects germination in *Bacillus subtilis*. J Bact 19; 5582-5589.
Spores suspended in lysozyme solution (200 µg lysozyme per ml in 10 mM potassium phosphate, 50 mM KCl, 1mM $MgCl_2$).
Incubation at 37°C for 30 min.
Heat activated at 70°C for 30 min.
Germination in 10 mM L-alanine at 37°C.

In addition to the protocol, it is important to take note of the following points
Typically, 10ul of cwlD spores are added to the lysozyme and buffer solution.
After the addition of L-alanine to the solution (which would initiate germination), the solution should be left in the incubator for 10 minutes.
After 10 minutes, the eppendorf tube containing the solution should be spinned down for approximately a minute.
Note: Another eppendorf tube containing water should be placed on the opposite site to balance out the weight.
After spinning down the solution, the supernatant should be removed and the spores should be resuspended in 1000ul of LB.
(Image missing)
Method B
Method B (slow method involving stripping of spore coat layers for improved germination ~ approx. 10% recovery)
Harwood, C. & Cutting, S. (1990). Modern microbiological methods: molecular biological methods for Bacillus. John Wiley and Sons Ltd., West Sussex, U.K.; 405-408.
Cleaning of spore surface by centrifugation (15,000 g, 20 min, 4 oC) using the following buffers:
TEP buffer , 0.5 M KCl, 1% glycerol
1 M NaCl (twice)
TEP buffer, 0.1 % SDS
TEP buffer
1M NaCl
dH2O, 0.01 % (w/v) Tween 80, 2 mM PMSF, 5 mM EDTA (twice)
(TEP buffer – 50 mM Tris-HCl pH7.4; 5 mM EDTA; 1 mM PMSF)
Removal of spore coat layers by alkali extraction:
Spores resuspended in 0.1 M NaOH
Incubate at 4 oC for 15 min (occasional vortexing)
Centrifuge (10,000 g, 10 min, 4 oC)
Pellet washed in dH2O (15,000 g, 10 min, 4 oC)
Heat activated at 70 oC for 30 min in Tris-HCl (pH 8.0 / 100 mM)
Germination in 10 mM L-alanine, 50 mM KCl.

Midiprep – Newcastle – 2009 http://2009.igem.org/Team:Newcastle/Project/Labwork/MoreProtocols
We follow the protocol from GenElute for midipreps. (NA0200S_NA0200). The list of plasmid kits can be accessed from Gen Elute's plasmid kits page.

Inducing competence in JM109 *E.coli* cells – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/MoreProtocols
For these *E.coli* cells a protocol devised by Promega (the company from which the competence agents were purchased) was used - see website (you will then need to open the Adobe file)

Preparing Competent *E.coli* cells for Heat Shock – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols

In general

This protocol is designed for 200 ml of cell culture which will result in 10-15 aliquots of 100 µl Ca2+-competent cells

Solutions required:

200 ml LB media (sterile)

150 ml pre-chilled 100 mM CaCl2 (sterile)

Pre-culture

Incubate plate overnight

Pick a single colony and inoculate 5 ml LB

Incubate overnight at 37°C

Culture

Inoculate 200 ml LB with 1ml of preculture

Incubate culture at 37°C on a shaker with 180 - 200 rpm

After 2 hours start measuring the OD600

When the culture reaches an OD600 between 0.1 and 0.2

Harvest cells

Carry out all steps on ice!!!

Transfer into one large centrifugation vial

Centrifuge cells 10 minutes at 4°C in a at 8'000 rpm

Carefully discard supernatant

Resuspend each pellet in 40 ml pre-chilled 100 mM CaCl2

Keep cells on ice for 40 minutes EXACTLY!!!

Centrifuge cells 10 minutes at 4°C at 8'000 rpm

Carefully discard supernatant

Resuspend each pellet in 1 ml pre-chilled 100 mM CaCl2

Freezing

To the the cell concentrate add 100% glycerol to give a final concentration of 0.1%

Aliquot 100 ul protions of the mix into sterile microfuge tubes

Shock-freeze cells in liquid nitrogen

Store samples at -80°C

Transforming DNA "Phil Style" – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols

Switch on heat block in flow to "LOW".

Go get cells out of -80˚C and leave on ice for 30 minutes.

Check to see if heat block is at approx 42-45˚C.

Add DNA 1-20 ul to cells after vortexing them.

Leave on ice for EXACTLY 30 mins.

Place tubes in heat block for EXACTLY 50 secs.

Transfer back to ice for 2 mins.

After 2 mins add 0.9 ml LB and incubate for 45-60 mins. At 37˚C.

Plate out 200 ul and 200ul of a 1:10 dilution and start praying.

Preparation of cells
Dilute a ON culture 1:200 into at least 200 ml LB
Grow to an OD600 between 0.1 and 0.2 (usually 2-3hrs)
Spin down cells
Resuspend in 40ml ice cold 0.1 M CaCl2 and leave on ice for 30 min.
Spin down cells and resuspend in 1 ml 0.1M CaCl2
Transfer cells to an 1.7ml tube and add 105 ul glycerol and make sure you get a homogeneous solution
Aliquot in 100 ul volumes into clean 1.7 ml tubes and shock freeze in liquid nitrogen
store at –80˚C until used up

Restriction Digests – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols
In general
Solutions required:
ddH2O (sterile and filtered)
Restriction enzyme and buffer
Your DNA

Simple Test Digests
For simple test digests the final volume should be 20 ul.
If you are testing vector DNA, quantify using a UV spec and dilute an amount down (usually 200 ul) to 0.05 ug/ul and use this as your working solution.

All digests must contain the following:
Sterile H2O giving a final volume of 20 ul (usually 7.5 ul)
2 ul 10x restriction buffer
10 ul of your DNA
0.5 ul Restriction Enzyme

All these should be added in the above order to prevent contamination.
Incubate at the appropriate temperature for 1 hour then run entire sample on a 0.8% agarose gel.

Cloning Experiments
For digests during a cloning experiment all final volumes should be 50 ul.
Sterile H2O giving a final volume of 50 ul
5 ul 10x restriction buffer
your DNA
1 to 2 ul Restriction Enzyme

For your vector DNA digest 20 ul of your working 0.05 ug/ul solution.
If your insert is a PCR reaction digest the lot after cleaning it up or for a subcloning digest 1-2 ug of plasmid DNA.
Incubate all reactions at the appropriate temperature for 3 hours.
For inserts (whether PCR products or fragments) run entire reaction on a gel in 2 lanes and extract DNA using Sigma Gel Extraction Kit.
For vectors use a standard ethanol precipitation to get rid of buffer and resuspend pellet in 20 ul ddH20.

Use DNA immediately for a Ligation (different protocol sheet).

Ligation Reactions – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols
In general
Before attempting a ligation you should read the information on the back
Ref: Molecular cloning vol 1 Sambrook et al.
Solutions required:
Digested DNA
T4 DNA ligase and buffer

Ligations
All ligations should have a final volume of 20 ul.
Your vector DNA should have a concentration of 0.05 ug/ul at the end of all manipulations.
N.B. Assume maximum recovery and use your starting concentration!

All ligations must contain the following:
Sterile H_2O giving a final volume of 20 ul
2 ul 10x ligation buffer
1 - 16 ul insert (see note)
1 ul 0.05 ug/ul vector DNA
1 ul T4 DNA ligase

Controls are very important here, especially cut vector with ligase and cut vector without ligase.
Incubate on the top shelf of a fridge overnight before transforming 10 - 20 ul into appropriate cells.

Note: INSERT DNA
The general rule is to have excess molar amounts of insert to vector.
If you cut your insert out of a gel and it is SMALLER than your vector you can take 16 ul of the elution without doing any quantification.
This also stands for PCR products (as they are almost always smaller than the vector). However, sometimes it is not necessary to elute from a gel.

Page 1.67 (sambrook)
...proportion of transformed bacterial colonies that carry recombinant plasmids. In this case, it is advisable to consider taking steps to reduce the background of colonies carrying nonrecombinant plasmids either by treating the linearized plasmid DNA with phosphatase or by adopting another cloning strategy so that the recombinant plasmid can be constructed by directional cloning.

Textbook: Molecular Cloning: A Laboratory Manual by Joseph Sambrook, David W Russell

Ethanol Precipitating – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols
Preparation of DNA
To a 50 ul solution of DNA add 5 ul 3M NaAc pH 5.2 and 140 ul 100% Ethanol (i.e. 0.1 Vol NaAc 2.5 Vols Ethanol).
Precipitate for 30 min (at RT or -80°C) before spinning at full speed for 15 minutes.
Wash the pellet with 500 ul 70 % Ethanol and spin for a further 10 mins.
Dry pellet in the speed vac for 3 to 5 mins.
Resuspend the pellet in required vol of required buffer.

Alkaline Dephosphorylation – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols

In general
Solutions required:
ddH2O (sterile and filtered)
Alkaline Phosphatase and buffer
Your DNA

Preparation of DNA
After a 3 hr digest of your DNA add 5 ul 3M NaAc pH 5.2 and 140 ul 100% Ethanol
Precipitate for 30 min before spinning at full speed for 15 minutes
Wash the pellet with 500 ul 70 % Ethanol and spin for a further 10 mins.
Dry pellet in the speed vac for 3 to 5 mins.
Resuspend the pellet in 50 ul H2O
Aliquot 8 ul into a clean tube and keep this is your phosphorylated control
Add 5 ul AP buffer and 3 ul Alkaline Phosphatase to the remaining 42 ul
Incubate at 37 °C for 1 to 3 hrs

Heat Inactivation and Preparation for ligation
Add the following to your dephosporylation reaction
10 ul 10x TNE
5 ul 10 % SDS
35 ul H2O
Incubate at 68°C for 15 min to inactivate the enzyme
Add 10 ul 3M NaAc pH 5.2 and 275 ul 100% Ethanol
Precipitate for 30 min before spinning at full speed for 15 minutes
Wash the pellet with 500 ul 70 % Ethanol and spin for a further 10 mins.
Dry pellet in the speed vac for 3 to 5 mins.
Resuspend the pellet in 20 ul H2O

Use DNA immediately for a Ligation (different protocol sheet).

Phil's mini method for Alkaline Lysis for Mini Prep – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols
Use a 3 - 5 ml culture either grown ON or day growth
spin and resuspend in 300ul of Sol.I+RNase
add 600 ul fresh Sol.II (2ml NaOH (1M) 1ml 10%SDS and 7 ml water)
5 min RT then add 250 ul Sol III - **SHAKE RIGOROUSLY!**
spin 20 min
1ml supernatant into new tube and add 600 ul Isopropanol
15 min spin and aspirate
add 500 ul 70% ethanol and spin for 5 min
aspirate and speed vac resuspending in 50 ul water

The DNA can then be used in a test digest using between 2 and 10 ul depending upon the origin of replication e.g. pUC19 use 2 ul and pACYC plasmids use 10 ul.

Solution I (can be stored at RT until Rnase A added)
50 mM Glucose
25 mM Tris.Hcl (pH 8.0)
10 mM EDTA (pH 8.0)
you can make it up to 500 ml

Add 250 ul of RNase A to 50 ml of Sol. I then store at 4°C

Solution III (for 100 ml)
5M Potassium acetate: 60ml
glacial acetic acid: 11.5 ml
H2O: 28.5 ml
Freezing Strains into the TPA Collection – Newcastle – 2009
http://2009.igem.org/Team:Newcastle/Project/Labwork/PhilsProtocols
In general
This protocol is designed to aid you in freezing strains
Solutions required:
2.0ml screw cap tubes (sterile)
DMSO (sterile)

Before Freezing
Make sure you have all your strains freshly streaked out on plates
Assign each strain to be frozen a TPA number completing the strain book FIRST (P.T.O for examples and help)
Pick a single colony and inoculate 5ml LB or PYE +/- antibiotic (if necessary) culture
Incubate cultures overnight at the correct temperature

Freezing
Label the correct number of screw cap tubes to freeze strains in duplicate
Add 150ul sterile DMSO to each tube using fresh tips each time
Add 1.5ml of each culture to each tube, mixing by pipetting up and down
Place in correct boxes in -80 degree Celsius freezer
NOTE: Box numbers start at the top left hand corner and go vertically down
Once in Freezer go back to strain book and date and initial all strains frozen.

Growing colonies in broth – METU-Gene – 2009 http://2009.igem.org/METU-gene/_Protocols
Prepare 5mL LB + Amp broth in a sterile Falcon tube.
Pick up a colony from the plate by a micropipette tip or sterile toothpick and put it in the falcon.
Incubate at 37C incubator for 14-16 hours.
Miniprep Plasmid Isolation (with Qiagen kit) – METU-Gene – 2009 http://2009.igem.org/METU-gene/_Protocols
1. Centrifuge the falcons at 4000rpm for 4-10 minutes.
2. Resuspend pelleted bacterial cells in 250uL Buffer P1(kept in +4C) and transfer to a 1.5mL eppendorf.
3. Add 250uL(microliter) Buffer P2 and invert the tube for ~6 times to mix (do not vortex). Solution should become blue (if indicator is added).
4. Add 350uL Buffer N3 and invert the tube immediately for ~6 times. Solution should become wtite and cloudy.
5. Centrifuge for 10 minutes at 13000 rpm. A compact white pellet will be formed.
6. Pour the supernatant to Qiagen spin column and centrifuge the column for 1 min. Discard the flow-through.
7. Wash the spin column by adding 0.75mL Buffer PE and centrifuge for 1 min.
8. Discard the flow-through and centrifuge for an additional 6.5-7min to remove residual ethanol in the wash buffer.

9. Place the Qiagen prep column in a clean 1.5 mL eppendorf. Add 32uL (can be modified according to the concentration aimed to be obtained) Buffer EB or water to the **center** of each Qiagen prep spin column and let stand for 5-10 minutes. Centrifuge for 1 min.
Store the minipreps at -20. The concentration obtained can be measured by Nanodrop.
Digestion – METU-Gene – 2009 http://2009.igem.org/METU-gene/_Protocols
For Vector: Mix (to a total of 20 uL):
7-7.5 uL mini-prepped vector DNA 7.5 uL distilled water 0.3 uL restriction enzyme 1 at 20 units/uL 0.3 uL restriction enzyme 2 at 20 units/uL 0.4 uL calf intestinal alkaline phosphatase (CIP) to prevent re-ligation of the vector to itself 2 uL 10x BSA 2 uL 10x appropriate NEB buffer (check from www.neb.com Double Digest Finder)
Insert: Mix (to a total of 10 uL):
7 uL mini-prepped 'vector' DNA 8.2 uL distilled water 0.4 uL restriction enzyme 1 at 20 units/uL 0.4 uL restriction enzyme 2 at 20 units/uL 2 uL 10x BSA 2 uL 10x appropriate NEB buffer
Incubate overnight at 37C.
Gel purify the insert using a Qiagen kit. Elute using 20 uL.
PCR purify the vector (can be gel purified too). Elute in 20 uL.
Ligation (with Roche Rapid Ligation kit) – METU-Gene – 2009 http://2009.igem.org/METU-gene/_Protocols
Mix:
1 uL digested vector 3 uL digested insert
The optimum molar ratio is 1:3, the volumes can be modified according to concentrations of the vector and insert. For sticky end ligations 1:5 ratio can be used.
Complete to 10 uL with 1X reagent 2 of the kit (diluted from 5X with distilled water), Vortex and spin.
Add 10 uL reagent #1.
Add 1 uL reagent #3.
Ligate for 10 minutes at room temperature.
Transform 2 uL of ligation mix in 25 uL DH5alpha competent cells.
Transformation – METU-Gene – 2009 http://2009.igem.org/METU-gene/_Protocols
Thaw competent E. coli on ice. Take 25uL cells into prechilled eppendorfs. Slowly add 2uL plasmid DNA. Do not vortex or make pipetting during the procedure.
Incubate on ice for 30 minutes (or more).
Heat shock at 42C water bath for 30 seconds (timing is critical).
Incubate for 2-5 min on ice.
Add 250 µL SOC medium (~10X volume) and incubate at 37 °C in shaker for 30-60 min.
Spread on LB+Amp plates (or any other selection) using glass beads or spreader.
Incubate overnight at 37°C.
Reference
Harvard Medical School, Silver Lab Protocols: [1] and manuals of the kits mentioned.
Preparation of competent E. coli – METU-Gene – 2009 http://2009.igem.org/METU-gene/_Protocols
*** Solutions:**
1) LB medium
2) SOB medium
For 500 mL: Tryptone 10 gr Yeast extract 25 gr NaCl 0.25gr
- Dissolve in 450mL and add 5 mL 0.25 KCl (1.86gr KCl in 100mL dH2O)
- Adjust the pH to 7.0 with 5N NaOH.
- Autoclave the medium.
- After autoclaving, add 2.5mL of 2M sterile MgCl2 (18.86 MgCl2 in 100mL)to the media.

3) TB solution
For 100mL Pipes or Hepes (1M) 10 mL CaCl2.2H2O (15mM) 0.221g KCl (250mM) 1.864g
- Dissolve in 80mLof dH2O and adjust pH to 6.7 with KOH.
- Add 1.09g MnCl2.4H2O (55mM) and dissolve completely. Complete the volume to 100mL.

- Filter sterilize the solution, do not autoclave.

*** Procedure:**

1) Streak DH5alpha on solid LB plates and culture overnight at 37C.
2) Inoculate 10-20 colonies (or 300uL LB culture if liquid medium is used) to 250mL of SOB medium with a loop in a 2L flask.
3)Grow until OD600 reaches 0.6 at 18Cor 37C with vigorous shaking at 200-250rpm.
4) Keep the culture inside the flask on ice for 10 min.
5) Transfer the culture to two seperate sterile centrifuge tubes and centrifuge at 2500g for 10 minutes at 4C.
6) Rsuspend the pellet in 80mL of ice-cold TB and incubate in ice for 10 min, then recentrifuge as previously.
7) Gently resuspend te pellet in 20mL of TB and add DMSO with gentle swirling to a final concentration of 7%.
8) Incubate in ice for 10 min and dispense by 0.4 mL into eppendorf tubes and immediately chill in liquid nitrogen.
9) Store frozen competent cells at -80C.

Reference: Inove et al. 1990.

PCR – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols

12.5 µL Phusion mastermix*
1 µL forward primer
1 µL reverse primer
0.5 µL template
10 µL demi water

*Phusion master mix contains:
200 µL 5x Phusion HF buffer
8 µL 25 mM dNTP's
282 µL MilliQ water
10 µL Phusion Polymerase

PCR Reaction
Hotstart
95 °C, 2 min.
25 cycles
95 °C, 30 sec.
61 °C, 20 sec.
72 °C, 1.5 min.
End
72 °C, 10 min.
4 °C, ∞

Plasmid isolation
Usually performed using Miniprep kits like NucleoSpin® Plasmid, (Machery nagel) or GeneElute™ Plasmid Miniprep Kit (Sigma-Aldrich). This is a consensus protocol.
Spin down ON Culture in table top centrifuge, 1 min. 13.000 RPM
Resuspend pellet, RNAse is added to degrade RNA (200 µL)
Add lysis buffer (200 µL), to lyse the cells and to release their contents
Add neutralization buffer (350 µL), proteins will denaturate
Centrifuge in table top centrifuge 10 min. 13.000 RPM
Add clear lysate in column provided in kit
Spin down 1 min. 13.000 RPM
Add wash buffer (usually needs EtOH to be added!) (500 - 750 µL)
Remove flow-through and spin again to remove residual wash buffer
Put column in clean 1.5 mL cup and add 15 - 50 µL MilliQ water or Tris buffer (pH=8.0)*
Incubate for 1 - 2 min.
Spin down 1 min. 13.000 RPM
*Less volume gives higher concentrations, supplied Tris buffer is claimed to give higher yields
Restriction
Mix

1 µL 10x fast digest buffer (Fermentas) or correct conventional buffer
0.5 µL Enzyme A*
0.5 µL Enzyme B*
8 µL DNA to be digested**
Incubate 0.5 - 1 h @ 37 °C (Fast digest do not require long incubations, when using conventional enzymes 1 h. should be maintained)
Purify cut plasmid using PCR clean up kit
* a combination of two of the following (when using biobrick standard) *Spe*I, *Eco*RI, *Pst*I and/or *Xba*I
** When digesting vectors, bring the digested vectors to 1% agarose gel and cut out with scalpel, purify using gel purification kit (NucleoSpin® Extract II, Machery nagel, Zymoclean™ Gel DNA Recovery Kit or similar) to an end volume indicated by the kit (End volume determines concentration, variations are possible) (alternatively, Phosphatase treatment of linearized vector)
Annealing synthetic oligo's
Phosphorylation of 5' ends & hybridization[1]
Mix:
3 µL 100 µM (anti-)sense oligo
1 µL 10 x PNK (polynucleotide kinase) buffer (Fermentas Buffer A)**
2 µL 10mM ATP **
1 µL T4 polynucleotide kinase (PNK)
3 µL MilliQ
(for selfcloser control, do not add oligo's. Instead 6 µL MilliQ in total)
Incubate @ 37 °C for 1.5 hours.
Mix
10 µL Sense mixture
10 µL Anti-sense mixture
3 µL 0.5 M NaCl
Place in boiling water for 3 min., and allow the reaction to cool to room temperature.
Upon reaching room temperature add restricted vector (see for ratio Ligation
If kept at low temperature before ligation heat up the annealing mixture up to 65 °C for 1 min. to prevent the formation of multimers
**Alternatively T4 DNA Ligase buffer can be used, already containing ATP
Ligation
Mix*
1 µL T4 ligase buffer
7.5 µL vector (purified from gel)
1 µL Insert
0.5 µL T4 ligase
Incubate
1h RT
or
ON @ 4 °C
*This is a consensus, calculations should be performed to have the ligations be done in a 5:1 - 10:1 (Insert:Vector) mass ratio.
Making competent cells
Competent cells: TOP10 & DB3.1
10 mL ON culture is used to inoculate LB, 100 µL ON culture per 20 mL*
Cultures are grown @ 37 °C until an OD_{600} of 0.2 ~ 0.3 is reached.
Cultures are spinned down 5 min. @ 4000 rpm, 4 °C
Supernatant is removed and pellet (per 20 mL culture) is resuspended in 5 mL chilled 0.1 M $CaCl_2$
Suspension is incubated on ice for 10 min.
Suspensions are spinned down 5 min. @ 4000 rpm, 4 °C

Supernatant is removed and pellet is resuspended in 1770 µL chilled 0.1 M $CaCl_2$ and supplemented with 230 µL 87% glycerol prior to making aliquots.
Cells are divided in 50 µL aliquots
Cells are snapfrozen in liquid nitrogen and stored @ -80 °C
* Cultures should be grown in the ratio 1:5 (medium:air), so 10 mL culture in a 50 mL greiner tube.
Transformation
Add 10 uL of ligation mixture or 1 uL isolated plasmid to competent cell aliquot
+ control: 1 µL pSB3K3 or pSB1AC3 plasmid (no death gene!), **- control**: 1 µL MilliQ*
Alternatively a single cut plasmid can be taken as a ligation control
*Alternative - control: 1 µL pSB1AC3 or pSB3K3 carrying ccdB deathgene
Incubate on ice for 15 - 30 min.
Heatshock 45 sec. @ 42 °C or 5 min. 37 °C
Let cells relax on ice for 1 - 2 min.
Add LB 200 µL (or 800 µL when spinning cells down, see below)
Incubate 37 °C, 250 RPM for 1 h
Plate out on LB-agar + Kanamycin (30 µg/ml for pSB3K3) or Ampicillin (100 µg/mL for pSB1AC3)
Plate out 50 µL & 200 µL (or 100 µL after spinning down and resuspending cells) of cell suspension
Grow ON @ 37 °C

Checking transformations
See if - control is empty for functioning antibiotics and death gene
See how many colonies on + control for functioning competent cells
See how many selfclosers and compare to samples (>10x on sample vs. selfcloser)
If enough transformants, inoculate 3 - 5 colonies in an ON culture
Alternatively perform colony PCR
Quality control – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols
Colony PCR
Put colony in 1 µL MilliQ water
Put colony suspension in microwave for 1 min. 1000 W
Use this as DNA template
PCR reaction

21 µL Taq mastermix*
1 µL forward primer
1 µL reverse primer
1 µL template
1 µL Taq polymerase

*Taq master mix contains:
100 µL Taq NH_4
8 µL dNTP's
80 µL MgCl
652 µL MilliQ water

PCR Reaction**
Hotstart
95 °C, 2 min.
25 cycles
95 °C, 30 sec.
61 °C, 20 sec.
72 °C, 1.5 min.
End
72 °C, 10 min.
4 °C, ∞

Put PCR product on agarose gel
** Indication, actual reaction program depends on primer set (Temperature of annealing) and the length of the template (Duration of elongation)
Restriction analysis
See also Restriction, however in checking the presence of a multitude of bricks more diverse enzymes can be used. Also the incubation time can be shortened (Pour an agarose gel, wait for it to solidify and put reaction on gel) because it is not required that everything is cut.
Membrane protein isolation

Use 20 mL of ON culture to start main culture in 1L LB medium containing 50 µg/mL ampicillin
Incubate 37 °C, 250 RPM until OD_{600} = 0.6, (approximately 2.5h of incubation, check the OD_{600} every hour)
Add inducer (e.g. [Team:Groningen/Protocols#IPTG|IPTG]])
Incubate 1 h, 37 °C, 250 RPM
Culture Wash.
Cool culture on ice
Spin down culture @ 8000 rpm, 10 min, 4°C
Wash pellet with 40 ml ice-cold 50 mM KPi, pH 7.0
Spin down culture @ 8000 rpm, 10 min, 4°C
Resuspend pellet in 12 ml 50 mM KPi pH 7.0
Homogenization
Sonication on ice
9 cycles of 15 sec. sonication, 45 sec. rest
Separation fractions
Spin down @ 8000 rpm, 10 min, 4 °C
Collect supernatant
Spin down at 90 000 rpm, 25 min, 4 °C
Resuspend pellet in 1 ml of 50 mM KPi pH 7.0 + 1M NaCl
Spin down @ 80 000 rpm, 25 min, 4 °C
Solubilization
Resuspend in 950 µl of solubilization buffer (50 mM KPi pH 8.0 + 400 mM NaCl + 20% glycerol) and 50 µl of 10% DDM
Incubate in 4°C with shaking for 30 min.
Spin down @ 80 000 rpm, 25 min in 4°C
Collect the supernatant
(Supernatant can be stored at this stage)
Add Ni-NTA resin (30 µL buffer A, 0.1% DDM (Bis(4-chlorophenyl)methane))
Incubate ON @ 4 °C
Spin down, 4 min. 3500 RPM
Remove supernatant
was resin with 1 mL buffer B, 0.1% DDM
Spin down, 4 min. 3500 RPM
Remove supernatant
Elute protein by buffer C, 0.1% DDM (50 µL)
Add protein loading buffer (with dithiothreitol (DTT))
Run 12% SDS-PAGE
Continue to Coomassie staining
Staining of SDS-PAGE gels with Coomassie Brilliant Blue
Heat gel in staining solution and shake for 10 min.
Poor off staining solution and add destain.
Heat gel in destaining solution and shake.
Replace destaining solution after 10 min and repeat until ready.
Measurements – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols
Fermentation
Performed in 2L autoclavable fermentor with dished bottom vessel stirred fermentor
Autoclave closed fermentor system
Inoculate 1.3 L LB (+100 µL Y30 antifoam) with 20 mL ON culture was used to
Airflow rate of 1 vvm
pH @ 7 (by addition of 4 M NaOH or 1 M HCl)
Temperature @ 37 °C

Agitation 400 to 800 RPM*
Oxygen concentration >50%
Take samples every 0.5 to 1 h. to determine optical density at 600 nm
50 mL samples in every growth phase (pre-exponential, early exponential, exponential, late exponential, steady state)
Spin samples down 35 min., 1000 RPM and remove supernatant
Continue Buoyancy test
*6-bladed flat disc turbine (Rushton type) impeller (60 mm diameter) at the bottom to disperse the bubbles coming from the sparger underneath and a 3-bladed marine impeller, vortex (60 mm diameter) halfway the broth volume to create an axial flow.
Buoyancy test
Continued from cultures (flask or fermentor) after centrifugation
Resuspend pellet in 1 - 5 mL saline solution
Determine OD_{600}
Dilute suspension to OD_{600} 1.5 with saline solution
Put homogeneous suspension in tubes, take care of descent lighting from behind (day light is best)
Record decrease of buoyancy (matter of hours in fermentation cultures, days in shakeflask cultures)
Metal uptake assay for *E. coli*[Kostal 2004]
Grow ON culture of *E. coli* @ 30 °C
Use *E. coli* + control vector, *E. coli* + pArsR-RFP, *E. coli* + pLac-fMT
Inoculate day culture 1:50, grow in 1L TB-Amp (100ml per time/[As(III)] sample)
Take OD_{600} samples every 1 - 1.5 h of *E. coli* + pLac-fMT
Induce *E. coli* + pLac-fMT at OD_{600} ~0.6 with 0.5 mM IPTG.
Harvest the cells @ stationary phase (after ~30 h) by spinning down @ 4000 RPM for 20 min. in Sorval centrifuge.
Wash 2 times with TB74S buffer
Resuspend in prewarmed (30 °C) TB74S buffer up to a OD_{600} of ~25
Take a 1 mL sample in small aluminum boxes and dry @ 104 °C for >4 h
Afterwards measure the dry weight of the sample and calculate the weight/volume of the entire sample.
For the concentration range:
Incubate 5 samples (of same time point) for 1h @ 30 °C with 0µM, 10 µM, 20 µM, 50 µM and 100 µM As(III).
For the concentration range:
Incubate 5 samples (of same concentration) @ 30 °C with 10 or 100µM As(III) for 0, 10, 20, 40, 60 min.
Harvest cells by spinning down.
Wash the cells with TB74S buffer
Resuspend in 10ml demi water.
Dry sample @ 65 °C for 2 days.
Store @ 4 °C or -80 °C
Determine the amount of As(III) in the cell at different stages and at different uptake concentrations using ICP-MS
Analysis of arsenic concentration of ICP-MS
Weigh 0.1g dried *E. coli* cells.
Add 5 ml 65% nitric acid.
For destruction the following microwave program was used:

	Stage 1	**Stage 2**
Power(max)	1200	1200
Power(%)	100	100

Ramp(min)	15	15
Hold(min)	0	30
Temp(°C)	140	210

Let the samples cool down.
Dilute the samples by adding demi water up to 50 mL
If needed, spin down 15 min. @ 4000rpm in a Sorvall centrifuge.
Measure the arsenic concentration by ICP-MS using both the standard mode (shows interference peak from multi-atomic molecule argon-chloride with the arsenic peak) and the collusion cell technology mode (doesn't show the interference peak but has a 10x lower resolution than standard mode).
Use a standard curve between 0 - 10 µg As/L and 0 - 100 µg As/L using a certified 1000 ppm (mg/L) stock
Fluorescence measurement
Dilute ON culture 1:20 in LB+Ampicillin in 50 mL greiner tube
Incubate 37 °C, 250 RPM until an OD_{600} ~0.5
Spin down 10 min. 4000 RPM, 4 °C
Resuspend pellet in LB+Ampicillin (half the volume used before)
Incubate @ 4 °C, 30 min.
Load samples onto a 96-wells plate, 250 µL
Induce by 1.25 µL of the following stock solutions

Metal					
$CuSO_4$	1 M	100 mM	10 mM	1 mM	0 mM
$ZnSO_4$	1 M	100 mM	10 mM	1 mM	0 mM
$NaAsO_2$	1 M	100 mM	10 mM	1 mM	0 mM
Final con.	**5000 µM**	**500 µM**	**50 µM**	**5 µM**	**0 µM**

Measure the fluorescence and OD_{600} every hour.
RFP is excited @ 580 nm and emission is measured at 609 nm.
Store the plates between the measurements in a shaking incubator @ 37 °C.
Fluorescence of resting cells with BBa_J61002-pArsR
This protocol was used to be able to correlate the determined fluorescence values with the arsenic concentrations measured by ICP-MS (after an arsenic uptake assay).
Cells were used as prepared for the (BBa_K190015) **arsenic uptake assay**.
Cultures with an OD_{600} of ~25 were induced with 100 µM $NaAsO_2$ for 60 to 160 min.
The fluorescence and OD_{600} were measured by a plate reader (Tecan, infinite 200, Tecan group, Switzerland).
Relative promoter units were calculated according to formula 9 from Kelly 2009.
Death assay – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols
Metal sensitivity assay[Lewinson 2009]
Measurement:
Grow selected strains ON in LB medium with or without antibiotic
Induce in culture with inducer (in our case 0.5 mM IPTG)
Strains used in our tests

Test 1:	**Test 2:**
WT (+pSB1AC3)	WT (+pSB1AC3)
pLac-HmtA	pLac-HmtA
pLac-GlpF	pLac-GlpF
pLac-GlpF-fMT	pLac-GlpF-fMT

	plow-GlpF-fMT
	pLac-GlpF

Measure OD_{600} of ON culture and dilute to an OD_{600} of 0.05 in LB+antibiotic & inducer (IPTG in our case). Inducer should be right concentration for use in microtiterplate (because you then dilute culture 150/200=1.33 times)
Add 150 ul of culture to 96 well microtiter plate (in triplo/quadruplo)
Add desired concentration of selected metal in 50 µL LB+antibiotic
Metals used in our test

Metal Concentration				
$NaAsO_2$	0 µM	1 µM	10 µM	50 µM
$CuSO_4$	0 µM	50 µM	250 µM	500 µM

Measure in Tecan Infinite 200 microplate reader (Tecan Group Ltd., Männedorf, Switzerland) or Tecan microplate *reader. Protocol:
Measure OD at 600 nm,
Every 15 minutes for 16-20hrs
Linear shaking, 6mm
37°C

Analysis:
Plot for different strains OD_{600} against time
Plot for different strains, the different metal concentrations against OD_{600} at 12 hours
List of solutions
Media – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols
LB(Agar)
10 g (Bacto)Trypton
10 g NaCl
5 g Yeast extract
(1.5% Agar, 15 g)
Dissolve in 1 L demi water
Autoclave
Store @ RT (LB) or 60 °C (LB-Agar)
TB medium
12 g Bacto-Tryptone
24 g Bacto-Yeast Extract
4 mL Glycerol [87%]
Dissolve in 900ml demi water
Separetely prepare 100 mL Kpi
0.17 M KH2PO4 (mw=136.09g/mol) (6.94g/300ml)
0.72 M K2HPO4 (mw=174.18g/mol) (7.62g/300ml)
dissolve in demi water
Autoclave and mix
Antibiotics – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols
Ampicillin
100 mg/ml Ampicillin (1000x) Stock
1 g of Ampicillin sodium salt in 10 mL of demiwater (or 50% EtOH)
Add NaOH or KOH to allow the Ampicillin to dissolve
Filter sterilize 0.2 µm filter and aliquot
Store -20 °C

Chloramphenicol
35 mg/ml Chloramphenicol (1000x) Stock
0.35 g in 10 mL 100% EtOH
Filter sterilize 0.2 µm filter and aliquot
Store -20 °C
Kanamycin
50 mg/ml Kanamycin (1000x) Stock
500 mg in 10 mL demi water
Filter sterilize 0.2 µm filter and aliquot
Store -20 °C
Chemicals – Groningen – 2009 http://2009.igem.org/Team:Groningen/Protocols
Buffer A
10 mM Imidazole
600 mM NaCl
50 mM KPi pH 8.0
10% Glycerol
0.1% DDM
Demi water
Buffer B
20 mM Imidazole
600 mM NaCl
50 mM KPi pH 8.0
10% Glycerol
0.1% DDM
Demi water
Buffer C
500 mM Imidazole
600 mM NaCl
50 mM KPi pH 8.0
10% Glycerol
0.1% DDM
Demi water
0.1 M $CaCl_2$
0.3319 g $CaCl_2$
Dissolve in 30 mL demi water
Destaining solution
16 % methanol
10 % acetic acid
74 % water
0.15 M NaCl (Saline solution, 0.9% NaCl)
9 g NaCl
Dissolve in 1 L demi water
4 M NaOH
160 g NaOH
Dissolve in 1 L demi water
~1 M HCl
500 mL demi water
500 mL HCl (37%, 11 M)
1 M IPTG
2.38 g Isopropyl-beta-D-thiogalactopyranoside (IPTG) in 10 mL demi water.
Filter sterilize with a 0.22 µm syringe filter.

Store in 1 mL aliquots at -20 °C.
Sodium Arsenite (III)
100mM Na-As solution
filter sterilize
Staining solution
0.25 % Coomassie Brilliant Blue R-250
50 % methanol
10 % acetic acid
40 % water
TB74S Buffer
0.605 g Tris (5mM)
8.76 g NaCl (150mM)
Dissolve in 1 L Demi water
Set pH with HCl to 7.4
10x TBE buffer
108 g Tris
55 g Boric acid
8.3 g EDTA
Dissolve in 1 L demi water
Adjust pH to 8.3

SDS-PAGE protocol – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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This protocol is designed to make an SDS-PAGE on proteins purified directly from an overnight cell culture. There is no need to make *in vitro* protein synthesis.

Protein purification
Lysis Buffer (2x) : for 100 ml
4g of SDS
20ml of glycerol
2ml of 2-Mercaptoethanol
70ml of stacking buffer (0.5 M Tris-HCl, pH=6.8)
100mg of Bromophenol blue
8ml of dd water

Make an overnight culture of the cell containing your protein of interest
1. Pellet the bacteria from the overnight culture @ 4000 rpm for 30 min. Do the centrifugation @ 4ºC
2. Discard the supernatant
3. Re-suspend the bacteria in 1 ml of dd water. Centrifiguate for 30 min/4000 rpm @ 4ºC
4. Discard the supernatant
5. Add approximately 1 equivalent of Lysis buffer to your cell pellet
6. Heat the solution for 10 min @ 95ºC
7. Add 1 equivalent of dd water
8. Heat for 10 min @ 95ºC
9. Centrifuge @ 11600 g for 10 min

-->Take the supernatant and apply the desired volume to a polyacrylamide gel (10 %)

Note : for coomassie blue staining, 5ul of the solution should be enough to have well-stained bands
Stain your gel wiht a coomassie blue solution (in MeOH and AcOH)-->incubate with soft shaking @ RT for about 2h

De-stain your gel using a 10% AcOH and 50% MeOH in water. The solution has to be changed every time it gets blue. You might need ot change the solution up to 20 times.

Note : for speeding the processes of staining and de-staining, you can put your gel+solution in the microwave for the couple of seconds. CAUTION to the MeOH vapors which are highly toxic. You should perform this process under a negative pressure fume hood.

M9 Medium – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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M9 minimal + AA + thiamine:

- 10 ml of M9 salts 10x
- 0.2 ml of MgSO4 (1M)
- 0.1 ml of CaCl2 (0.1 mM)
- 10 ml of glucose (20%) -> we actually now think it's better to put 4% so 20 ml
- 200 mg of AA mix
- 800 ul of uracile
- 800 ul of histidine
- 800 ul of leucine

M9 salts 10x (-> 500 ml)

- 30 g Na2HPO4 * 7H2O
- 15 g KH2PO4
- 2.5 g NaCl
- 5 g NH4Cl

Illumination Process – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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Material
LED blue (458nm) 3mm (Distrelec ref number: 63 12 48)
LED red (750nm) 5mm (Roithner LaserTechnik LED750-03AU)
Resistor network 56 Ohms (Distrelec ref number: 71 52 75)

Resistor network 82 Ohms (Distrelec ref number: 71 52 81)
DC Power supply of 5V

Configuration
As the forward voltage of the blue LEDs is 3.5V and the nominal current is 20 mA, we mounted each blue LED in serial with a resistance of 82 Ohms.
The forward voltage of the red LEDs is 1.9V and the nominal current is 100mA. So we mounted each red LED in serial with a resistance of 56 Ohms.

Procedure
The initial cell culture is made in dark state. Then we illuminate them in the incubator, with constant shaking. To do so, we use small erlenmeiers to have a sufficient amount of culture for the experiments and so that the cells are in a sufficient medium to grow. We aslo use the red LEDs while loading our samples for further experiments as the LovTAP respond only to a wave lenght.

Transformation – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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Material
50 µl competent cells
LB-agar plates with corresponding antibiotics
Water bath at 42°C

Procedure
1. Thaw cells on ice for 20min
2. Add DNA (ligation product) to the cells, mix.
3. Incubate 20min on ice
4. Heat shock 45s @ 42°C
5. Incubate 20min on ice (optional)
6. Add 500 µl SOC and incubate 1h @ 37°C (shaking)
7. Spread over LB-agar plate
8. Incubate overnight @ 37°C

Ligation Protocol – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

Reaction mix

T4 ligase buffer	1 µl
T4 ligase	0.5 µl

6:1 molar ratio of insert to vector (~10 ng of vector).
Complete with dH2O up to 10 µl.

Molar ratio

$$\text{insert mass in ng} = 6 \cdot \left[\frac{\text{insert length in bp}}{\text{vector length in bp}}\right] \cdot \underbrace{\text{vector mass in ng}}_{10ng}$$

Notes
In general, we can take 1 µl of vector for 3 µl of insert. But you can change the molar ratio in case the sizes of the insert and the vector are really different.
To control ligation specificity, you need to have a **control with the vector only**.

Incubation
1h at Room Temperature.

Digestion Protocol – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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We want to have all the DNA preps at **700 ng/µl**, for the vector as for the insert. If the DNA preps are not concentrated enough, you can add more DNA and increase the volume of the reaction, respecting the buffers concentration (10x). Complete with dH2O if needed.

Reaction Mix

DNA	7 µl (700 ng of 100 ng/µl prep)
NEB buffer 10x	1 µl
BSA 10x	1 µl
Enzyme 1	0.5 µl
Enzyme 2	0.5 µl
dH2O	0 µl
Total	**10 µl**

Dephosphorylation
Add 1 µl of artic phosphatase to the vector to prevent ligation of the vector on itself.

Incubation
Vector: 2h at 37°C → add artic phophatase → 20min at 37°C
Insert: 1h20min at 37°C
Enzyme properties - *www.neb.com*

	NEB 1	**NEB 2**	**NEB 3**	**NEB 4**	**BSA**	**Inactivation**
EcoRI	100	100	100	100	optional	20 min at 65°C
SpeI	75	100	25	100	X	20 min at 80°C
XbaI	0	100	75	100	X	20 min at 65°C

PstI	75	75	100	50	X	20 min at 80°C
NotI	0	50	100	25	X	20 min at 65°C

Klenow fragment synthesis – EPF Lausanne – 2009
http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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This protocol has been designed in order to get *ready-to-ligate* small DNA fragments from scratch. The procedure doesn't require any purification step, in order not to loose DNA material due to the small sizes of the fragments. Therefore, it includes a dephosphorylation step, which is crucial for the maximization of the ligation that follows. The all expermiment is performed in a single tube with successive additions of new reactants.

The amount of DNA at the beginning of the reaction is calculated in order to fit the desired amount required for the ligation step that follows the end of the klenow fragment synthesis.

Design your DNA oligos
Fix your target DNA concentration that you want at the end (in ng/ul)
Multiply by 50 to get the total mass of DNA that you want in your tube at the end (the protocol is designed to have a final volume of 50ul)
Out of the M.W. of your DNA fragment, calculate the number of mol that you would expect at the end --> it gives you the the nb of mol that you will need for each DNA oligos (each oligo must have the same nb mol as for the DNA fragment)
Out of the concentration of your oligos, you can calculate the volume of each required

Step 1 : Klenow fragment synthesis
3.64ul of NEB buffer (choose this one according to the restriction enzymes you will use later. The klenow fragment enzyme works in all NEB buffer)
0.37ul of BSA 100x
X ul for each primer according to concentration
Y ul of MQ
-->The final volum at this stage should be 36.4ul
Thermal cycling
94ºC for 5min
Ramp down the temperature to 5ºC below your annealing temperature. CAUTION : ramp the temperature down at a MAXIMUM of 0.1ºC/s (to avoid secondary structure formation in your primers)
Set the temperature @ 37ºC

Step 1'
Add 1ul of the Klenow fragment enzyme
Add 1.6ul of dNTPs (final concentration of 1mM each). Stock solution of dNTPs : 25mM each
-->The final volume at this stage should be 39ul
Thermal cycling
Incubate 1h30 @ 37ºC
Inactivate the enzyme 20min @ 75ºC

Ramp down the temperature to 37ºC (!!! 0.1ºC/s !!!)

Step 2 : Digestion
Add 0.5ul of each enzyme
-->volume at this stage should be 40ul

Thermal cycling
Incubate 2h @ 37ºC
Inactivate the enzymes 20min @ 80ºC
Ramp down the temperature to 37ºC (!!! 0.1ºC/s !!!)

Step 3 : Dephosphorylation
Add 5ul of Antartic phosphatase buffer (10X)
Add 5ul of Antartic phosphatase enzyme (*)
-->Final volume should be 50ul
Thermal cycling
Incubate 2h @ 37ºC
Inactivate the enzyme 10 min @ 65ºC
Ramp down the temperature to 25ºC (!!! 0.1ºC/s !!!)

(*) This amount of enzyme has been calculated using the following. According to NEB : 1ul needed to dephosphorylate 1-5mg of pUC19 (~2690 bp) in 30 min. So, calculate your molar bp ratio compared to pUC19 and multiply by your supposed DNA mass. The result will give you by how much you have to multiply the phosphatase volume (in ul). You can also let the incubation run for longer (like here 2h) and so use less phosphatase (assume the relationship between ul of enzyme and time of incubation is a linear function). CAUTION : if you use more enzyme volume, your final volume will also be bigger. Make sure to adjust your phosphatase buffer accordingly!

1.5 Steps PCR Protocol – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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1. Do the following mixing:

dNTP	1 µl
5x Buffer + MgCl2	10 µl
Primer Mix	1 µl
template	0.5 µl
HiFi Plus DNAp	0.5 µl
dH2O	36.5 µl
Final Volume	**50 µl**

2. Make it run **10** cycles:

1	94°C	4:00
2	94°C	0:30

3	55°C	1:00
4	72°C	2:00
5	Cycle 2-4 10 times	
6	72°C	7:00
7	4°C	∞

3. We now add the standard iGEM primers which finish the extension, and do the following cycles:

1	94°C	4:00
2	94°C	0:30
3	55°C	1:00
4	72°C	2:00
5	Cycle 2-4 25-30 times	
6	72°C	7:00
7	4°C	∞

PCR with *Taq Platinium* Protocol – EPF Lausanne – 2009
http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

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1. Add the following components to a sterile 0.5-ml microcentrifuge tube. Volumes are for a single 50-µl reaction, and can be scaled as needed. Prepare a master mix of common components for multiple reactions.

Components	**Volume**	**Final concentration**
10X PCR Buffer, Minus Mg	2,5 µl	1X
10 mM dNTP mixture	1 µl	0.2 mM each
50 mM MgCl2	1.5 µl	1.5 mM
Primer mix (10 µM each)	1 µl	0.2 µM each
Template DNA	≥1 µl	(as required)
Platinum® Taq DNA Polymerase	0.2 µl	1.0 unit*
Autoclaved, distilled water	to 25 µl	Not applicable

*1.0 unit is sufficient for amplifying most targets. In some cases, more enzyme may be required (up to 2.5 units).

2. Cap the tubes, mix, and centrifuge briefly to collect the contents.

3. Incubate tubes in a thermal cycler at 94°C for 30 seconds to 2 minutes to completely denature the template and activate the enzyme.
4. Perform 25–35 cycles of PCR amplification as follows:

Denature	94°C for 30 seconds
Anneal	55°C for 30 seconds
Extend	72°C for 1 minutes per kb

5. Maintain the reaction at 4°C after cycling. The samples can be stored at –20°C until use. Analyze the products by agarose gel electrophoresis.

PCR protocol – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

Primers
Make primers pair mix with 3µM concentration (total).

Reaction mix	
Plasmid	0.5ul
Primers pair (3µM)	1ul
Thermo POL buffer	2.5µl
dNTP	0.5 µl
Taq EPFL polym	0.25 µl
H20 (MQ)	20.25 µl
Total	**24.5 µl**

We used **2** tubes of PCR of 25µl each.
Cycles
Program PCR: count 1min/kb
94°C – 120''
94°C – 45'' |
56°C – 45'' | → 30x
68°C – 120'' |
72°C – 420'' |
10°C – ∞

PCR purification
PureLink™ PCR Purification Kit – Invitrogen

Binding DNA
1. Add 4 volumes of PureLink™ Binding Buffer with isopropanol (above) or Binding Buffer HC with isopropanol (above) to 1 volume of PCR (50-100 µl). Mix well.

2. Remove a PureLink™ Spin Column in a Collection Tube from the package.
3. Add sample with appropriate Binding Buffer from Step 1 to the PureLink™ Spin Column.
4. Centrifuge the column at room temperature at 10,000 × g for 1 minute.
5. Discard the flow through and place the spin column into the collection tube.
6. Proceed to Washing DNA, next protocol.

Washing DNA
1. Add 650 µl of Wash Buffer with ethanol to the column.
2. Centrifuge the column at room temperature at 10,000 × g for 1 minute. Discard the flow through from the collection tube and place the column into the tube.
3. Centrifuge the column at maximum speed at room temperature for 2–3 minutes to remove any residual Wash Buffer. Discard the collection tube.
4. Proceed to Eluting DNA, below.
Eluting DNA
1. Place the spin column in a clean 1.7-ml PureLink™ Elution Tube supplied with the kit.
2. Add 30 µl of Elution Buffer (10 mM Tris-HCl, pH 8.5) or sterile, distilled water (pH >7.0) to the center of the column. Here we purify the 2 tubes in one column.
3. Incubate the column at room temperature for 1 minute.
4. Centrifuge the column at maximum speed for 2 minutes.
5. The elution tube contains your purified PCR product. Remove and discard the column. The recovered elution volume is ~48 µl.
6. Store the purified PCR product at -20°C or use PCR product for the desired downstream application.

Miniprep Protocol – EPF Lausanne – 2009 http://2009.igem.org/wiki/index.php?title=Team:EPF-Lausanne/Protocols/Miniprep

Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge

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This protocol is designed for purification of high-copy plasmid DNA from 3x 5 ml overnight cultures of E. coli in LB (Luria-Bertani) medium.

Procedure
1. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet. If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy. If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form.

5. Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting. In this step, the 3 tubes have to pass trough the column.

6. Centrifuge for 30–60 s. Discard the flow-through.

7. Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through. This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.

9. Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer. Important: Residual wash buffer will not be completely removed unless the low-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 30 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center.

We obtain a final concentration comprised between 250 and 400 ng/ul.

Gel electrophoreses – DTU Denmark – 2009 http://2009.igem.org/Team:DTU_Denmark/protocols
Materials and chemicals
Agarose
1X TAE buffer
MiliQ water
Ethidium Bromide 0.625 mg/ml (Amresco)
Mini Sub Cell GT (BioRad)
Hyperladder I (Bioline) (Appendix 6.1.1)
5x DNA Loading Buffer, Blue (Bioline)
Procedure
Preparation of 1% agarose gel For a final volume of 500 ml, 5 g of agarose was dissolved into 500 ml of TAE buffer and mixed with a magnetic stirrer. The agarose was melted in microwave for 5 min. The agarose is completely melted when the solution is totally clear (no veil).
Preparation of DNA Loading buffer was added to DNA. When mixing small amounts this can be done on a piece of parafilm.

Agarose gel electrophoresis Capacity of small wells app.: ~ 15 µl, big wells: ~ 50µl.. Standard 75 mV 400 mA 1 hour / 45 min. Standard amount of hyperladder loaded: 5 µl (4 µl hyperladder 1 µl loading buffer).
Imaging the gel Gel Doc 2000 (Bio Rad). Quantity One 4.0.2 (Bio Rad). The exposure varies.
Hyperladder™ I
Hyperladder™ I was used throughout the study as ladder for all gels
PCR amplification – DTU Denmark – 2009 http://2009.igem.org/Team:DTU_Denmark/protocols
The following recipe was used for amplifying genes used for cloning with the Phusion-polymerase. If more than one PCR-reaction was made, a mastermix containing the polymerase, dNTP, PCR-buffer and water could be made giving more precise concentrations in the mix.

It is critical that the DNA Polymerase is the last component added to the PCR mixture, since the enzyme exhibits 3´→5´ exonuclease activity that can degrade primers in the absence of dNTPs.
When running PCR reactions with Taq the PCR buffer is 10x so only 5µl needs to be added
Phusion polymerase
Phusion Polymerase has the highest fidelity of any commercial thermo stable polymerase (50X greater than Taq). This is the reason that this enzyme was used for all cloning procedures described in this report. Taq is mostly used for colony PCR where the purpose is to detect the presence of a certain sequence and not to use the PCR product for further cloning.

T [ºC]	Time		
98	30	sec	
98	30	sec	Repeat 30 times
57*	30	sec	
72	15-60**	sec	
72	8	min	
12	Hold		

Table 2: Standard PCR program with Phusion polymerase.
* for primers > 20nt, anneal for 10 – 30 seconds at a Tm +3°C of the lower Tm primer. for primers ≤ 20nt, use an annealing temperature equal to the Tm of the lower Tm primer for calculating of melting temperature see Primer design. **15 sec per kb for low complexity DNA (e.g. plasmid, lambda or BAC DNA). 30 sec per kb for high complexity genomic DNA
Taq polymerase

T [ºC]	Time		
94	2	min	
94	30	sec	Repeat 30 times
56*	30	sec	
72	4**	min	
72	8	min	
12	Hold		

Table 3: Standard program with Taq Polymerase.
***Annealing temperatures should be chosen to match the Tm values of the primer pair.**
****use 1 minute/kb**
Colony PCR
For each colonyPCR to be performed a PCR tube is filled with 35 µl MiliQ Water. A sterile tooth pick which has touched a desired colony is stirred around in the water for the bacteria to stay in the water. Then primers, buffer, dNTP and DNA Polymerase (usually Taq) is added in the same amounts as stated earlier for PCR reactions
A similar program as the before mentioned standard is used, with the modification of starting with 3 minutes at 98º C to get the bacteria to lyse so the DNA is available for the primers to anneal.

T [ºC]	Time		
94	3	min	
94	30	sec	Repeat 35 times
52	30	sec	
72	4	min	
72	10	min	
12	Hold		

Table 4: Typical colony PCR program.

Primer design

The annealing temperature (T_m) of a primer is an important value to set in a PCR reaction is by definition the temperature at which one half of the DNA duplex will dissociate to become single stranded. At temperatures lower than the melting temperatures unspecific binding is possible so best PCR results is achieved at an annealing temperature close to the lowest melting temperature of the two primers. This means that primer pairs that have similar melting temperatures will yield a PCR reaction with reduced background noise by unspecific binding of the primers to the template.

T_m can be calculated using the "Oligo analyzer" www.genelink.com/tools/gl-downloads.asp

Or be approximated using: $T_m(°C) \approx 2(N_A+N_T) + 4(N_G+N_C)$

The primers described in this report is designed using http://fokker.wi.mit.edu/primer3/input.htm, which is a handy tool to find primer pairs to amplify a specific sequence and discards potential primers that have a tendency to e.g. self anneal. Usually a primer length of 22 was used based on experience. Primers was ordered at www.sigma.com.

DNA sequencing – DTU Denmark – 2009 http://2009.igem.org/Team:DTU_Denmark/protocols

Preparing and sending DNA

Description from http://www.starseq.com/nomenu.php?nomenu=1&ln=7n8

Mix Each PCR tube contains DNA, MQ H_2O and a single sequence primer in a total volume of 6µl. Add 3-5 µl plasmid and 1µl primer – see Table 5.

Label legible, with indelible black pen (e. g. Staedtler permanent lumocolor; Art. Nr. 318-9; EAN 40 07817 304563 or similar). Note your abbreviation on the lids and number them continuously.

Send Protect the reaction containers with a box or something similar and send together with order form in a padded envelope to: StarSEQ, GENterprise GMBH, Johann-Joachim-Becher-Weg 30a, 55099 Mainz, Germany´

Table 5: Relevant sequencing informations

DNA	dissolved in water or TrisHCl (10mM), pH 7-8
Amounts	PCR product: 200 bp: 50 ng, 500 bp: 100 ng; 1 kb: 200 ng
Plasmid DNA	400 ng - 700 ng
Cosmid DNA, PACs, BACs	>1 µg
Primer	10 pmol, i.e. 1 µl of a 10 µMolar primer solution
Melting temperature	52 - 60°C
Optimal length	18 - 25mer
Tube	200 µl PCR tubes with flat lids (e. g. Starlab, Art. Nr. I 1402-8100 or similar); Close only, no Parafilm

Analyzing retrieved files

The files return as SEQ and AB1 files. AB1 files can be opened with the freeware program FinchTV. Open all files. In each file the sequence that look reliable (normally from base 20 to ~700) is copied and pasted in a txt file.

Assemble the contigs using Vector NTI from Invitrogen

Use the "Assemble" function, and click"Open New Assembly Project".

Add fragments (txt. files)
Select fragments and assemble.
Digestion with restriction enzymes – DTU Denmark – 2009
http://2009.igem.org/Team:DTU_Denmark/protocols
Materials and chemicals
MiliQ water
Restriction enzymes 10,000 U/ml
NEBuffer 10X (1,2,3,4)
Bovine serum albumin (BSA) 100X (10X)
DNA
All restriction enzymes, enzyme buffers and bovine serum albumin (BSA) used in this study were purchased through New England Biolabs. Information about specific enzyme conditions e.g. type of NEBuffer and whether BSA was needed or not was obtained through the NEB website: http://www.neb.com/nebecomm/products/category1.asp?#2.
Initial concentrations of NEBuffers were provided as 10X and diluted to 1X in the final mix. BSA was provided as 10 mg/ml (100X) and should optimally have a final concentration of 100 µg/ml (1X). In this study the original BSA solution had been diluted to 10X which then was used as stock. Enzymes that do not require BSA should not be affected if BSA is present.

Procedure

Short protocol MiliQ H_2O was added first, buffer next, BSA when required, then the DNA solution, and finally the enzyme. The reaction mixture was mixed by gently pipetting up and down or by flicking the tube.
Amount of enzyme Generally, 10 U (1 µl) enzyme is normally added to 1 µg of purified DNA in a final volume of 50 µl. Thus, the amount of enzyme depends on the DNA concentration which can be verified by gel electrophoresis. A little more enzyme was added when double digestion was performed with enzymes requiring different types of buffers for optimal activity. E.g. 30 U of AsiSI and 40 U of KpnI was used with NEBuffer 2, because KpnI has got only 75% activity in this buffer and AsiSI had 100%.
Incubation In the 1st hour of incubation most of the DNA will be digested according to NEB, but for most of our reactions a more complete digestion was obtained by incubating “over night”. When this was done new enzymes were added halfway. All reaction were incubated overnight at 37º C.

Enzyme	Recognition site	Used for
***Kpn*I**		Cloning
***Asi*SI**		Cloning, Restriction Analysis
***Apa*LI**		Restriction Analysis, linearized BGHA P8 before transformation
***Not*I**		Restriction Analysis

Table 6: Restriction endonucelases used in this study.

More information

NEB restriction enzymes:
http://www.neb.com/nebecomm/products/faqCategory1.asp#661

NEBuffers:
http://www.neb.com/nebecomm/products/productB7000.asp
Ligation – DTU Denmark – 2009 http://2009.igem.org/Team:DTU_Denmark/protocols

1 µl ligase	
4 µl 10x ligase buffer	
X µl plasmid, cut	X = 50-100 ng plasmid
Y µl PCR product, cut	Y = 300 ng PCR product

Z µl MQ up to 40 µl total	

Everything is mixed, and incubated – either at room temperature for 2 hours, or overnight using the idea of Lund *et al (26)* of incubating on PCR machine with the following PCR program

T [ºC]	Time		
10	30	sec	
30	15	sec	Repeat 99 times
30	15	sec	
10	30	sec	
10	30	sec	
16	Hold		

Table 7: Ligation program on PCR machine

After the incubation, the mix can be stored at -18˚C. Lund et al (26) showed that ligation with cohesive ends carried out on with the temperature shifts could give an increased ligation yield of 7.7 times. This is because ligase works best at 30º C but template annealing is most effective at 10º C.
Miniprep for plasmid purification – DTU Denmark – 2009
http://2009.igem.org/Team:DTU_Denmark/protocols
(Sigma GenElute™ Plasmid Miniprep Kit, from manual)
1. Harvest & lyse bacteria
a. Pellet cells from 1 – 5 ml overnight culture 1 min (1 ml from TB or 2xYT; 1-5 ml from LB medium). Discard supernatant.
b. Resuspend cells in 200 µl Resuspension Solution. Pipet up and down or vortex.
c. Add 200 µl of Lysis Solution. Invert gently to mix. Do not vortwx. Allow to clear for ≤ 5 min.
2. Prepare cleared lysate
a. Add 350 µl of Neutralization Solution (S3). Invert 4- 6 times to mix.
b. Pellet debris 10 min. at max speed.
3. Prepare binding column
a. Add 500 µl Column Preparation Solution to binding column in a collection tube.
b. Spin at ≥ 12.000 x g, 1 min. Discard flow-through.
4. Bind plasmid DNA to column
a. Transfer cleared lysate into binding column.
b. Spin 30" – 1 min. Discard flow-through.
5. Wash to remove contaminants
a. Optional (*EndA*$^+$ *strains only*): Add 500 µl Optional Wash Solution to column. Spin 30" – 1 min. Discard flow-through.
b. Spin 1 min to dry column
6. Elute purified plasmid DNA
a. Transfer column to new collection tube.
b. Add 100 µl Elution Solution. Spin 1min.
DNA purification from gel – DTU Denmark – 2009 http://2009.igem.org/Team:DTU_Denmark/protocols
Using illustra™ GFX PCR DNA and Gel Band Purification Kit. Protocol from manual has been slightly altered by eluting using MiliQ Water and not elution buffer.
1. Sample Capture
a. Weigh a DNase-free 1.5 ml micro centrifuge tube and record the weight.
b. Using a clean scalpel, long wavelength (365 nm) ultraviolet light and minimal exposure
time, cut out as small an agarose band as possible containing the sample of interest.

Place agarose gel band into a DNase-free 1.5 ml microcentrifuge tube.
c. Weigh the microcentrifuge tube plus agarose band and calculate the weight of the agarose slice.
d. Add 10 µl Capture buffer type 2 for each 10 mg of gel slice.
e. Mix by inversion and incubate at 60°C until the agarose is completely dissolved. Mix by inversion every 3 minutes.
f. For each purification that is to be performed, place one GFX MicroSpin column into one Collection tube.

2. Sample Binding

a. Centrifuge Capture buffer type 2- sample mix briefly to collect the liquid at the bottom of the tube.
b. Transfer 600 µl Capture buffer type 2- sample mix onto the assembled GFX MicroSpin column and Collection tube.
c. Incubate at room temperature for 1 minute.
d. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.
e. Discard the flow through by emptying the Collection tube. Place the GFX MicroSpin column back inside the Collection tube.
f. Repeat Sample Binding steps b. to e. as necessary until all sample is loaded.

3. Wash & Dry

a. Add 500 µl Wash buffer type 1 to the GFX MicroSpin column.
b. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.
c. Discard the Collection tube and transfer the GFX MicroSpin column to a fresh DNase-free 1.5 ml microcentrifuge tube (supplied by user).

4. Elution

a. Add 10–50 µl Mq water to the center of the membrane in the assembled GFX MicroSpin column and sample Collection tube.
b. Incubate the assembled GFX MicroSpin column and sample Collection tube at room temperature for 1 minute.
c. Spin the assembled column and sample Collection tube at 16 000 × g for 1 minute to recover the purified DNA.
d. Proceed to downstream application. Store the purified DNA at -20°C.

DNA purification from enzymatic reaction – DTU Denmark – 2009
http://2009.igem.org/Team:DTU_Denmark/protocols
Using illustra™ GFX PCR DNA and Gel Band Purification Kit. Protocol from manual has been slightly altered by eluting using MiliQ Water and not elution buffer.

1. Sample Capture

a. Add 500 µl Capture buffer type 2 to up to 100 µl sample.
b. Mix thoroughly.
c. For each purification that is to be performed, place one GFX MicroSpin column into one Collection tube.

2. Sample Binding

a. Centrifuge Capture buffer type 2-sample mix briefly to collect the liquid at the bottom of the tube.
b. Load the Capture buffer type 2-sample mix onto the assembled GFX MicroSpin column and Collection tube.
c. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.
d. Discard the flow through by emptying the Collection tube. Place the GFX MicroSpin column back inside the Collection tube.

3. Wash & Dry

a. Add 500 µl Wash buffer type 1 to the GFX MicroSpin column.
b. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.
c. Discard the Collection tube and transfer the GFX MicroSpin column to a fresh DNase-free 1.5 ml microcentrifuge tube (supplied by user).

4. Elution
a. Add 10–50 µl Mq-water to the center of the membrane in the assembled GFX MicroSpin column and sample Collection tube.
b. Incubate the assembled GFX MicroSpin column and sample Collection tube at room temperature for 1 minute.
c. Spin the assembled column and sample Collection tube at 16 000 × g for 1 minute to recover the purified DNA.
d. Proceed to downstream application. Store the purified DNA at -20°C.
Transformation of chemically competent *E. coli* – DTU Denmark – 2009
http://2009.igem.org/Team:DTU_Denmark/protocols
Defrost the frozen cells on ice. Use 50µl cells pr transformation for transformation of plasmids and 100 µl of cells for transformations of ligation mix.
Add an appropriate volume of plasmid1[1] (often 1µl) to 50µl cell suspension. If transforming with a ligation mix add half of the ligation mix (5-10 µl ligation mix) to the 100µl cells.
Keep on ice for 30 minutes
Heat chock for 90 seconds at 42°C.
Transfer quickly to ice and keep them for 5 minutes.
Plate 100 µl cell suspension on selective medium (LB-amp) and incubate at 37°C O/N. Store the rest of the transformed cells in the fridge.
Between 5 and 6 the following can be done:
Add 1ml LB medium. Use 0,5 ml for ligation mixtures. Mix.
Incubate samples for 1 hour at 37°C.
High Efficiency Yeast Transformation – DTU Denmark – 2009
http://2009.igem.org/Team:DTU_Denmark/protocols
DAY 1
Inoculate the yeast strain in 2-5ml of liquid YPD and incubate O/N at 30°C on a rotary shaker. Leave a 50 mL shakeflask in the incubater with YPD, so it will be 30 C in the morning.
In the morning, inoculate a shake flask containing 50ml YPD with the 2-5ml cultures. Incubate 3-5 h at 30°C on a rotary shaker. (Option: take 1 mL of o/n culture and leave it for 5 hours)
NB: For a transformation with a basic plasmid, the O/N culture is plenty.
DAY 2
Harvest the cells by centrifugation at 3000g for 5 min (for the centrifuge in 220 this is equal to 4220 rpm). You need the rotor F-16 from the first floor that can take falcon tubes. The rotor code should be 30.
Wash the cells in 25ml sterile water. Centrifuge at 3000g for 5 min and resuspend cells in 1ml water.
Boil a 1.0 ml sample of carrier DNA for 5 min and chill in an ice/water bath while harvesting the cells. *It is not necessary to boil the carrier DNA every time. Keep the aliquot in freezer and boil again after 3-4 freeze-thaws.* (They can usually be found in lab 015)
Transfer the cell suspension to a 1.5ml micro centrifuge tube and centrifuge for 30s. Remove the supernatant with a micropipette.
Resuspend the cells to a final volume of 1ml with water and vortex vigorously to resuspend the cells. Put the cells on ice.
Pipette 100µl samples (10^8 cells) into 1.5ml micro centrifuge tubes, one for each transformation and one extra for each culture for SC plating. Centrifuge at top speed for 30s and remove the supernatant with a micropipette. (NB: Remember to set water baths at 42).
Make sufficient Transformation Mix corresponding to the number of transformations that need to be performed (count 1 extra). Keep the mix on ice/water.
All volumes are mentioned in µl

(for a deletion you will need approx 300-500 ng of each fragment)

 Place Xµl of plasmid on top of the cells. Add (360-X)µl of Mix to each transformation tube and resuspend the cells by vortex mixing vigorously.

 Incubate the tubes at 42°C for 40 min

 Micro centrifuge at top speed for 30s and remove the transformation mix with a pipette.

 Redissolve in 200µl of sterile water into each tube. Stir the pellet by pipetting and vortex.

 For transformation with an integrative plasmid, linear construct or nucleotide, plate 100µl onto 2 selective plates.

Incubate the plates at 30°C for 2-3 days.

Producing competent cells – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols
Starting from a single colony on a plate:
Transfer colony into 50ml liquid LB media and leave in a 200rpm shaking incubator overnight
Take 10ml of the culture and innoculate into one litre LB and grow in shaking incubator until OD600 of 0.2-0.3 (4 hours?)
Put culture on ice for 30 minutes
Centrifuge at 4000g for 6 minutes
Remove supernatant and resuspend cells in an equal volume of ice-cold 0.1mM HEPES
Repeat centrifugation
Resuspend cells in 0.5 volume ice-cold 0.1mM HEPES
Repeat centrifugation
Resuspend cells in ice-cold 10% glycerol (20ml)
Combine to form two tubes of 40ml glycerol
Repeat centrifugation
Resuspend in ice-cold glycerol (3ml)
Divide cells into 100ul aliquots and store at -80
(Cells should be at a final volume of ~3 x 10^10 cells.ml^-1)
Competent cells Transformation – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols
Electrocompetent cells thawed on ice
Prepare vector DNA on ice
Biobricks
With pipette tip, punch hole through foil cover into designated well
Add 20uL DIW
We will be removing about 5uL; the rest needs to go in an eppendorf, labeled with biobrick number, and stored at -20°C
Violacein and melanin need to be thawed
Vector DNA pipetted into chilled 1mm separation electrocuvette = 4 total
5uL of biobricks
0.5uL of melanin and violacein plasmid
Add 45 uL Competent cells
Tap electrocuvette gently to evenly spread mixture in the electrocuvette gap with no air bubbles
Thoroughly dry the cuvette
1.68 kV passed across cuvette, 5.1-5.4 time constant at 200 ohms and 25 uF

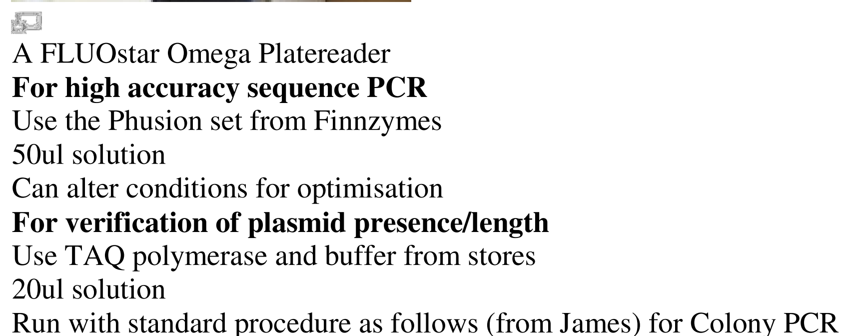
Add 0.25 mL SOC liquid medium to electrocuvette
Incubate electrocuvettes at 37 degrees C for 60 minutes
Pipette 150uL onto a (warmed) selective LB agar plate, spread with blue spreader
Orange genes biobrick: ampicillin
Promoter for orange genes biobrick: ampicillin
Melanin: ampicillin, copper, and tyrosine
Violacin: trimethoprim
Do 1:10 dilution with SDW into a new eppendorf
Pipette 150uL onto a selective LB agar plate, spread with blue spreader, 4 separate inoculums
Carotene extraction with acetone – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols
Adopted from *Yuan et al. (2006)*:
1. Incubate *E.coli* in 5ml LB with antibiotics at 37 oC for 20 hours.
2. Harvest cells using centrifugation at 4000 rpm for 10 minutes.
3. Re-suspend cells in 300 ul acetone and ***vortex for 5 minutes****** *(***: original protocol recommended "Homogenise cells with glass beads in Bead-Beater for 30s (Biospec products)", but we did not have the equipment. Vortex is used for homogenisation instead).*
4. Centrifuge sample at 14,000 rpm for 1 minute. Collect supernatant.
5. Measure absorption using spectrophotometer at 450 nm. Normalise data to cell density (OD 600 nm).
This is performed using **Omega Microplate Readers** from BMG-Labtech.
CAUTION: Acetone may corrode plastic microplates and cause severe damage to the equipment if left in the plate reader for too long. With microplate readers, dilute acetone extract 10 times with water (to give 200ul) before loading into plastic wells.
Agarose gel electrophoresis – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols
Gel preparation
To prepare 1% agarose gel (say 200ml), add 2g of agarose powder to 200 ml of 1X TAE buffer (obtained by diluting 10X TAE stock buffer with water) and heat in microwave until all powder desolves. Gel stains should be added when the agarose becomes cool enough to touch.
For ethidium bromide gels, add 2 uL EtBr to 20 mL of agarose (makes 1 gel).
Electrophoresis setting
For electrophoresis, set constant voltage at 80V (with current at approximately 3 mA) and run for 30--60 minutes (or until sufficient separation of DNA).

Finnzymes Phusion (TM) PCR – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols
Shuna's protocol
PCR Master Mix
25ul 2x Phusion Master Mix
0.5uM of each primer
~5ng of DNA
Make up to 50ul with SDW
PCR Cycle
Thirty seconds at 98 degrees
Thirty cycles:
98 degrees for 5-10 seconds
67 degrees for 10-30 seconds (depending on primer)
72 degrees for 30 seconds/kb
Final extension at 72 degrees for 5-10 minutes Hold at four degrees.
James's protocols:
Phusion master mix
2.5 uL forward primter
2.5 uL reverse primer
0.5 uL template

25 uL 2x Phusion master mix
optional 1.5 uL DMSO
make up to 50 uL with SDW
Using Phusion enzyme
2.5 uL forward primter
2.5 uL reverse primer
0.5 uL template
1 uL dNTPs
0.5 uL Phusion polymerase
10 uL x5 buffer (use buffer GC for high GC content, has shown to give higher product yeilds)
make up to 50 uL with SDW

PCR procedures – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols

G-Storm and Finnzymes PCR Machines



A FLUOstar Omega Platereader

For high accuracy sequence PCR

Use the Phusion set from Finnzymes
50ul solution
Can alter conditions for optimisation

For verification of plasmid presence/length

Use TAQ polymerase and buffer from stores
20ul solution
Run with standard procedure as follows (from James) for Colony PCR

Reaction Mixture

Template: 1 uL from O/N culture (1-2 uL if colony picked straight from plate into water)
VF2: 1uL
VR: 1uL
Eco-Taq: 0.2 uL
10X buffer: 5 uL
dNTPs: 0.4 uL (stock is 10 mM)
make up to 20 uL volume with H20

Reaction procedure
95 degrees C for 2 minutes
33 cycles of:
95 degrees C for 30 seconds (denaturation)
65 degrees C for 30 seconds (annealing)
72 degrees C, 1000bp/min (elongation)
72 degrees C for 5 minutes
hold at 4 degrees C
Gels
Run on EtBr for good quality viewing (make agarose gel). This is viewed in the red-room downstairs.
Run on SYBR-safe gel if the DNA is required. This can be viewed under blue light in the covered dark-area.
Glycerol Stocks – Cambridge – 2009 http://2009.igem.org/Team:Cambridge/Protocols
For -80
Cells in 10-15% glycerol, 1:4 ratio of glycerol:culture
0.5mL 80% glycerol
2mL fresh culture
For -20
Cells in 20-40% glycerol, 1:1 ratio of glycerol:culture
1mL 80% glycerol
1mL fresh culture
Add cells to glycerol, vortex, put on ice immediately, then store.

Plates preparation - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Autoclave LB medium with 1.5% agar.
Cool (at about 50°C, to prevent agar polymerization).
Before pouring the plates add antibiotic (Ampicillin 1000x [ ] or Kanamicin 200x [ ]).
Put about 20ml of medium per plate.
Leave it solidify and store at 4°C.

Up

Transformation - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Thaw the competent cells in ice (do not refreeze).
Add 0.1-0.3µg of plasmidic DNA or the respective amount of the ligation reaction into microfuge tubes on ice.
Add 80µl of cells.
Keep on ice for 20min.
HeatShock at 42°C for 60sec without agitation.
Keep on ice for 2min.
Add 0.9 ml of LB medium at room temperature.
Incubate at 37°C for 1hr with agitation.
Pellet the cells and discard most of supernatant, leaving about 100µl.
Resuspend the pellet and streak on plates containing appropriate antibiotics.
Incubate the plates overnight at 37°C.

Up

Inoculation - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Put 5 ml of LB media in a 15 ml tube.
Add the appropriate antibiotic.
Pick one colony from the plate with the inoculation loop
Immerse the loop into the LB solution.
Incubate the plates overnight (12 hours) at 37°C.

Up

Miniprep - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Pellet for 10 mins at 4400 rpm and discard most of supernatant.
Resuspend pelleted bacterial cells in 250µl of Buffer P1 and tranfer to a microcentrifuge tube.
Add 250µl of Buffer P2 and mix thoruoghly by inverting the tube 4-6 times.
Add 350µl of Buffer N3 and mix immediately and thoruoghly by inverting the tube 4-6 times.
Centrifuge for 10 min at ~18,000 x g in a table-top microcentrifuge.
Apply the supernatant (from step 4) to the QIAprep spin column by decanting or pipetting.
Centrifuge for 30-60 s. Discard the flow-though.
Recommended: Wash the QIAprep spin column by adding 0.5 ml of Buffer PB and centrifuging for 30-60 s. Discard the flow through.
Wash QIAprep spin column by adding 0.75ml of Buffer PE and centrifuge for 30-60 s.
Discard the flow through and centrifuge for an additional 1 min to remove residual wash buffer.
To elute DNA, place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 30µl of Buffer EB (or water) to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Up

Digestion reaction - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

E/S cut
enzyme 1 --> ECO R1
enzyme 2 --> SPE
buffer --> ECO R1
X/P cut
enzyme 1 --> Xba
enzyme 2 --> Pst 1
buffer --> 3
S/P cut
enzyme 1 --> SPE
enzyme 2 --> Pst 1
buffer --> 2
E/P cut
enzyme 1 --> ECO R1
enzyme 2 --> Pst 1
buffer --> ECO R1

Mix:
0.5 µl of BSA
0.5µl of each enzyme

3µl of buffer
5µl of DNA
20.5µl of H2O(most pure)
Mix and then spin down.
1h at 37 °C.
20 min at 80 °C to inactivate rescriction enzymes.
Put in ice.
Start gel run preparation.

Up

Gel preparation - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

150ml of Buffer TBE 1x.
Add the appropriate quantity of Agarose for the desired thickness
0.7%= 1g of agarose
0.7-1% (from 400 bases to 3Kb DNA fragments)
0.5% (6-7 Kb fragments)
2-3% (50-100 base long fragments)
Microwaves for 2 min.
Cool down under flowing water.
Add 10µl of EtBr.
Prepare the gel tray and set in place the wide-thoot comb.
Pour the gel in the gel tray (work in the hood for safety purpose)
Get rid of bubbles and let solidify.

Up

Gel Electrophoresis - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Put the solidified gel in the apposite run container.
Check that TBE fully covers the gel.
Gently extract the comb.
Add the Loading buffer in the digested DNA (6 ul of loading 6X in 30 ul of digestion volume).
Make the deposit in the wells of the samples and loading of reference (a part of loading should be prepared to scale by reference).
Close the container.
Start the run:
50/100 V --> until separate bands
120 V --> until half run
140 V--> until end of run.

Up

Gel extraction - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.

After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
Add 1 gel volume of isopropanol to the sample and mix.
Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold.
To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube.
Recommended: add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
Centrifuge the column in a 2 ml collection tube (provided) for 1 min at 18,000 x g.
Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
To elute DNA, add 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl of elution buffer to the center of the QIAquick membrane, let the column stand for 1 min and then centrifuge for 1 min.
If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Up

Ligation reaction - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Ligation with one insert and one vector --> f.v.=20µl
8µl of insert
4µl of vector
1µl of T4 ligase
4µl of Buffer 5X
3µl of H_2O mQ
Ligation with two insert and one vector --> f.v.=30µl
4µl of vector
8µl of insert 1
8µl of insert 2
1µl of T4 ligase
6µl of Buffer 5X
3µl of H_2O mQ
Conservation:
40 min at Room Temperature, or
3 hours at 15°C

Up

Chemiocompetent cells - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Take some colonies of DH5α cells from a fresh streaked plate and inoculate into 125ml of Soc medium. Grow the cells overnight at 25°C (it is advised to grow them slowly in order to have them better synchronized). It takes approximately 20 hours. It is advisable to grow the cells at 25°C overnight and then to shift them at 37°C. Bacteria are ready for harvesting when OD600 is between 0.37 and 0.4. Higher OD will lead to less competent cells (it is important to harvest the bacteria when they are still in the logarithmic phase of growth).

transfer the culture into 50 ml falcon tubes.
Centrifuge at maximum speed at 4°C for 10 min.
Re-dissolve all the cells in 40ml of Transformation buffer.
Incubate on ice for 10 min.
Spin down the cells (at maximum speed) at 4°C for 10 min.
Re-dissolve the pellet in 10ml of Transformation buffer.
Add 700µl of DMSO.
Aliquot (250µl) and freeze at -80°C.

Up

Mediums and buffers - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Soc medium
To prepare 200 ml of SOC medium dissolve in ultrapure water:
Tryptone 4g
Yeast extract 1g
1N NaCl 2ml
1N KCl 0.5ml
5M NaOH 40 µl to adjust the pH to 6.8
After autoclave add 2ml each of 2M Mg-salt (1M $MgSO_4$ and 1M MgCl) and 2M glucose.

LB medium
To prepare 1L of LB medium dissolve in ultrapure water:
Tryptone 10g
Yeast extract 5g
NaCl 10g
5M NaOH 200µl
Autoclave and store at room temperature.

Transformation Buffer
(to be always made fresh)
To prepare 60 ml dissolve in ultrapure water:
(15mM) $CaCl_2$ 0.13g
(250mM) KCl 1.12g
(10mM) Pipes 0.18g
Adjust pH with KOH to 6.7.
Add (55mM) $MnCl_2$ 0.65g.
Filter with 0.22µm filter.

M9 medium
Dissolve 56.4g in 1l of distilled water.
Autoclave for 15 min at 121°C.
This convenient 5x concentrate can be stored and diluted as needed to prepare 5l of 1x M9 minimal salts.
For M9 medium:
Aseptically mix 200ml of 5X M9 minimal salts 5x[ ] with 643.9 ml of sterile water, if necessary, cool to 45-50°C.
Aseptically add 20ml of sterile 1M glucose and 2mL of sterile 1M magnesium solfate,0.1ml of 1M sterile calcium chloride, 34 ml Thyamine Hydroclhoride and 100 ml 2% casa-amino acids.

Up

Antibiotics stocks preparation - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols
Ampicillin
Dissolve ampicillin in ultrapure water 100mg/mL (stock 1000x [ ], working concentration 100 µg/mL).
Aliquot and store at -20°C.

Kanamycin
Dissolve Kanamicin in ultrapure water 50mg/mL (stock 1000x [ ], working concentration 50µg/mL).
Aliquot and store at -20°C.

Up

IPTG stocks preparation - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

IPTG (Isopropyl β-D-1-thiogalactopyranoside)0.1 M stocks.

Up

Fluorescence test - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

Growth O/N for 12h in M9 medium (5ml) of:
transformed *E. coli* with appropriate antibiotic;
In the morning, measure OD.
centrifuge 1ml of bacterial culture at 4400rpm for 3min at 25°C;
discard the supernatant;
resuspend the cell pellet in some microliters of M9 medium.
slide preparation and image analisis.
IPTG induction:
centrifugate bacteria culture at 4400rpm for 3min at 25°C;
discard the supernatant;
resuspend cell pellet in M9 medium and IPTG with appropriate antibiotic (1000x [ ]);
Incubate at 37°C.
Test fluorescence after the chosen time intervals.

Up

M9 supplemented media - Bolongia – 2009 http://2009.igem.org/Team:Bologna/WetlabProtocols

For 1L of 1X media
200 mL 5X M9 minimal salts
Dissolve 56.4 g Bacto M9 minimal salts, 5X from Difco in 1L H_2O
Separate into 200 mL aliquots
Autoclave to sterilize. 121°C for 15 minutes.
34 mL 10 mg/mL thiamine
Dissolve 10 mg per mL of H_2O
Use a 0.22 µm filter to filter-sterilize
10 mL 40% glycerol
Add 80 mL glyerol to 120 mL of H_2O
Autoclave to sterilize
20 mL 10% Casamino acids
Dissolve 50 g Bacto Casamino acids from Difco in 500 mL H_2O

Autoclave to sterilize
2 mL 1M $MgSO_4$
Dissolve 24.65 g $MgSO_4 \cdot 7H_2O$ in 100 mL H_2O
Autoclave to sterilize
100 µL 1M $CaCl_2$
Dissolve 14.7 g $CaCl_2 \cdot 2H_2O$ in 100 mL H_2O
Autoclave to sterilize
733.9 mL H_2O
Sterilize deionized water in autoclave
Combine above solutions using sterile technique. (May notice some precipitation during preparation but precipitate should go back into solution once volume is brought up to 1L with sterile H_2O.) Add antibiotic as appropriate and store at 4°C

Transformation – Aberdeen Scotland – 2009
http://2009.igem.org/Team:Aberdeen_Scotland/WetLab/Protocols
The following are different transformation methods used as part of our project
Transformation of TSS competent cells
1. Thaw 100µl of TSS/cells on ice
2. Add 1µl of biobrick DNA, pipette gently to mix(100pg – 1 ng of plasmid in a volume of 1-2µl)
3. Incubate on ice for 30 minutes
4. Add 0.9ml SOC at 4°C
5. Incubate for 1 hour at 37°C in shaker
6. Spread 100 µl onto agar plate, containing appropriate antibiotic (see note ii.)
7. Centrifuge remainder of cells at 200rpm / 5 min in a microfuge
8. Remove all except 50 µl of supernatant: resuspend gently with a pipette
9. Plate on a fresh agar plate, containing antibiotic
10. Invert plates and incubate overnight at 37°C

Notes; i) Antibiotic concentrations in LB:
Ampicillin - 100 µg ml^{-1}
Tetracycline - 10 µg ml^{-1} in ethanol
Chloramphenicol - 30 µg ml^{-1} in ethanol
Kanamycin - 30 µg ml^{-1} in dH_2O
ii) Antibiotic concentrations in LB-agar:
Ampicillin - 100 µg ml^{-1}
Tetracycline - 5 µg ml^{-1} in ethanol
Chloramphenicol - 15 µg ml^{-1} in ethanol
Kanamycin - 30 µg ml^{-1} in dH_2O+
Transformation of SCS1 Supercompetent Cells – Aberdeen Scotland – 2009
http://2009.igem.org/Team:Aberdeen_Scotland/WetLab/Protocols
Protocol is the same for XL-1 Blue competent cells which we also used.
1. Pre-chill Eppendorf tubes on ice (one for each transformation and one for pUC control). Preheat SOC medium to 42C.
2. Thaw the supercompetent (or competent in Xl-1 Blue) cells on ice. When thawed, gently mix and alliquot 53µl of cells into pre-chilled tube.
3. Add 0.9µl of B-mercaptoethanol to each aliquot of cells
4. Swirl contents gently. Incubate on ice for 10 minutes, swirling again every 2 minutes.
5. Add experimental DNA to each aliqout expect for one single tube to which 1µl of pUC control DNA is

added.
6. Incubate on ice for 30 minutes. 7. Heat-pulse the tubes in a 42C water bath for 45 seconds. The duration of this is critical for miximal efficiency.
8. Incubate tubes on ice for 2 minutes.
9. Add 0.9ml of preheated SOC medium and icubate the tubes at 37C for 2 hours in shaking incubator.
10. 100µl was plated onto agar plates and 2.5µl of pUC control was plated onto an Amp resistant agar plate.

Transformation of Calcium Chloride competent cells – Aberdeen Scotland – 2009
http://2009.igem.org/Team:Aberdeen_Scotland/WetLab/Protocols
1. Inoculate one E.coli colony in 5ml of LB medium (liquid) over night.
2. Take 1ml of the overnight culture and add it to 100ml of pre-warm LB liquid in a 1L flask.
3. Incubate until the optical density is about 0.5 at 600 nm (~3 hrs).
4. Split 100 ml culture into 25 ml aliqouts and incubate on ice for 10min. All the subsequent steps should be carried out at 4°C and cells should be kept on ice as much as possible.
5. Centrifuge for 10min at 3500rpm (4°C).
6. Remove supernatant carefully by pouring and pipetting of the reminder. Place on ice immediately.
7. Resuspend each pellet in 2.5 ml of chilled TSS buffer.
8. Add 100 µl to Eppendorf tubes on ice. Freeze those aliqouts in a dry ice/ethanol bath and sotre at -80°C.

Mini Preps – Aberdeen Scotland – 2009
http://2009.igem.org/Team:Aberdeen_Scotland/WetLab/Protocols
Our mini preps were prepared using QIAprep Spin Miniprep Kits from Qiagen. The protocol is from the handbook, using a Microcentrifuge.
Protocol
1. Culture E.coli overnight in 5ml LB medium in a shaking incubator at 37C
2. Centrifuge culture at 4000 rpm for 10 minutes.
3. Resuspend pelleted bacterial cells in 250µl Buffer P1 and transfer to a microcentrifuge tube.
4. Add 250µl Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
5. Add 350µl Buffer N3 and mix immediately and thoroughly by inverting tube 4-6 times.
6. Centrifuge for 10 min at 13,000 rpm in a table-top microcentrifuge.
7. Apply supernatant to QIAprep spin column by decanting/pipetting.
8. Centrifuge for 60s at 13,000 rpm and discard flow-through.
9. Wash column by adding 0.5ml Buffer PB, centrifuge for 60s and discard flow-through.
10. Wash column by adding 0.75ml Buffer PE, centrifuge for 60s and discard flow-through.
11. Centrifuge for an additional minute and discard flow-through.
12. To elute DNA, place QIAprep column in clean 1.5ml microcentrifuge tube. Add 50µl Buffer EB (or water) to center of each QIAprep spin column, let stand for 2 min and centrifuge for 1 min.

Preparing Competent Cells – Aberdeen Scotland – 2009
http://2009.igem.org/Team:Aberdeen_Scotland/WetLab/Protocols
Due to limited success using the Calcium Chloride method, we also utilised the TSS method in our experiments.
Preparing competent cells – TSS Method
1. Inoculate one E.coli colony in 5ml of LB medium (liquid) over night.
2. Take 1ml of the overnight culture and add it to 100ml of pre-warm LB liquid in a 1L flask.

3. Incubate until the optical density is about 0.5 at 600 nm (~3 hrs).
4. Split 100 ml culture into 25 ml aliqouts and incubate on ice for 10min. All the subsequent steps should be carried out at 4°C and cells should be kept on ice as much as possible.
5. Centrifuge for 10min at 3500rpm (4°C).
6. Remove supernatant carefully by pouring and pipetting of the reminder. Place on ice immediately.
7. Resuspend each pellet in 2.5 ml of chilled TSS buffer.
8. Add 100 µl to Eppendorf tubes on ice. Freeze those aliqouts in a dry ice/ethanol bath and store at -80°C.
Preparing competent cells – Calcium Chloride Method

1. Inoculate one E.coli colony in 5ml of LB medium (liquid) over night.
2. Take 1ml of the overnight culture and add it to 100ml of pre-warm LB liquid in a 1L flask.
3. Incubate until the optical density is about 0.5 at 600 nm (~3 hrs).
4. Aseptically transfer 50ml into a Falcon tube (repeat that step) and store on ice for 10min to cool the cultures to 0°C.
5. Centrifuge at 4000rpm for 10min at 4°C.
6. Take off supernatant and leave the tubes in an inverted position for about one minute to drain the reminder.
7. Resuspend the pellet in 10ml of ice-cold 0.1M $CaCl_2$.
8. Centrifuge at 4000rpm for 10min at 4°C.
9. Take off supernatant and leave the tubes in an inverted position for about one minute to drain the reminder.
10. Resuspend in 2ml of ice-cold $CaCl_2$ (0.1M) for each 50ml of original culture.
11. At this point the cells can be split into aliqouts of 200µl and be frozen at -70°C.

Roche: Rapid DNA Ligation Kit – Aberdeen Scotland – 2009
http://2009.igem.org/Team:Aberdeen_Scotland/WetLab/Protocols
Preparations:
Prepare 1x conc. DNA dilution buffer.
Prepare a total of 30-50 ng of DNA using a 1:3 molar ratio of vector : insert.
1. Dissolve Vector DNA and Insert DNA, in carefully mixed DNA dilution buffer.
2. Carefully mix T4 DNA Ligation buffer and subsequently add 10ul of DNA ligation buffer to mixture.
3. Add 1ul of T4 DNA Ligase to the mixture.
4. Incubate mixture for 5 minutes at 15-25*C
6. Store Ligation Mixture WITHOUT Heat Inactivating the Enzyme at -15 to -25 *C

Recipe for Competent Cells – Waterloo – 2009
http://2009.igem.org/Team:Waterloo/Notebook/Protocols
Materials
Frozen stock of DH5 alpha
250mL Erlenmeyer Flask (w/ caps)
LB tubes
Sterile LB
Instructions
1. From frozen stock DH5 alpha, inoculate 5mL LB tubes and grow overnight at 37˚C
2. Autoclave 250 mL Erlenmeyer flask with 50 mL LB broth for tomorrow. Don't forget to loosen to cap if you are not using tin foil.

3. Next morning: add culture into the autoclaved 250mL Erlenmeyer Flask with 50 mL LB broth.
4. Grow at 37 ˚C for 2-3 h to OD 0.4-0.6 i.e. log phase. Ask a lab leader to look at it to save time, or otherwise use a spectrophotometer to read it at absorbance at 600nm.
5. Transfer cultures to a falcon tube and spin at 4000 rpm for 15 min at 4˚C
6. Resuspend in 20 mL of 0.1 M $MgCl_2$ (preferred) or $CaCl_2$ (note: swirl until cells are completely resuspended, do not vortex.)
7. Spin again.
8. Resuspend in 10mL of 0.1 M $CaCl_2$
9. Incubate on ice for 4 hours
10. Spin again and prepare $CaCl_2$-glycerol solution.
___ Culture tubes
(___+1) x 0.86mL $CaCl_2$
(___+1) x 0.14mL 100% glycerol
11. Do the following in 4˚C walk-in:
- Resuspend a culture tube with 2mL of the $CaCl_2$-glycerol solution, and then resuspend a second culture tube with this.
- Aliquot into 200 µL or 500µL

<u>Transformation</u> – Waterloo – 2009 http://2009.igem.org/Team:Waterloo/Notebook/Protocols

Before you start

Cells lose their competence once thawed, so be sure to:
a) keep them on ice at all times.
b) use cells immediately after thawing.
c) discard any unused thawed cells.

Materials

Competent cells
DNA
Ice + Ice bucket
1.5mL microfuge tube
Glass Spreader
Ethanol
Agar Plate with desired antibiotic resistance
Alcohol burner

Instructions

1. Thaw competent cells on ice. Include 2 extra ones for a Negative (no DNA) and Positive control.
2. In a 1.5 mL microfuge tube, add 50 µL of cells and 1 µL of DNA, and pipette up and down to mix.
3. Incubate on ice for 30 min.
4. Transfer directly to 42C heat block for 45 s to “heat shock”.
5. Transfer to ice for 2 min.
6. Add 500 µL of LB.
7. Incubate at 37C for 1 h on the shaker.
8. Centrifuge tubes at 13,000rpm for 2 mins.
9. Decant most of the supernatant. Leave approximately 50 µL.
10. Prepare and label plates with date and construct names. Let the plates dry in the flow hood if necessary.

For each sample, the following steps must be carried out quickly in the laminar flow hood to ensure proper spreading of cells.

11. In the laminar flow hood, resuspend pellet in remaining liquid by pipetting up and down.
12. Transfer to center of plate.

13. Dip glass spreader in ethanol and flame.
14. Let the glass spreader cool by gently touching the agar.
15. Spread liquid around plate until it appears dry.
16. Seal plates with Parafilm or put in a bag.
17. Incubate inverted in 37 degrees Celsius incubator overnight.

Inoculation Protocol – Waterloo – 2009 http://2009.igem.org/Team:Waterloo/Notebook/Protocols

Materials

DNA Samples
Ampicillin (2µL/sample)
Kanamycin (2.5µL/sample)
Liquid LB Media (1 tube/sample)

Instructions

1. Label all tubes.
2. Do the following in the flow hood:
- Pipette the correct amount of antibiotic into the corresponding DNA tubes. 2 µL for ApR, 2.5 µL KmR.
- Dip the sterile stick into the DNA and into the corresponding media tube.
3. Incubate at 37 degrees Celsius overnight on the agitator.

Glycerol Stock Preparation – Waterloo – 2009
http://2009.igem.org/Team:Waterloo/Notebook/Protocols

Before you start

- It is important to use aseptic technique when carrying out the procedures

Materials

5 mL culture tube
60% Glycerol
Sterile screw-top cryotube

Instructions

1. Inoculate a 5 mL culture tube.
2. Incubate overnight on the shaker.
3. Pipette 500 µL of 60% glycerol culture into a sterile screw-top cryotube.
4. Piptette 500 µL of culture into the cryotube.
5. Label and date cryotube.
6. Vortex.
7. Cover the label with clear tape.
8. Store in -80°C freezer.
9. Add strain to database.

Ligation (10µL recipe with 3 insert : 1 vector ratio) – Waterloo – 2009
http://2009.igem.org/Team:Waterloo/Notebook/Protocols

Materials

MQ water
Ligase buffer
Ligase
Eppendorf tube

Vector DNA
Insert DNA
Instructions
1. Calculate ligation volumes. Total volume should be 10 µL and insert to vector ratio between 3:1 –5:1.
2. In one tube mix
- Vector DNA
- Insert DNA
- 10X ligase buffer
- Ligase
- MQ if needed
3. Leave on the bench for 1 hour (or even overnight)

Gel Electrophoresis – Waterloo – 2009 http://2009.igem.org/Team:Waterloo/Notebook/Protocols
Materials
Agarose
1X TAE buffer
Gel Red
1:10 diluted 1kb DNA ladder
Loading Dye
Gel Rig
Instructions for 0.8% Agarose 100 mL gel
Prepare the Gel
1. Measure 0.8 g of agarose on weigh paper.
2. Measure 100mL of TAE buffer using a graduated cylinder into a flask.
3. Add agarose to flask. Swirl to dissolve as much as possible.
4. Microwave for 20 seconds, swirl. Repeat until agarose is dissolved. Allow argrose to cool down to room temperature.
5. Add 2 µL of Gel Red into the gel.
6. Cover the ends of the gel tray with masking tape to seal them.
7. Pour the gel into the tray.
8. Drop the combs into the slots to form the wells.
9. Allow the gel to set.
10. Remove the combs and rinse with distilled water.
Preparing samples
1. Pipette 10µL each digested sample into a labeled microfuge tube (for gel extraction use 20 µL).
2. Add 2µL of loading dye to each sample (for gel extraction use 4 µL)
3. Find the diluted 1kb DNA ladder.
4. Organise the tubes in loading order.
5. Record loading order
Running the Gel
1. Place the tray in the rig with the wells closest to the far side.
2. Add TAE buffer until the surface of the gel is just covered.
3. Pipette 10 µL of the digestion mixture and diluted ladders into the appropriate wells.
4. Put the cover on. Black at the back and red at the head.
5. Connect the wires to an available power supply.
6. Set the power supply to 120V and start it.
7. Check that bubbles are forming near the electrodes.
8. Wait about 45 minutes. The dye front should be 3/4 of the way down the gel.

<u>DNA Extraction</u> – Waterloo – 2009 http://2009.igem.org/Team:Waterloo/Notebook/Protocols
Materials
All required materials provided in the BioBasic DNA Extraction Kit (K0513)
Instructions
1. Follow the instructions given in DNA Extraction Kit.
2. Nanodrop the construct.

<u>Digestion of Plasmid DNA</u> – Waterloo – 2009 http://2009.igem.org/Team:Waterloo/Notebook/Protocols
Before you start
- Know what is the total digestion volume mix. From that you can determine the enzyme concentration which will have to be at or below 10%. You can also calculate the buffer volume based on the concentration of the buffer needed
Materials
MQ water (V_{total} - V_{buffer} - V_{DNA} - $V_{enzymes}$)
Buffer (depends on required concentration: For 10x buffer, add 1/10 V_{total}
DNA (1 µL)
Enzymes (0.5 µL each/digest)
Microfuge tube (1/digest)
Instructions
1. In one tube, mix the above materials in the given order.
- Keep the enzymes cold as much as possible. Do not leave them out for longer than necessary.
2. Vortex briefly.
3. Centrifuge 2-3 seconds.
4. Digest 1 hour at 37ºC.

<u>Colony PCR</u> – Waterloo – 2009 http://2009.igem.org/Team:Waterloo/Notebook/Protocols
Materials
- 20 µL of Master Mix
<u>Calculations for Master Mix</u>
___ number of colonies (and control)
(___+1) x 0.5µL dNTPs
(___+1) x 15µL PCR H2O MQ
(___+1) x 2.5µL Taq Buffer
(___+1) x 0.5µL fwd primer
(___+1) x 0.5µL rev primer
Instructions
1. Prepare agar plate with the correct antibiotic. Draw a grid with as many squares as the colonies you're screening.
2. Label PCR tubes for each colony and one control (no DNA or colony)
3. Add 6µL of PCR H2O MQ to each PCR tube
4. Pick a colony from the agar plate with a pipette tip and resuspend in 6 µL PCR H2O. (if inoculating LB, resuspend in 7 µL PCR H2O)
5. Add 1 µL of resuspended DNA to a grid square. Incubate overnight at 37C.
6. Optional: Add 1 µL of resuspended DNA in corresponding LB tube and inoculate overnight
7. Prepare master mix. Vortex master mix before adding Taq. Once you added Taq mix gently.

8. Add 20 µL of Master Mix to each PCR tube.
9. Centrifuge briefly in the PCR machine. Use the pulse option.
10. Start the PCR machine on the desired cycle.

1-2-3 Miniprep (Resuspension, Lysis, Neutralization) – Waterloo – 2009
http://2009.igem.org/Team:Waterloo/Notebook/Protocols

Before you start

Prepare solutions 1, 2, and 3. Only solution 2 needs to be made fresh. Solutions 1 and 3 can be made in excess and stored at 4 degrees Celsius.

Solution 1

1. Combine
- 11 mL of 50 mM glucose
- 8.33 mL of 25mM Tris·Cl adjusted to pH 8.0
- 6.67 mL of 10mM EDTA adjusted to pH 8.0
2. Autoclave
3. Add RNase to make the concentration 450 µg/mL
4. Store in 4°C fridge

Solution 2

160 µL MQ water/sample
20 µL 10% SDS/sample
20 µL 2N NaOH/sample

Solution 3

1. Combine
- 60 mL of 5M KAc
- 11.4 mL glacial acetic acid
- 28.5 mL MQ water
2. Store in 4°C fridge

Materials

- Solution 1 (250 µL/sample)
- Solution 2 (250 µL/sample)
- Solution 3 (350 µL/sample)
- 1.5 mL microfuge tubes (3/sample)
- Isopropanol (600 µL/sample)
- 70% ethanol (500 µL/sample)

Instructions

1. Label microfuge tubes.
2. Pour ~1.5mL cell culture in a microfuge tube till it's almost full.
3. Centrifuge for 30 seconds at 13,000 rpm.
4. Decant the supernatant.
5. Repeat steps 2-4 until all cells have been pelleted.
6. Resuspend cells thoroughly in 250µL of solution 1. Incubate for 2 minutes.
7. Add 250µL of solution 2.
8. Mix gently by inverting 6 to 8 times.
9. Incubate for exactly 5 minutes.
10. Add 350µL of solution 3. Mix gently by inverting 4 to 6 times.
11. Incubate on ice for 5 minutes.
12. Centrifuge for 10 min at 13,000 rpm at 4 degrees Celsius. While waiting, label new sets of tubes.
13. Transfer supernatant to a new 1.5 mL tube. Discard old tubes.
14. Add 600µL of isopropanol. Invert 6-8 times to mix.

15. Spin for 10 min at 13,000 rpm to pellet DNA.
16. Discard supernatant without disturbing pellet.
17. Add 500µL of ice-cold 70% ethanol. Mix gently by inverting 6-8 times to wash.
18. Spin for 10 min at 13,000 rpm to pellet DNA.
19. Carefully decant the supernatant without disturbing the pellet.
20. Leave tubes open in inverted position to dry, or put in speed-vac.
21. Resuspend DNA in MiliQ water for storage in -20 degrees Celsius.

Plate Pouring Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
For 1 L ~ 60 plates
20 g LB powder (follow label directions)
Add 15 g/L agar, fill to 1L water
Autoclave, let cool till touchable - Don't let solidify!
Add antibiotic:
Amp: from 50 mg/mL stock, to a final concentration of 250 ug/mL, so 2.5 mL per 500 mL beaker
Kan: from 12 mg/mL stock, to a final concentration of 30 ug/mL, so 1.25 mL per 500 mL beaker
Chl: from 35 ug/mL stock, to a final concentration of the same, so 1 mL per 1 L beaker
Agitate and pour plates

Top 10 Preparation of Competent Cells Protocol – VictoriaBC – 2009
http://2009.igem.org/Team:VictoriaBC/Labprotocols
Taken from **http://openwetware.org/wiki/TOP10_chemically_competent_cells**

Preparing seed stocks
Streak TOP10 cells on an **SOB** plate and grow for single colonies at 23°C
room temperature works well
Pick single colonies into 2 ml of SOB medium and shake overnight at 23°C
room temperature works well
Add glycerol to 15%
Aliquot 1 ml samples to Nunc cryotubes
Place tubes into a zip lock bag, immerse bag into a dry ice/ethanol bath for 5 minutes
This step may not be necessary
Place in -80°C freezer indefinitely.

Preparing competent cells
Inoculate 250 ml of **SOB** medium with 1 ml vial of seed stock and grow at 20°C to an OD600nm of 0.3
This takes approximately 16 hours.
Controlling the temperature makes this a more reproducible process, but is not essential.
Room temperature will work. You can adjust this temperature somewhat to fit your schedule
Aim for lower, not higher OD if you can't hit this mark
Centrifuge at 3000g at 4°C for 10 minutes in a flat bottom centrifuge bottle.
Flat bottom centrifuge tubes make the fragile cells much easier to resuspend
It is often easier to resuspend pellets by mixing *before* adding large amounts of buffer
Gently resuspend in 80 ml of ice cold CCMB80 buffer
sometimes this is less than completely gentle. It still works.
Incubate on ice 20 minutes
Centrifuge again at 4°C and resuspend in 10 ml of ice cold CCMB80 buffer.
Test OD of a mixture of 200 µl SOC and 50 µl of the resuspended cells.

Add chilled CCMB80 to yield a final OD of 1.0-1.5 in this test.
Incubate on ice for 20 minutes
Aliquot to chilled screw top 2 ml vials or 50 µl into chilled microtiter plates
Store at -80°C indefinitely.
Flash freezing does not appear to be necessary
Test competence (see below)
Thawing and refreezing partially used cell aliquots dramatically reduces transformation efficiency by about 3x the first time, and about 6x total after several freeze/thaw cycles.

Measurement of competence – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
Transform 50 µl of cells with 1 µl of standard pUC19 plasmid (Invitrogen)
This is at 10 pg/µl or 10^{-5} µg/µl
This can be made by diluting 1 µl of NEB pUC19 plasmid (1 µg/µl, NEB part number N3401S) into 100 ml of TE
Hold on ice 0.5 hours
Heat shock 60 sec at 42C
Add 250 µl **SOC**
Incubate at 37 C for 1 hour in 2 ml centrifuge tubes rotated
using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
For our plasmids (pSB1AC3, pSB1AT3) which are chloramphenicol and tetracycline resistant, we find growing for 2 hours yields many more colonies
Ampicillin and kanamycin appear to do fine with 1 hour growth
Plate 20 µl on AMP plates using sterile 3.5 mm glass beads
Good cells should yield around 100 - 400 colonies
Transformation efficiency is (dilution factor=15) x colony count x 10^5/µgDNA
We expect that the transformation efficiency should be between $5x10^8$ and $5x10^9$ cfu/µgDNA

Materials
Detergent-free, sterile glassware and plasticware (see procedure)
Table-top OD600nm spectrophotometer
SOB
CCMB80 buffer
10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
80 mM $CaCl_2.2H_2O$ (11.8 g/L)
20 mM $MnCl_2.4H_2O$ (4.0 g/L)
10 mM $MgCl_2.6H_2O$ (2.0 g/L)
10% glycerol (100 ml/L)
adjust pH DOWN to 6.4 with 0.1N HCl if necessary
adjusting pH up will precipitate manganese dioxide from Mn containing solutions.
sterile filter and store at 4°C
slight dark precipitate appears not to affect its function

Preparation of Competent Cells Protocol – VictoriaBC – 2009
http://2009.igem.org/Team:VictoriaBC/Labprotocols
BCMB 301B Protocol

Cells must be kept cold at all times.

1.) An overnight culture of E. coli DH5α is subcultured by diluting 1/100 in LB medium and grown in a shaking water bath at 37oC until the OD600 is between 0.4 and 0.6 (2-3 hr).
2.) Chill 20 mL of this subculture on ice for 15 min.
3.) Centrifuge cells for 5 min at 7500 rpm.
4.) Decant supernatant and resuspend pellet in 1/5 growth volume (4 mL) of CM1 buffer.
5.) Centrifuge cells for 5 min at 7500 rpm.
6.) Decant supernatant and resuspend pellet in 1/50 growth volume (0.4 mL) of CM2 buffer.
7.) Transfer two 200 uL aliquots to small sterile test tubes, and two 200 uL aliquots to epi tubes. Keep competent cells on ice.

Buffer preparations

CM1:
NaOAc = 0.1 mL of 1 M pH 5.6 stock to produce 10 mM
MnCl2 = 0.5 mL of 1 M stock to produce 50 mM
NaCl = 0.2 mL of 1 M stock to produce 5 mM
Top to 10 mL with water and chill on ice.

CM2:
NaOAc = 0.1 mL of 1 M pH 5.6 stock to produce 10 mM
Glycerol = 0.5 mL of 99.5% stock to produce 5%
MnCl2 = 0.05 mL of 1 M stock to produce 5 mM
CaCl = 0.7 mL of 1 M stock to produce 70 mM
Top to 10 mL with water and chill on ice.

Rehydration Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
Biobrick parts are shipped from the registry in a dehydrated from. As such they must be rehydrated before they can be used.
Puncture a hole through the foil with a pipette tip into the well that corresponds to the Biobrick - standard part that you want
Add 15 µL of diH20 (deionized water)
Let the water sit for 5 minutes
Take 2 µL DNA and transform into your desired competent cells, plate out onto a plate with the correct antibiotic and grow overnight. Your goal here is to obtain single colonies.

Transformation Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
1) Add 2ul of rehydrated plasmid to 100ul of competent cells, let sit on ice for 30 minutes
2) Heat shock at 42 degrees for exactly 2 minutes in a hot water bath
3) Chill on ice for 1-2 minutes
4) Add 400ul of pre-warmed LB
5) Incubate at 37 degrees for 1 hr 15 min under medium agitation for amp plates, and 2 hr for kan and cm plates
6) Plate 200 ul and incubate at 37 degrees

Broth Culture Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
Next Day Broth Culture Protocol:
A.
From a single colony on a plate, use a sterile stick to pick and then swirl in 3 mL of LB + antibiotic.

Antibiotic quantities:
Amp: add 15uL to 3 mL LB
Kan: add 7.5uL to 3 mL LB
Cm: add 3uL to 3 mL LB

From: **http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000248&r=1880**
Pick a single colony from a freshly streaked selective plate and inoculate a culture of 1–5 ml LB medium containing the appropriate selective antibiotic. Incubate for 12–16 h at 37°C with vigorous shaking.
Growth for more than 16 h is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube or flask with a volume of at least 4 times the volume of the culture.

B.
From a single colony on a plate, use a sterile stick to pick and then swirl in 50 mL of LB.

IPTG induction protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols

Made 105uL of 1M IPTG from 0.025g IPTG in 105uL sterile deionized water.

Taken from **http://openwetware.org/wiki/IPTG**
Isopropyl-beta-D-thiogalactopyranoside
Induces transcription from promoters regulated by LacI repressor.
Molecular weight is 238.31 g/mol. The chemical formula is **here**.
Dissolve 1g in 4196 µL deionized water to make 1M solution.
Filter sterilize with syringe and 0.22µm filter
Generally a 1mM solution is an effective amount to induce the pLac promoter region. It should be noted that this may vary over cell strains. For example:
lacIq: cell strains which overproduce LacI repressor (ie. ***E. coli*** **cell strain** type **D1210**, aka. **BBa_V1003**)
For including IPTG in LB agar plates, a typical amount people recommend is 0.1-0.5mM IPTG.

Qiagen Miniprep Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
From **http://openwetware.org/wiki/Miniprep/Qiagen_kit**

Procedure
Pellet 1-5mL of an overnight culture by spinning 1.5 mL at 8000 for 10 min.
Resuspend pelleted bacterial cells in 250 µl Buffer P1 (kept at 4 °C) and transfer to a microcentrifuge tube.
Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
Add 250 µl Buffer P2 and gently invert the tube 4–6 times to mix.
Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
Even if all you care about is the plasmid DNA, don't vortex it - this will cause smearing on your gel and hide the plasmid band.
Add 350 µl Buffer N3 and invert the tube **immediately** but gently 4–6 times.

To avoid localized precipitation, mix the solution gently but thoroughly, immediately after addition of Buffer N3. The solution should become cloudy.
Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
A compact white pellet will form.
Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.
Centrifuge for 30–60 s. Discard the flow-through.
Spinning for 60 seconds produces good results.
(Optional): Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.
This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.
Although they call this step optional, it does not really hurt your yield and you may think you are working with an endA- strain when in reality you are not. Again for this step, spinning for 60 seconds produces good results.
Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.
Spinning for 60 seconds produces good results.
Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.
IMPORTANT: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions. *They are right about this.*
Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.
If you are concerned about the concentration of the DNA, you can alternatively add 30 µL water to the center of the column, incubate at room temperature on the bench for 5 mins and then centrifuge for 1 min. This will increase the concentration of DNA in your final sample which can be useful in some cases. See notes below for why you should elute in water rather than the Buffer EB they recommend if you plan to sequence your sample. Even if you are not sequencing, it may be beneficial to elute in water. For instance, if you elute in buffer EB and you are using this DNA in a restriction digest, then the additional salts in your sample can affect the salt content of your digest. This may matter with some finicky enzymes.

Agarose Gel Electrophoresis Protocol – VictoriaBC – 2009
http://2009.igem.org/Team:VictoriaBC/Labprotocols

Materials

Do not autoclave solutions containing isopropanol or MOPS; use sterile filtration if necessary.

Buffer P1 - Alkaline prep buffer
50 mM Tris-HCl pH 8.0
10 mM EDTA
100 µg/ml RNaseA
The buffer and RNaseA can also be ordered from Qiagen separately (catalog numbers 19051 and 19101).
(Denatures DNA and protein, chromosomal DNA separated from plasmid because it's bound to cell wall. Plasmid DNA remains in clear supernatant.)

Buffer P2 - Lysis buffer
200 mM NaOH

1% SDS
Check Buffers P2 and P3 for salt precipitation and redissolve at 37 degrees C if neccessary.
(This is why we mix slowly, to prevent contamination of plasmid with chromosomal DNA (There is no other step that separates them).)

Buffer P3 (not for spin columns, but for Qiatips, midi, maxi, giga kits)
3.0 M potassium acetate pH 5.5

Buffer N3 - Neutralizing buffer
4.2 M Gu-HCl
0.9 M potassium acetate
pH 4.8

Buffer PB - Endonuclease wash
5 M Gu-HCl
30% ethanol
(maybe add 10mM Tris-HCL PH 6.6, and that is better)
(Removes endonucleases which may degrade target DNA.)

Buffer PE - Ethanol wash
10 mM Tris-HCl pH 7.5
80% ethanol
(Removes salts.)

Buffer QBT equilibration buffer
750 mM NaCl
50 mM MOPS pH 7.0
15% isopropanol
0.15% triton X-100

Buffer QC wash buffer
1.0M NaCl
50 mM MOPS pH 7.0
15% isopropanol

Buffer QF elution buffer
1.25M NaCl
50 mM Tris-HCl pH 8.5
15% isopropanol

Buffer QN
1.6M NaCl
50 mM MOPS pH 7.0
15% isopropanol
Buffer FWB2
1M potassium acetate, pH 5.0

Cryopreservation Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
Materials
40% glycerol solution
Cryogenic vials

Method

Make a 1/100 dilution subculture of an overnight subculture, or pick from overnight plates and inoculate
Allow to grow from some hours, so it will be in logarithmic growth phase
Add 1 ml of 40% glycerol in H_2O to a cryogenic vial.
Add 1 ml sample from the culture of bacteria to be stored.
Gently vortex the cryogenic vial to ensure the culture and glycerol is well-mixed.
Alternatively, pipet to mix.
Use a tough spot to put the name of the strain or some useful identifier on the top of the vial.
On the side of the vial list all relevant information - part, vector, strain, date, researcher, etc.
Store in a freezer box in a -80C freezer. Remember to record where the vial is stored for fast retrieval later.

Notes

While it is possible to make a long term stock from cells in stationary phase, ideally your culture should be in logarithmic growth phase.

Agarose Gel Electrophoresis Protocol – VictoriaBC – 2009
http://2009.igem.org/Team:VictoriaBC/Labprotocols
Gel preparation
1.) Prepare 500 mL of 1X TAE buffer (10 mL from 50X TAE: 2M Tris, 1M Acetic acid, 50 mM EDTA)
2.) Prepare a 60 mL 1% powdered agarose gel (0.6g) (or 80mL with 0.8g for larger tray)
3.) Combine agarose and TAE in 125 mL flask and plug with scrunched up Kimwipe.
4.) Dissolve in microwave for 50 sec., swirl.
5.) If cloudy, reheat for 15 sec.
6.) Repeat with 5 sec. heating times until crystal clear. Be Careful!
7.) When it has cooled enough to touch add 8 uL of a 2 mg/mL solution of ethidium bromide.
8.) Immediately pour in to the tray and insert comb.
9.) Set for at least 20 min.

Electrophoresis – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
1.) Mix 2 uL undigested plasmid DNA with 2 uL 6X sample buffer and 8 uL dH2O. (For digested DNA use 4 uL DNA with 2 uL 6X sample buffer and 6uL dH2O.)
2.) Load the mixed 12 uL samples into lanes.
3.) Apply constant voltage of 100V and run for 45 min in 1X TAE.

Method 2.
EtBr soak for 10-20 minutes after gel has run instead of adding to gel itself.
Final concentration of 0.2 ug/mL, so for 500mL, add 10uL of 10mg/mL EtBr.

We're thinking that the **DNA ladder** from Ivitrogen (**this page**) corresponds to our ladder.

3A Assembly Protocol – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
Taken from **http://ginkgobioworks.com/support/**

Red - Destination Plasmid
Blue/Purple - Upstream Part
Green/Yellow - Downstream Part

Digest – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
Vortex everything before adding
1. Prepare 3 tubes for: Upstream, Downstream and destination Plasmid
2. Add 22.5 uL sterile, deionized water (=42.5 uL - 500 ng DNA with DNA at 25 ng/uL)
3. Add 20 uL DNA (500 ng at 25 ng/uL)
4. Add 5 uLNEBuffer 2
5. Add 0.5 uL BSA
6. Add 1 uL 1st restriction enzyme
7. Add 1 uL 2nd restriction enzyme
=This should equal 50 uL.
8. Mix by flicking tube
9. Incubate at 37oC for 15 min
10. Incubate at 80oC for 20 min

You can now freeze these digests at -20oC or proceed
(You can also run a 1% agarose gel with 20 uL of the digest to test for digested part lengths)

Ligate – VictoriaBC – 2009 http://2009.igem.org/Team:VictoriaBC/Labprotocols
1. Agitate 10X T4 DNA Ligase Reaction Buffer until all precipitate is in solution
2. Prepare a tube for each addition
3. Add 11 uL sterile, deionized water
4. Add 2 uL from each digest: Upstream, Downstream, and Plasmid (6 uL total)
5. Add 2 uL 10X T4 DNA Ligase Reaction Buffer
6. Add 1 uL T4 DNA Ligase
=This should equal 20 uL
7. Incubate at room temperature for 10 min
8. Incubate at 80oC for 20 min

You can now freeze these ligation mixes at -20oC or proceed to Transformation.

Cleaning Miniprep Spin Columns Protocol – VictoriaBC – 2009
http://2009.igem.org/Team:VictoriaBC/Labprotocols
- soak column 24-48 hr in 1M HCl - try make sure no air bubbles at filter surface
- store in acid to inhibit contamination - say 0.1M HCl
- before use, rinse and reequilibrate
- 3-5x 0.75mL dH_2O spin thru, discard
- 1x 0.75mL PE spin thru, discard and spin again

full reference to come
stub: BioTechniques 42:186-192 (February 2007)

Spectroscopy Data Collecting Protocol – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
1. Aliquot 1mL of overnight culture to absorbance cuvette
2. Take optical density absorbance reading to acquire concentration data (to getidea about growth kinetics)
3. Sonicate 200mL ofsample (4x10s)to release cellular contents
4. Blank spectrometer with LB
5. Load sonicated sample into quartz cuvette and take absorbance scan from350-500nm

6. Acquire peaks and check absorbance values attarget areas

Western Blotting for SAA Secretion – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
Sample collection
1. Prepare overnight broth cultures of E. coli cellstransformed with the SAA expression construct and pSB1AC3 plasmid backbone (control cells)
2. Re-suspend 500ul of each culture in 50ml LBmedia in a 125mL bottle. Incubate the bottles on 37°Cshaker.
3. Collectsamples at 0hrtime-point by transferring 3ml ofthe each 50ml culture into a 10mlfalcon tube.
4. Pellet cells by centrifugation (4000rpm, 15minutes) atroomtemperature.
5. Transferthe supernatantinto a new 10mlfalcon tube. Store cell pellets and supernatants at -20°C.
6. Repeatstep 3 to 5 to collectsamplesfor 2hr, 4hr, and 12hrtime-point.
Sample preparationsfor SDS-PAGE
7. Transfer 55ul ofthe supernatantsamplesinto amicrocentrifuge tube. Add 11ul of 6X SDS sample bufferinto each tube. Boilsamples at 100°C for 5minutes.
8. Prepare 10X dilutions ofthe supernatantsamples bymixing 5.5ul of original supernatants, 49.5ul of ddH2
O, and 11ul of SDS sample bufferinmicrocentrifuge
tubes.
9. Resuspend the cell pelletsin 2ml of ddH2
O.
10. Repeatstep 7 and 8 to prepare cell lysate samples.
11. Repeatstep 7 and 8 to prepare positive controlsamples - HDL derived from inflamedmice (obtained fromDr.Deeley'slab atQueen's Cancer Research Institute)
Western blotting
Gelrecipe:
7.5% gel(for 1mmspacer)
reagents separation gel(for 4) stacking gel(for 4)
H2
O 20ml 6ml
Buffer 10ml 2.5ml
Acryl(30:08) 10ml 1.5ml
10% APS 400ul 100ul
TEMED 40ul 10ulQueen's Genetically Engineered Machine Team 2009
2
12% gel(for 1mmspacer)
reagents separation gel(for 4) stacking gel(for 4)
H2
O 7ml 6ml
Buffer 5ml 2.5ml
Acryl(30:08) 8ml 1.5ml
10% APS 200ul 100ul
TEMED 20ul 10ul
Gel wasrun at 20-30mA per gel, and the voltage should be lowerthan 150V. Wait until the dye front hasran offthe gel.
12. cut 2 pieces of whatman filter paperto 5.5X8.5cm
13. cut a piece ofmembrane to 5.5X8.5cm
14. soak themembrane in MeOH
15. lay themembrane on top ofthe transfer buffer

16. when the gel isfinished running,take the apparatus apart.
17. remove the stacking gel and wash the separation gel in transfer bufferfor 10min.
18. wetthe transfer apparatus with transfer buffer.
19. wetthe filter paper and place one on the apparatus.
20. place themembrane on the filter paper.
21. place the gel on themembrane.
22. place the lastfilter on top
23. wet withmore transfer buffer
24. close the apparatus
25. run 40min at 100mA/gel, but do notletthe voltage >25V.
26. rinsemembrane in TBST
27. Block in 5% skimmilk for 1hr.
28. Incubate nitrocellulose blots with 1° antibody (whole SAA) at 4°C overnight.
29. Wash blots 3X each 15minutesin TBST.
30. Incubate blots with 2° anti-rabbit antibody for 1hr at room temperature.
31. add luminescence kit and agitate for 30s/side
32. expose and develop x-ray film.
33. to strip: 0.2N NaOH for 10min at room temp.

Protocols:RestrictionDigestion – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
Determine the order ofthe two parts you will be putting together;the one in front will be referred to asthe insert, while the one behind will be referred to asthe vector. Both the vector and the insert need to have their own separate tube, atleastin the beginning.
RestrictionDigest Protocol
In the Insert Tube...
600 ng ofDNA (To figure outthe volume,the calculation is 600 / concentration of plasmid. This gives you volume in µL).
Water,so thatthe volume of bothDNA and waterin the tube is 35 µL total
4 µL of React 1 Buffer
0.5 µL of EcoR1
0.5 µL of Spe1
1 µL BSA
In the vector Tube...
250ng ofDNA (To figure outthe volume,the calculation is 250 / concentration of plasmid. This gives you volume in µL).
Water,so thatthe volume of bothDNA and waterin the tube is 35 µL total
4 µL of React 2 Buffer
1µL BSA
0.5 µL of Xba1
0.5 µL of Pst1
Put both tubesinto the 37°C water bath for one hour. After, place theminto the 65°C heating block for 10minutes. This deactivates any enzymesin the tube (which is ok, because by now they've done allthey need to). Take the insert out, and putitin a -20°C freezer.

PCRProtocol – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
Protocolforthermocycler:
- lid temp: 105°C preheating: on
1. 50.0°C 5min
2. 95.0°C 5min
3. 95.0°C 30sec

4. 56.0°C 30sec
5. 72.0°C 30sec
6. 10.0°C 15min
PCR reaction recipe:
6.0ul 10XPCR buffer
0.4ul dNTP
37.1ul ddH2
O
2.0ul FWDprimer
2.0ul REV primer
2.0ul DNA template
0.5ul Taq polymerase

Quick Ligase ligation – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
Materials
2xQuick ligase buffer(in 40µl aliquots;these are 1-time use since freeze-thaw cycles degrade the ATP in the buffer).
Quick Ligase fromNEB
ddH2O
Purified, linearized vector(likely inH2
Oor EB)
Purified, linearized insert(likely inH2
Oor EB)Italic text
Procedure
For 10µlreaction
Larger volumes can be scaled up if needed
5 µL 2XQuick ligase buffer
0.5 µlQuick ligase
6:1 Molarratio ofinsertto vector(~10ng vector). Try to keep totalDNA concentration <100ng/rxn for optimal efficiency.
Add (4.5 - vector and insert volume)µl ddH2
O
Method
1. Add appropriate amount of deionizedH2
Oto sterile PCR tube
2. Add in appropriate amounts of vector and insert.Heatthemixture to 42*C for 2min to free up sticky ends(can set up a thermocyclerforthis).
3. Add 5 µL of 2X ligation bufferto the tube.
Pipette buffer up and down before pipetting to ensure thatitis well-mixed.
4. Add 0.5 µL ofQuick ligase. PIPETTE halfthe volume ofthemixtureUP AND DOWNto ENSURE MIXINGOF THE ENZYME.
Also,the ligase, likemost enzymes, isin some percentage of glycerol which tendsto stick to the sides of yourtip.Justtouch yourtip to the surface ofthe liquid when pipetting to ensure accurate volume transfer.
5. Letthe 10 µL solution incubate atroomtemp for 5-10min.
6. Denature the ligase at 65°C for 10min.
7. Store at -20°CQueen's Genetically Engineered Machine Team 2009
2
T4 ligase ligation – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
Materials
T4DNA Ligase

10x T4DNA Ligase Buffer ->make sure itsmells bad (like "wet dog"); ifit doesn't smell, itmight be bad.
Deionized,sterileH2
O
Purified, linearized vector(likely inH2
Oor EB)
Purified, linearized insert(likely inH2
Oor EB)Italic text
Procedure
10µl Ligation Mix
Largerligationmixes are also commonly used
1.0 µL 10X T4 ligase buffer(use 10µl aliquotsin -20 freezer;repeated freeze-thaw cycles can degrade the ATP in the bufferthat's criticalforthe ligation rxn)
6:1 Molarratio ofinsertto vector(~10ng vector)
Add (8.5 - vector and insert volume)µl ddH2
O
0.5 µL T4 Ligase
Method
1. Add appropriate amount of deionizedH2
Oto sterile PCR tube
2. Add in appropriate amounts of vector and insert.Heatthemixture to 42*C for 2min to free up sticky ends(can set up a thermocyclerforthis).
3. Add 1 µL ligation bufferto the tube.
Pipette buffer up and down before pipetting to ensure thatitis well-mixed.
4. Add 0.5 µL T4 ligase. PIPETTE halfthe volume ofthemixtureUP ANDDOWNto ENSURE MIXINGOF THE ENZYME.
Also,the ligase, likemost enzymes, isin some percentage of glycerol which tendsto stick to the sides of yourtip.Justtouch yourtip to the surface ofthe liquid when pipetting to ensure accurate volume transfer.
5. Letthe 10 µL solution incubate at 16*C for 1hr.
6. Denature the ligase at 65°C for 10min.
7. Store at -20°C

Protocol:Heat Shock Transformation – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols

Thaw 100 µL of competent cells(pertransformation) on ice just before they are needed
AddDNA (2ul)to thawed cells andmix by flicking the side ofthe tube. Leave on ice for 30minutes
Heatshock for 2minutes at 42 degrees Celsius or 5minutes at 37 degrees Celsius
Place on Ice for 5minutes
Add 500ul 2XTYmediumto each tube
Incubate for 30 to 60minutes with shaking at 37 degrees Celsius.(Note thatfor Kanamycin containing plasmids always use one hour)
Spin down to remove allsupernatant except approximately 100 µL
Plate approximately 30 µL on each oftwo antibiotic plates
Grow overnight at 37 degrees Celsius

Forthis protocol we used a couple of controls
Positive Control- pBluescriptin TOP10 cells on ampicillin plates
Negative Control- TOP10 cells grown on ampcillin plates

Glycerol Stock Preparation
Adapted fromButanerds ProtocolsfromtheUniversity of Alberta iGEM Protocols pdf
What you will need
- **Overnight bacterial growth**
- **screw captubes**
- **glycerol**

Protocol
- **Pipet 0.5mL of 50% glycerol into 3 1.5 screw cap tubes**
- **Add 0.5mL of overnight culture to each tube**
- **Pipet up and down to gentlymix**
- **Flash freeze in liquidN2 or dry ice/ethanol bath**
- **Place in -80 C freezer when frozen**

Electroporation Protocol – Queens – 2009 http://2009.igem.org/Team:Queens/Protocols
Setup:
- 0.1cmcuvette gap
- Set pulse at 1.8 kV
- Check actual volts
- Time constant at 5 "ms"
Procedure
1. Prepare 1ml SOCmedia (cold)
2. load cuvette and pressrun
3. immediately add 1ml SOC
4. Incubate 1hr at 37°C unless using temp sensitive cells
5. plate accordingly.
Notes:
- DNA should not contain high salt concentration
• Ifso,must use PCR purification kit
- 1ulDNA: 40ul electrocompetent cells
- All equipmentshould be chilled (includingmedia)
SOC "Superrich media"
- 100ml ddH2
O
- 2g tryptone
- 0.5g yeast extract
- 0.06gNaCl
- 0.36g glucose (dextrose)
- 0.02g KCl
- 0.2g MgCl2 - 6H20
- 0.25g MgCl2 - 7H20

Anchor Binding Capacity – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
What you will need:
Paramagnetic Streptavidin Beads
Annealed Anchor
Binding Capacity
Prepare a 20 μM solution of the Anchor (same as "Annealing Anchor/Terminator" but using 30 μL water instead of 80 μL)
Set up a set of microcentrifuge tubes with 10, 20, 30, 40, 50, 60, 70, 80 μL of 4 mg mL^{-1}beads dispensed into the respectively labelled tubes.

Wash the beads twice with 100 µL Wash/Binding buffer and once with 100 µL water, then aspirate water off the beads.
Prepare 4 µL of the 20 µM Anchor solution to separate tubes (corresponding to which bead-containing tube they will be added to) along with enough water to bring the final solution volume up to half the dispensed bead volume. (i.e. to the 40 µL tube add 4 µL Anchor + 16 µL water).
Add half of this solution to the tubes containing the washed beads. Retain the other half.
Suspend the beads in the DNA solution.
Let bind at room temperature for 10 minutes.
Pellet the beads with a magnet and aspirate the solution.
Take readings at A_{260} with a UV-Vis Spectrophotometer of the DNA solutions prior to binding to bead (the half you retained) and after binding to bead (the aspirated solution). (ng of DNA can also be found by in-gel band intensity quantification methods)
Calculation
Using the A_{260} readings, calculate respective ng of DNA by multiplying volume of DNA applied to beads.
Calculate the difference of DNA for each set of readings per bead volume to give ng of DNA bound to bead.
Substitute the ng DNA bound into the following equation to get pmol of DNA:
$(\text{ng DNA}_{bound} \times 10^9) / (21519.1 \text{ g mol}^{-1} \times 10^{12})$
Divide this number by the µL of beads used to get binding capacity:
$\mu\text{L Beads} \times 10^3 \times 4 \text{ mg mL}^{-1}$
Disclaimer
By doing the binding capacity in this manner we found that binding capacity is highly dependant on DNA and bead concentration (see the graphs "here"). Thus, we simply used the calculated binding capacity for 40 µL of beads since this is the volume we use for all builds. A better binding capacity assay should be developed, but due to time constraints this method proved good enough since multiple runs of this experiment yielded numbers with small standard deviation:
$209 \pm 20 \text{ pmol mg}^{-1}$

Linearizing BioByte Constructs by I-SceI Digestion – Alberta – 2009
http://2009.igem.org/Team:Alberta/Protocols
What you will need:
I-SceI
Circular Biobyte Construct
Digestion:
For a 20 µL reaction:
5 µL DNA sample
2 µL NEB 10x I-SceI Buffer
2 µL 10x BSA
10.5 µL ddH2O
0.5 µL I-SceI (5U/µL)

Incubate at 37ºC for 1 hour

Rapid Bead-based Byte Assembly On-chip – Alberta – 2009
http://2009.igem.org/Team:Alberta/Protocols
What you will need:
Annealed Anchor
Annealed Terminator
Prepared BioBytes

Magnet
Streptavidin-Coated Magnetic Microsphere "Beads" (4mg/mL)
Wash/Binding Buffer - with no NaCl
T4 DNA Ligase/Buffer
USER™ (1 U/µL)
Mineral Oil

Preparation:

1. Pipette 4.2 µL of each Byte into separate 1.5 mL microcentrifuge tubes (1 Byte per tube). As well, to each tube, also add 0.5 µL of ligase and 0.3 µL of ligase buffer.
2. The beads are stored at 4ºC, vortex briefly to resuspend the beads and dispense 2 µL of them into a tube. Add 3 µL of washing buffer. Return the stock of beads to 4ºC immediately.
3. Apply a magnet to the side of the tube, wait until solution clears completely, aspirate supernatant.
4. Wash beads by adding 5 µL of washing buffer and resuspend by flicking.
5. Apply the magnet as before, wait until solution clears completely, aspirate supernatant.
6. Repeat steps 4 & 5 once more.

On-chip protocol for construct creation and release

Remember: Add AB Bytes to BA Bytes and BA Bytes to AB Bytes. If you want a circular construct, include the Terminator as a Byte.

7. Fill the center chamber of the microfluidic chip with washing buffer. Observe the channels fill. If a channel is blocked, use another chip or use a pipette on the corresponding outer chamber to "suck" the washing buffer through from the center chamber.
8. Fill the central washing chamber to a higher level than the top of its chamber to induce Laplace flow.
9. Dispense 2 µL of beads into an empty chamber
10. Dispense 5 µL of the Anchor into a chamber.
11. Dispense 5 µL of each Byte (including the added ligase) into separate chambers. If any chambers have filled with washing buffer, pipette it out, then pipette the Byte in right away.
12. Using the magnet underneath the chip (either by hand, or with the robot), guide the beads out of their chamber, through the channel, through the washing chamber, and into the anchor chamber.
13. Mix every 2 minutes by moving the magnet side to side until 10 minutes is up.
14. Guide the beads to the washing chamber, then into a Byte chamber.
15. Mix every 2 minutes. After 15 minutes, guide the beads to the wash chamber, then to the next Byte chamber.
16. Repeat the previous step for the remaining Bytes.
17. After the last Byte is done, recover the beads by pipette into a 1.5 mL microcentrifuge tube. Wash the beads as in steps 4 to 5, resuspending the beads in 5 µL of dH20.
18. Add 1 µL 10x Pfu Buffer + 0.5 µL USER.
19. Incubate @ 37ºC 1 hr
20. Remove the beads by applying magnet, wait for solution to clear, aspirate supernatant into a fresh tube.
21. If circularizing: heat to 95ºC for 1 min, cool to RT slowly (let it sit on the bench).

Rapid Bead-based Byte Assembly – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols

What you will need:

Annealed Anchor
Annealed Terminator
Prepared BioBytes
Magnet
Streptavidin-Coated Magnetic Microsphere "Beads" (4mg/mL)
Wash/Binding Buffer
T4 DNA Ligase/Buffer

USER™ (1 U/µL)
Competent Cells
LB
Ice
Agar Plates
Preparation:
1. Prepare X 1.5 microcentrifuge tubes, where X is the number of constructs to be made.
2. The beads are stored at 4ºC, vortex briefly to resuspend the beads and dispense 40 µL into each tube. Return the stock of beads to 4ºC immediately.
3. Apply the magnet to the side of the tube, wait until solution clears completely, aspirate supernatant.
4. Wash beads by adding 75 µL of "Wash/Binding Buffer" and resuspend by flicking.
5. Apply the magnet as before, wait until solution clears completely, aspirate supernatant.
6. Repeat steps 4 & 5 once more.
Anchor
7. Apply 20 µL of Anchor (A or B) to the bead solution
8. Incubate at room temperature for 10 minutes
9. Apply the magnet, wait until solution clears completely, aspirate supernatant.
10. Wash twice as in steps 4-6.
11. Wash once more with 75 µL 1X ligase buffer.
BioByte Addition and Construct Release
12. Add 20 µL of the first Byte with 5' end complementary to the anchor (AB Byte to A anchor and BA Byte to B anchor), resuspend beads, then add 2.3 µL 10x Ligase Buffer + 1 µL Ligase.
13 Incubate at room termpature for 20 minutes, every few minutes resuspend the beads by flicking the tube. BE GENTLE and tap the droplets back to the bottom of the tube after mixing.
14. Wash twice as in steps 4-6 with Wash Buffer and once more with 1X ligase buffer.
15. Repeat steps 12-14 for each BioByte to be added, always adding a Byte with a 5' end complementary to the 3' end of the previous end. Add AB Bytes to BA Bytes and BA Bytes to AB Bytes.

If you wish to circularize:
16. Add 20 µL of Terminator (A or B depending on previous Byte), resuspend beads, then add 2.3 µL 10x Ligase Buffer + 1 µL Ligase.
17. Incubate at room temperature for 20 minutes
18. Wash twice with Wash Buffer and once more with 1X ligase buffer.

Release from the bead:
19. Resuspend beads in 20 µL dH2O.
20. Add 2.5 µL 10x Pfu Buffer + 1 µL USER.
21. Incubate @ 37ºC 1 hr
22. Remove the beads by applying magnet, wait for solution to clear, aspirate supernatant into a fresh tube.
23. If circularizing: heat to 95ºC for 1 min, cool to RT slowly (let it sit on the bench).
Transformation
1. Add 5-10 µL circular construct to pre-chilled 1.5 mL microcentrifuge tube.
2. Chill on ice.
3. Add 75 µL or 100 µL competent cells to tube; mix gently.
4. Incubate on ice for 30 min.
5. Heat shock at 42ºC for 30-90 s.
6. Sit on ice for at least 2 min.
7. Add 400 – 800 µL LB broth to tube.
8. Incubate at 37ºC for 1 hour, with shaking.
9. Plate onto LB plates with correct antibiotics (usually 20 µL and 200 µL or rest).

Byte Production via PCR and USER Digestion – Alberta – 2009
http://2009.igem.org/Team:Alberta/Protocols
This procedure allows for the amplification of any gene or part in the pAB and pBA plasmids. The procedure is the same for both pAB and pBA Byte Amplifcation with the only difference being the universal primers (for pAB Bytes use pAB+ and pAB-, for pBA Bytes use pBA+ and pBA-).
What you will need:
PFU Turbo Cx (Stratagene)
FWD/REV Primers
DNA Template
dNTPs
ddH2O
USER™
Gel Purification Kit
EtOH (100%, 70%)
NaOAc 3M pH 5.2
Byte PCR and Digestion:
1. In a PCR tube mix the following (Assuming a 100 µL Reaction Volume):
4 µL Template (pAB or pBA – 1.5 ng/µL)
4 µL Forward Universal Primer (10 µM)
4 µL Reverse Universal Primer (10 µM)
2 µL PFU Cx (2.5 U/µL)
10 µL PFU Cx Buffer (10X)
10 µL dNTPs (2mM)
66 µL ddH2O
2. Run the PCR reaction under the following conditions:
95˚C (2 Minutes)
30 Cycles of:
95˚C (30 Seconds)
56˚C (1 Minute)
72˚C (2 Minutes)
72˚C (5 Minutes)
3. Add 1 µL USER™ (New England Biolabs) to each PCR tube and incubate at 37˚C for 1 hour.
4. Run the PCR product on an agarose gel.
5. Gel column purify to isolate PCR product (add the 10 µL 3M sodium acetate prior to loading onto column)
6. (ethanol precipitation may be required).
Ethanol Precipitation
1. In a 1.5 mL microcentrifuge tube add 1/10th volume of 3M sodium acetate, pH 5.2 to DNA solution to be precipitated.
2. Add 2x volume 100% ethanol to DNA + sodium acetate solution.
3. Incubate at 0 or -20˚C for at least 30 minutes (the longer, the better).
4. Centrifuge at 4˚C for 30 min at 14000 rpm.
5. Aspirate supernatant carefully.
6. Add 750 L 70% ethanol to tube, vortex briefly to wash pellet.
7. Centrifuge at 14000 rpm for at least 10 minutes at 4˚C.
8. Aspirate supernatant carefully.
9. Resuspend pellet in minimal volume of ddH2O or 10 mM Tris-HCl pH 8.5 buffer.

Wash/Binding Buffer – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
What you will need:

NaCl
EDTA
Tris-HCl
pH meter
Recipe:
0.5 M NaCl
20 mM Tris HCl (pH 7.5)
1 mM EDTA

Annealing Anchor and Terminator Bytes – Alberta – 2009
http://2009.igem.org/Team:Alberta/Protocols
Instructions on how to anneal the complementary strands to obtain the same concentrations we used in our experiments (10µM).
What you will need:
Anchor oligos (complementary)
Terminator oligos (complementary)
Procedure:
To make a 10 µM Anchor solution with an A(B) overhang one must anneal Anchor_A(Anchor_B) and Anchor_Comp. To make a 10 µM Terminator solution with an A(B) overhang one must anneal Term_A(Term_B) and Term_Comp.
Combine 10 µL of Anchor/Term_(A or B) and Anchor/Term_Comp 100 µM stock solutions into an eppendorf tube.
Add 80 µL water or TE, mix.
Heat solution to 95C for 2 minutes.
Let cool to room temperature slowly.

Sanger Sequencing Reaction – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
Procedure
In a 0.2 ml PCR tube add
template 5.0 ul (200 ng/ul)
VF primer 1.0 ul
dilute buffer 2.5 ul (reads BD dilute buffer on tape of cap) in PCR/Sequencing box
BD sequence mix 1.5 ul (reads BD)
Mix well.
Select program 'seq-dye' on PTC 200 thermal cycler
Run program. It will take about 2 hrs.
Remove tube from PCR machine and transfer 10 ul rxn mix to 1.5 ml Eppendorf tube. Add
1.5 ul Blue NaOAc/EDTA
40 ul 95% ethanol
Let sit on ice 15 min
Centrifuge 10 min max speed
Should see a small blue dot at bottom of tube
Discard supernatant
Wash pellet with 500 ul of 70% ethanol
discard supernatant and spin briefly to bring down the residual liquid. Draw liquid off with a P10 pipette tip. *Do not disturb the pellet.*
Air dry for 10 min

Place in -20 C freezer to be delivered to MBSU 4th floor microbiology M534. Rxns delivered before 2pm will normally be returned the next day.

Vector Dephosporylation – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols

What you will need:

Antarctic phosphatase

10x Antarctic phosphatase buffer

Procedure

Add 1/10 volume of 10x antarctic phosphatase reaction buffer to 1-5 ug of DNA cut with any restriction endonuclease in any buffer

Add 1 ul of Antarctic phosphatase and mix

Incubate 5 min at 37 C

Heat inactivate for 5 minutes at 65 C

Proceed with ligation

Notes

Vector dephosphorylation can be useful in cutting transformation background, but it is very harsh. It chews off 5' phosphates, but it also keeps chewing on the DNA ends, reducing total transformation efficiency. Try without dephosphorylation first, and minimize exposure to phosphatase if you must resort to using it. See also Sambrook.

Colony PCR – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols

What you will need:

Eppendorf tubes

Sterile toothpicks

PCR tubes (small tubes)

10X PCR buffer

25 mM MgCl2

5mM dNTPs

Taq polymerase

milliQ water

PCR primers (specific for each reaction)

Procedure

Set up one Eppendorf tube for each colony you are testing. Add 75 uL milliQ water to each tube.

Label each colony you are testing by circling it and numbering it on the back of the agar plate.

Remove 20-50% of the each colony with a sterile toothpick and twist the toothpick in the water in the corresponding tube. These tubes are your template tube. Use the same toothpick to streak for single colonies on an appropriate plate. Divide the plate in quarters – streak one colony per quarter

Pipette 4 uL of each template into a corresponding PCR tube.

Make a master PCR mix using the following recipe: for each reaction, add

2.5 uL 10X PCR buffer

2.5 uL 25 mM MgCl2

1.0 uL dNTPs

1.0 uL forward primer

1.0 uL reverse primer

12.5 uL milliQ water

0.5 uL Taq polymerase (remember to add enzyme last!)

Notes

This recipe is for one reaction. If you are doing 10 reactions, multiply each volume by 11; you will need a little extra for pipetting error. Remember to mix your master mix thoroughly.
Add 21 uL of your master mix to each of the tubes containing template.
Load samples into PCR machine. Run program 'Colony PCR' or Col56e2 on the PTC 200 thermal cycler.

Ligation – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
What you will need:
Vector
Insert
Milli Q buffer
5X ligase buffer
T4 DNA ligase
Procedure
Volumes of vector and insert may vary but a typical 20 ul reaction might be
milliQ water 11 ul
5x ligase buffer 4 ul
vector (200 ng/ul) 1 ul
insert (200 ng/ul) 3 ul
ligase 1 ul
Incubate at R/T for 3 hrs or overnight at 12-16 C

Gel Extraction – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
What you will need:
Clean scalpel
Transilluminator
Eppendorf tube
Gel extraction kit
55degC water bath
Vortex
Microfuge
Procedure
Place the gel on the transilluminator. Put on face shield and gloves.
Turn on transilluminator and quickly make four slices; one on each side of the band you want to cut out and turn transilluminator off. Keep exposure to a minimum.
Pry out the gel 'cube' and place it in an eppendorf tube.
Follow instructions in kit manual.

Agarose Gel Electrophoresis – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
What you will need:
1X TAE
Graduated cylinder
250 mL flask
Agarose
Gel forming tray
Ethidium bromide
Procedure:
Dilute stock of 50X TAE to 1X with ddH2O.

Measure 70 mL of buffer for small gels (large gels 170 ml).
Transfer buffer to 250 mL flask (or 500 ml).
Weigh out enough agarose to make 1% gel. (1% of 70 mL is 0.70 g)
Transfer agarose to flask. Form an improvised cap by inverting 50 ml flask into neck.
Melt agarose in microwave, stirring ever 15-20 seconds. This should take about 2 min.
Allow agarose to cool.
While agarose is cooling, assemble gel pouring apparatus by inserting gate into slots. Use a pasteur pipet to run a bead of molten agarose along the inner and outer edges of the gates to help seal the box and prevent leaks.
Allow gel to cool until flask can be handled comfortably.
Place comb in the gel rig.
Pour agarose into gel tray.
Allow to solidify completely. While the gel is solidifying, prepare the samples. Add your sample and 2uL of 10x OG loading dye to a tube, then make the total volume of the tube up to 20 uL. Or 2.2 ul of 10x OG to 20 ul sample.
Pour 1X TAE over gel so that gel is covered by 3-5 mm of buffer.
Load samples into lane. Do not forget to load 1kb+ ladder into one of the lanes.
Hook electrodes to gel apparatus. Nucleic acids are negatively charged, so they will run to the positive (red) terminal.
Pipette 10 uL ethidium bromide (from 10 mg/ml stock) into the buffer at the bottom of the gel. Mix well
Turn on the gel. Run for 60 min @ 90V. Check with handheld UV Source.
Place gel in plastic wrap.
Carry to G311.
With bare hands log in as Gen 420 with password Molecular1.
Double click on the Genesnap from Syngene icon.
Click on the Green Button to start live image.
Put one glove on your left hand and place gel on transilluminator. Now do not touch anything with your left hand.
With your right hand slide the door down completely.
The transilluminator image on the screen should turn purple.
Use the arrows on the exposure button to increase the exposure time until the gel and bands are clearly visible.
If necessary use the zoom arrows to increase or decrease the size of the gel.
Reposition the gel if necessary – open the door with your right hand and move the gel with your gloved left hand.
To fine focus the image use the eye arrows.
When the image is sized and focused properly capture it by clicking the red button.
Print a photograph by clicking the printer icon in the tool bar at the top.
Record your use on the sheet. Supervisor=iGEM and your initials.
Log off.
Remove your gel and clean the transilluminator with water and dry with paper towels.
Take the gel back to the lab and dispose of properly.

Notes

Ethidium bromide is carcinogenic!

Restriction Digest – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols

What you will need:

Restriction enzyme buffer
Restriction enzymes
milliQ water

DNA to be digested

Procedure:

Select a restriction enzyme buffer that is appropriate for BOTH of the enzymes you are using. See information sheets at front of lab for correct buffer and concentration.

The total volume of all enzymes in the reaction should be less than 10% of the final reaction volume.

Enzymes usually are supplied at 10U/ul and 1ul will be more than enough for our digests.

Add components in the following order:

Water

Buffer

DNA

Enzyme I

Enzyme II

An example of a typical 25 ul reaction would be

milliQ water 15.5 ul

10x Tango Buffer 2.5 ul

DNA (200 ng/ul) 5.0 ul

XbaI 1.0 ul

PstI 1.0 ul

Incubate at 37 C for two hours (longer is okay too).

Notes

FastDigest enzymes use a single uniform buffer, and claim to work in 5 min.

Plasmid Miniprep – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols

What you will need:

Overnight culture

Qiagen plasmid miniprep kit

Procedure:

Transfer 1.5 ml of culture to Eppendorf.

Spin down at 6000 rpm.

Discard supernatant and follow procedure in kit handbook

Determine concentration of purified plasmid with NanoVue spectrophotometer in G311.

Notes

Low speed pellet is easier to resuspend.

Step 2 in Qiagen, the alkaline lysis with buffer P2, should be brief to avoid damaging DNA.

Glycerol Stock – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols

What you will need:

Overnight with bacterial growth

Screw cap tubes

Glycerol

Procedure:

Pipet 150 uL 50% glycerol into 3* properly labeled 1.5 ml screw cap tubes.

Add 350 uL of overnight culture to each tube.

Pipet up and down to gently mix.

Flash freeze in liquid N2 or dry ice/methanol bath

Place in -80 C freezer when frozen.

Notes

If we make extra,then we have them if we need to send them out or want to store in several locations.

5 mL Overnight
What you will need:
10 mL culture tube Use 16mm x 160mm or 16mm x 125 mm
5 mL LB
5uL 1000X antibiotics
Single colonies on a plate (Best not to start O/N from glycerol stocks)
Procedure:
Add 5 mL non-contaminated LB. Do this first, then add antibiotic.
Pipet 5uL of each antibiotic into culture tube.
Select single colony using sterile toothpick or a flamed loop that has been cooled
Place toothpick or loop in culture tube, stir.
Remove toothpick or loop and place culture tube in incubator at 37 C overnight shaking vigorously (250 rpm).
Notes
For I0500, culture overnight; then add 1 mM IPTG in morning and continue culture for another 3 hrs. Then miniprep.
Ampicillin: use 10 ul, all others use 5 ul.

Transformations – Alberta – 2009 http://2009.igem.org/Team:Alberta/Protocols
What you will need:
"Competent" XL10 Gold cells (100 uL per transformation)
1 uL of plasmid you want to transform
1 mL of LB (non-contaminated) per transformation
Plate with correct antibiotics (3 per transformation)
Procedure:
Obtain XL10 Gold* cells from the –70 C freezer (GXXX, grey Nunc freezer, 2nd shelf from bottom) and allow to thaw on ice. Aliquots are about 200 ul. Take out only what you will use.
Pipet 1uL of the plasmid (or 5 ul of ligation reaction) you want to transform into an Eppendorf tube. Add MilliQ water to 10 ul.
When XL10 cells are thawed, pipet 100 uL into the tube with the plasmid. Pipet up and down to mix gently. Place tube on ice 30 minutes.
After incubating on ice for 30 minutes, place cells in incubator set to 42 C for 90s. (Use the water bath near the front sink in G308 – its temperature will be most accurate. This step must be done for EXACTLY 90 s).
Return the tube to ice for 2 min.
Add 1mL (1000uL) of LB to the tube.
Incubate at 37 C for 20-30 min.
Spread cells on plate with appropriate antibiotic: plate 200ul, 20ul in 80ul of LB broth, and 2ul in 98ul of LB broth. Let dry.
Place plates inverted in incubator at 37C overnight.
Notes
The competent cells we have made up are XL10-Gold – if we need to transfer them to DH5alpha at a later date we can retransform the final BioBricks
Can try 200 ul of cells instead of 100 ul to attempt to increase efficiency of transformation.

Assembly protocol **– USTC – 2009** http://2009.igem.org/Team:USTC/Standard_%26_Protocol
Minipreps
Performed with BIO BASIC INC. EZ-10 Spin Column Plasmid DNA MiniPreps Kit BS414
Digestion
The digestion enzymes we use are listed:
Pst I Fermentas ER0611 3000U
EcoR I Fermentas ER0271 5000U
Spe I Fermentas ER1251 1500U
Xba I Fermentas ER0681 400U
Gel Extraction
Performed with BIO BASIC INC. EZ-10 Spin Column DNA Gel Extraction Kit BS354
Ligation
For short segments:
TakaRa DNA Ligation Kit Ver2.0 Code D6022
BIO BASIC INC. FAST LIGATION KIT BS512
For long segments ligation:
TaKaRa DNA Ligation Kit LONG Code D6024
Transformation
Colony PCR

Measurement protocol **– USTC – 2009** http://2009.igem.org/Team:USTC/Standard_%26_Protocol
Constitutive promoter measurements
1. Streak a LB plate of the strain which contain one of the parts listed in pSB1A3 .
2. Inoculate two 3ml cultures of supplemented M9 Medium and antibiotic(Ampicillin 0.1mg/ml) with single colony from the plate.
3. Cultures were grown in test tubes（BIO BASIC INC.12ml Polypropylene Round-bottom Culture Tubes With Graduations And Dual Cap Cat.No:TD444） for 16hrs at 37℃ with shaking at 200rpm.
4. Cultures were diluted 1:100 into 3ml fresh medium and grown for 3hrs.
5. Measure the fluorescence(SHIMDZU SPECTROFLUOROPHOTOMETER RF-5301PC, 250ul quartz cell path length 10mm,501 nm excitation,514 nm emission,1.5nm slit width) and absorbance (HITACHI UV-VIS spectrophotometer U-2810 ,200ul quartz cell,path length 10mm,600nm,1.5 nm slit width) every 30 minutes in the next 4hrs.
Hybrid promoter response to AHL
1. Streak a LB plate of the strain which contain one of the parts listed in pSB1A3 .
2. Inoculate two 3ml cultures of supplemented M9 Medium and antibiotic(Ampicillin 0.1mg/ml) with single colony from the plate.
3. Cultures were grown in test tubes（BIO BASIC INC.12ml Polypropylene Round-bottom Culture Tubes With Graduations And Dual Cap Cat.No:TD444） for 16hrs at 37℃ with shaking at 200rpm.
4. Cultures were diluted 1:1000 to tubes of 3ml fresh medium and grown for 4.5hrs.
5. Stock concentration of the cognate AHL, 3-oxohexanoyl-homoserine is diluted and added to different tubes to yield different final concentrations (1E-5,1E-7,1E-8,1E-9,1E-10M).To ensure the same response time , the AHL should be added with a time interval of 2mins between tubes, so do the measurements procedure.
6. Measure the fluorescence(SHIMDZU SPECTROFLUOROPHOTOMETER RF-5301PC, 250ul quartz cell path length 10mm,501 nm excitation,514 nm emission,1.5nm slit width) and absorbance ((HITACHI UV-VIS spectrophotometer U-2810 ,200ul quartz cell path length 10mm,600nm,1.5 nm slit width) for the first time 30 minutes after adding AHL. Repeat measurement every 30 mins in the next 4hrs.
Hybrid promoter response to AHL&aTc
1. Streak a LB plate of the strain which contain one of the parts listed in pSB1A3 .

2. Inoculate two 3ml cultures of supplemented M9 Medium and antibiotic(Ampicillin 0.1mg/ml) with single colony from the plate.
3. Cultures were grown in test tubes（BIO BASIC INC.12ml Polypropylene Round-bottom Culture Tubes With Graduations And Dual Cap Cat.No:TD444） for 16hrs at 37℃ with shaking at 200rpm.
4. Cultures were diluted 1:1000 to 11 tubes 3ml fresh medium and grown for 4.5hrs.
5. Stock concentration of the cognate AHL, 3-oxohexanoyl-homoserine and aTc (anhydrotetracycline) are diluted and added to different tube to get different final concentrations listed in the table below:

Tube No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
c(AHL)/M	0	1.00E-06	1.00E-06	1.00E-06	1.00E-06	1.00E-06	1.00E-04	1.00E-04	1.00E-04	1.00E-04	1.00E-04
C(Atc)/ng/ml	0	0	2	20	200	2000	0	2	20	200	2000

To ensure the same response time , the AHL and aTc should be added with a time interval of 2mins between tubes, so do the measurements procedure.
6. Measure the fluorescence(SHIMDZU SPECTROFLUOROPHOTOMETER RF-5301PC, 250ul quartz cell path length 10mm,501 nm excitation,514 nm emission,1.5nm slit width) and absorbance ((HITACHI UV-VIS spectrophotometer U-2810 ,200ul quartz cell path length 10mm,600nm,1.5 nm slit width) for the first time 30 minutes after adding AHL and aTc. Repeat measurement several hours a time until OD600 reach to 0.8,it will take about 7hours in average.
M9 Medium
M9 media: for 1L 1X media
1 X M9 salt:
Na_22HSO_4 33.9g/L
$KaH_2SO_4$15g/L
NaCl 2.5g/L
NH_4Cl 5g/L
1. Dissolve 11.3 g Bacto M9 minimal salts in 970mL water.
2. Autoclave to sterilize. 121°C for 20 minutes.

8 mL 50% glycerol
1. Add 4 mL glyerol to 4 mL of H_4O
2. Filter sterilize.

2ml 1mol/L $MgSO_4$
1.Dissolve 24.65g $MgSO_44.7H_2O$ in 100ml water.
2. Autoclave to sterilize. 121°C for 20 minutes.

0.1ml 1mol/L $CaCl_2$
1.Dissolve 1.11g $CaCl_2$ in 10ml water.
2. Autoclave to sterilize. 121°C for 20 minutes.

2g Casamino acids
Dissolve in 1 X M9 salt directly

20ml thiamine
1. Dissolve 0.337 g Casamino acids in 20mL water;
2. Autoclave to sterilize. 121°C for 20 minutes.
Combine above solutions using sterile technique and store at 4°C.
Standard – USTC – 2009 http://2009.igem.org/Team:USTC/Standard_%26_Protocol

This standard defines the measurement of the PoPS of a specific Biobrick. It defines how to measure PoPS in a relative unit , not a absolute value.

Strains:
The system should be measured in the strain of Top10.

Plasmid:
The Biobrick parts measured must be supplied in the plasmid pSB1A3.

Reporter:
The Part BBa_I13504 is chosen as the reporter of all the PoPS output of a Biobrick.

Medium:
The cultures should be grown in the M9 medium. The recipe of M9 medium :
1X M9 salts:
Na_2HSO_4 33.9g/L
KaH_2SO_4 15g/L
NaCl 2.5g/L
NH_4Cl 5g/L
Dissolve in 1L H_2O
1mM thiamine hydrochloride ;
0.4% glycerol ;
0.2% casamino acids ;
2mM $MgSO_4$;
0.1mM $CaCl_2$;

Measurement detail
a. Colonies should be picked from a streaked LB plate and grown for 16~18hrs. Growth condition:37℃,200rpm.
b. Dilute the culture 1:100 when measure a constitutive promoter and 1:1000 for a regulatable promoter.
c. The culture should be grown for 3~4hrs before the measurement begin.
d. Measure fluorescence and OD600 in at most 30mins after sampling.
e. For the regulatable promoter , it is necessary to ensure the same response time. The time interval between
the stimulation and measurement should be exactly the same.
f. All the measurement should be made in the linear range of the equipments. It is necessary to test it before
your experiment.

Data process:
a. A relative fluorescence unit ustc_st.1 is defined to describe the fluorescence intensity independent from the measurement equipment.
b. The part BBa_ K176009 is selected as the fluorescence standard. The fluorescence intensity of BBa_K176009 was measured as the cultures were grown.
c. Define flu,od as the raw data of fluorescence intensity and OD600. Fit flu/od value correspondent to OD600. Take the correspondent value of flu/od to OD600=0.5 *std*as the defined standard 1000 units.

d. All the other raw data $\frac{Flu}{OD}_{raw}$ should be converted to standard flu/od value as shown in equation(1):

$$\frac{Flu}{OD}_{std} = \frac{\frac{Flu}{OD}_{raw}}{std} * 1000$$

equation(1)

Microbiological Culture – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
General Protocol
Essential requirements for growth
1) supply of suitable nutrients
2) source of energy
3) water
4) appropriate temperature
5) appropriate pH
6) appropriate levels (or absence) of oxygen
1x LB medium (Luria-Bertani medium)
tryptone, 10 g
yeast extract, 5 g
NaCl, 10 g
deionized H2O, to 950 ml
Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume to 1 liter with H2O. Sterilize by autoclaving.
Tips
1) Remember to do PARALLEL experiments at the same time!
2) Bacteria-free technique is a must in the wetlab, especially for the induction of protein expression, and the identification of positive clone.
3) Bear in mind that bacteriophage is just nearby!
4) DO NOT add antio-biotics when recombination bacteria are first picked out and transplanted from a plate to a culture bottle.
Molecular Cloning
Isolation of Plasmid DNA – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
General Protocol
1)Bacteria culture: Inoculate the single colony harboring plasmid into 20 ml LB medium containing antibiotics, 37℃, 12-16 hours.
2)Pellet 1–3 ml of cells by centrifugation for 1–2 minutes at 12000rpm. Decant the supernatant. (1.4ml each time and collect about 3 ml culture of E.coli in 1.5ml eppendorf tube.) Completely resuspend the cell pellet in 100 µl solution 1 by vigorous vortex.
3)Add 150 µl solution 2, mix by inverting the tube 4-6 times gently and incubate the tube on ice for 1-2 minutes (do not exceed this period!). The cell suspension should be clear immediately.
4)Add 150 µl solution 3, invert the tube gently several times, place the tube at room temperature for 5 minutes, and centrifuge at 12000rpm for 15 minutes.
5)Add 420 µl binding buffer to the mini-spin column. Then transfer the supernatant of procedure 3 to the same mini-spin column. Mix the supernatant and binding buffer with pipette carefully. Then place the column in another tube, centrifuge at 12000rpm for 30 and then discard waste liquid in the tube.
6)Add 750 µl wash buffer to the column, and centrifuge at 12000rpm for 1minute.
7)Repeat procedure 5. Then centrifuge at 12000rpm for 2minutes. Eliminate wash buffer as thoroughly as possible. The ethanol in wash buffer will impact the following enzyme-catalyzed reactions.
8)Carefully move the column into another clean tube. Add 50 µl Elution buffer or water into the column, place it at room temperature for 5minutes, andcentrifuge at 12000rpm for 1 minute.
Tips

1)Elution buffer should be added in the middle of adsorption material to guarantee all the plasmid DNA recovered.
2)To increase the recovery efficiency, increase the elution volume or elution times if it is necessary
3)0.5ml RNaseA（10mg/ml）can be added to the purified plasmid to eliminate RNA thoroughly.
4)If the molecular weigh of purified plasmid exceeds 10kb,the column may be placed in 70℃ water bath for 3-5 minutes to ensure plasmid DNA totally recovered.
Transformation of Recombinant DNA – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
General Protocol
1)Pipette competent cells suspension into the tubes, 100 µl each tube (If the competent cells are taken from -70℃, perform following steps immediately after thawing).
2)Add 10 µl of recombinant plasmid into the tube which is the tube with competent cells.
3)Mix the solutions gently, keep on ice for 20-30 minutes.
4)Heat shock by transferring the tubes to a water bath of 42℃ for 1 to 2 minutes.
5)Immediately return the tube to the ice bath. Keep on ice for 2 minutes.
6)Add 0.9 ml of LB (with no antibiotics added) into each tube. Incubate the tubes for 45 minutes to 1 hour at 37℃ to allow the cells to express their antibiotic gene product.
7)Spread about 200 µl of the resulting solutions (do dilution if necessary) on LB plates (with corresponding antibiotic added). After complete absorption of liquid LB, upside down the plates and incubate the plates at 37℃ overnight.
Tips
1)Never spread the transformation solution until you have assured that the glass stick is cooled down!
Ethanol Precipitation – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
General Protocol
1)Add 2 volume of 100% ethanol and 1/10 volume of 3M NaAc (pH=5.2) to the enzymatic digested solution.
2)Place the tube at -20℃ refrigerator for 20min.
3)Centrifuge at 4℃, 12,000rpm for 10 min.
4)Carefully discard the supernatant and add 100µl 70% ethanol.
5)Centrifuge and discard the supernatant again.
6)Desiccation at room temperature for 15 minutes.
7)Add 10 µl distilled water to dissolve DNA.
Tips
PCR – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
General Protocol

1. Standard PCR

PCR System			
Reagent	Concentration/Activity	Volume (50uL System)	Volume (100uL System)
10x Pyrobest buffer II	10x	5	10
Pyrobest		0.3	0.5
dNTPmix	10mM each	1	2
Primer 1	10uM	1	2
Primer 2	10um	1	2
Template DNA	changeable	0.5	1
MgCl2(Deletable)	0.2M	0.5	1
ddH2O		40.5	81

(Pyrobest DNA polymerase from Takara Co.Ltd.)

PCR Program		
Step	Condition	Time
1	95℃	5min
2	95℃	30sec
3	[Tm(fu)-4]℃	30sec
4	72℃	DNA length/kb/min
5	RETURN TO STEP 2	30-35 cycles
6	72℃	10min
7	4℃	HOLD

2. Fusion PCR

The basic system is similar to common PCR. There are some notes to raise the fusion efficiency:
a. Complementary region length: 15-20bp
b. Raise the annealing temperature in the fusion step.

Fusion PCR Program		
Step	Condition	Time
1	95℃	5min
2	95℃	30-50sec
3	{Tm(fu)+[(-2)~5]}℃	40-80sec
4	72℃	DNA length/kb/min
5	RETURN TO STEP 2	10-15 cycles
6	72℃	5min
7	Add amplification Primers	
8	95℃	2-5min
9	95℃	30sec
10	[Tm(fu)-4]℃	30sec
11	72℃	DNA length/kb/min
12	RETURN TO STEP 2	25-30 cycles
13	72℃	10min
14	4℃	HOLD

Tips
Protein Isolation and Identification – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
Protein Isolation for prokaryotes
1) Bacteria of positive clone are grown in sterilized medium containing 1% anti-biotics at 37 °C.
2) At OD600 = 0.8, or overnight, cells are induced by addition of IPTG and left at 25 °C overnight.
3) The suspension is then centrifuged (30 min, 4500 rpm, 6 °C) and the pellet resuspended in lysis buffer (20 mM imidazol, 0.25M NaCl) and the cell suspension sonicated two cycles, using an ultrasonic processor. For each cycle, the working time/ pausing time is 4s/ 6s.

4) The cells are pelleted by centrifugation at 12000rpm for 50 min at 10 °C, using a high-speed centrifuge.
5) The supernatant is loaded on a Ni-NTA column, washed with lysis buffer and eluted with Buffer B (400 mM imidazol).
6) Freeze the fractions at the temperature of -20 °C, for other detections.
Protein Isolation for eukaryotes – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
1) The cells are pelleted by centrifugation at 800rpm for 5 min at 4 °C. And wash with ice-cold PBS twice.
2) Lysis with TEN-T buffer at 4 °C for 1 hour. And then centrifuge at 12000rpm for 15 min at 4 °C.
3) The suspension is kept at the temperature of -20 °C for other detection, while the pellet is discarded.
Protein Identification (SDS-PAGE) – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
Pouring the separation gel
1) Assemble gel sandwich according to the manufacturer's instructions, or according to the usage of alternative systems. For Mini-Gel, be sure that the bottom of both gel plates and spacers are perfectly flush against a flat surface before tightening clamp assembly. A slight misalignment will result in a leak.
2) Combine solutions C and B and water in a small Erlenmeyer flask or test tube.
3) Add ammonium persulfate and TEMED, and mix by swirling or inverting container gently (excessive aeration will interfere with polymerization). Work rapidly at this point because polymerization will be under way.
4) Carefully introduce solution into gel sandwich using a pipet. Pipet solution so that it descends along a spacer. This minimizes the possibility of air bubbles becoming trapped with the gel.
5) When the appropriate amount of separating gel solution has been added (in the case of the Mini-Gel, about 1.5cm from top of front plate or 0.5cm below level where teeth of comb will reach), gently layer about 1cm of water on top of the separating gel solution. This keeps the gel surface flat.
6) Allow gel to polymerize (30-60 min). When the gel has polymerized, a distinct interface will appear between the separating gel and the water, and the gel mold can be tilted to verify polymerization.
Pouring the stacking gel
1) Pour off water covering the separating gel. The small droplets remaining will not disturb the stacking gel.
2) Combine Solution C and B and water in a small Erlenmeyer flask or a test tube.
3) Add ammonium persulfate and TEMED and mix by gently swirling or inverting the container.
4) Pipet stacking gel solution onto separating gel until solution reaches top of front plate.
5) Carefully insert comb into gel sandwich until bottom of teeth reach top of front plate. Be sure no bubbles are trapped on ends of teeth. Tilting the comb at a slight angle is helpful for insertion without trapping air bubbles.
6) Allow stacking gel to polymerize (about 30 min).
7) After stacking gel has polymerized, remove comb carefully (making sure not to tear the well ears).
8) Place gel into electrophoresis chamber.
9) Add electrophoresis buffer to inner and outer reservoir, making sure that both top and bottom of gel are immersed in buffer.
Protein sample preparation
Protein sample is diluted with an equal volume of sample buffer. In the experiment, take 10ul samples and 10ul sample buffer. The mixture is heated for 5 min at 100°C. Introduce sample solution into well using a Hamilton syringe. Layer protein solution on bottom of well and raise syringe tip as dye level rises. Be careful to avoid introducing air bubbles as this may allow some of sample to be carried to adjacent well. Rinse syringe thoroughly with electrode buffer or water before loading different samples. Include molecular weight standards in one or both outside wells. A slab gel is especially useful for molecular weight determinations since the sample and molecular weight standard proteins can be run under identical conditions on a single gel. There are a number of commercially available SDS-PAGE molecular weight standards which give a good spread of molecular weight lines in a gel.
Running a gel

1) Attach electrode plugs to proper electrodes. Current should flow towards the anode.
2) Turn on power supply to 200V.
3) The dye front should migrate to 1cm from the bottom of the gel in 30-40 min for two 0.75mm gels (40-50 min for 1.5mm gels).
4) The high electrical current used in gel electrophoresis is very dangerous. Never disconnect electrodes before first turning off the power source. If using an electrophoresis apparatus, which is not completely shielded from the environment, always leave a clearly visible sign warning that electrophoresis is in progress.
5) Turn off power supply.
6) Remove electrode plugs from electrodes.
7) Remove gel plates from electrode assembly.
8) Carefully remove a spacer, and inserting the spacer in one corner between the plates, gently pry apart the gel plates. The gel will stick to one of the plates.

Stain and destain the gel

1) Stain the gel in staining solution for overnight at RT.
2) Destain the gel, change fresh destaining solution several times.

Reagens

BUFFER SYSTEM

(A) Lower buffer: 18.17g Tris, 0.4g SDS, pH 8.8 (HCl), added H2O to 100ml (1.5mol/L Tris-HCl buffer).
(B) Upper buffer: 6.06g Tris, 0.4g SDS, pH 6.8 (HCl), added H2O to 100ml (0.5mol/L Tris-HCl buffer).
(C) 30% Acrylamide: 30g Acrylamide, 0.8g Bisacrylamide, added H2O to 100ml.
(D) 10%(w/v) Ammonium persulfate (fresh): 0.1g ammonium persulfate, added H2O to 1ml.
Electrophoresis Buffer: 3g Tris, 14.4g glycine, 1g SDS, added H2O to 1 liter H2O, Distilled water.
Sample buffer: 1g SDS, 5ml Glycerol, 50mg Bromophenol blue, 2.5ml Mercaptoethanol, 5ml Upper buffer, added H2O to 50ml.

Staining and destaining solution

Staining solution:
0.25g Coomassie Blue R-250 was dissolved in 50ml methanol, added 8ml acetic acid and 42ml H2O to 100ml.
Destaining solution:
200ml methanol, 70ml acetic acid, added H2O to 1000ml.

Separation Gel Preparation

Table 1 12.5% Running gel (Separating gel)

Reagen	Volumn
A	4.5ml
C	7.5ml
H2O	5.9ml
TEMED	0.02ml
D(fresh)	0.07ml
total	18ml

Stacking Gel Preparation

Table 2 3% Stacking gel

Reagen	Volumn
B	2.0ml
C	0.8ml

H2O	5.2ml
TEMED	0.01ml
D(fresh)	0.04ml
total	8.05ml

Cell Culture – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
General protocol
HeLa cells frozen in liquid nitrogen are revived in routine method, and inoculated in RPMI-1640 blended with 10% fetus cattle serum, cultured in six-well culture plate. The plates are put in the incubator with the condition of 37℃, 5%CO2. The growth of cells is observed every day. 70% of the cells confluence is digested with EDTA and then is made into cell suspension.
Plasmid DNA Transfection – Tsinghua – 2009 http://2009.igem.org/Team:Tsinghua/Protocol
Use the following procedure to transfect DNA into mammalian cells in a 6-well format. All amounts and volumes are given on a per well basis. Prepare complexes using a DNA (µg) to Lipofectamine™ 2000 (µl) ratio of 1:2 to 1:3 for most cell lines. Transfect cells at high cell density for high efficiency, high expression levels, and to minimize cytotoxicity.
1) Adherent cells: One day before transfection, plate 0.5-2 x 105 cells in 500 µl of growth medium without antibiotics so that cells will be 90-95% confluent at the time of transfection.
Suspension cells: Just prior to preparing complexes, plate 4-8 x 105 cells in 500 µl of growth medium without antibiotics.
2) For each transfection sample, prepare complexes as follows:
a. Dilute DNA in 50 µl of Opti-MEM® I Reduced Serum Medium without serum (or other medium without serum). Mix gently.
b. Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount in 50 µl of Opti-MEM® I Medium. Incubate for 5 minutes at room temperature. Note: Proceed to Step c within 25 minutes.
c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine™ 2000 (total volume = 100 µl). Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy). Note: Complexes are stable for 6 hours at room temperature.
3) Add the 100 µl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
4) Incubate cells at 37°C in a CO2 incubator for 18-48 hours prior to testing for transgene expression. Medium may be changed after 4-6 hours.
5) For stable cell lines: Passage cells at a 1:10 (or higher dilution) into fresh growth medium 24 hours after transfection.
Add selective medium (if desired) the following day. To obtain the highest transfection efficiency and low cytotoxicity, optimize transfection conditions by varying cell density as well as DNA and Lipofectamine™ 2000 concentrations. Make sure that cells are greater than 90% confluent and vary DNA (µg): Lipofectamine™ 2000 (µl) ratios from 1:0.5 to 1:5.
Tips
1) Do not add antibiotics to media during transfection as this causes cell death.
2) Maintain the same seeding conditions between experiments.
3) Remember to test serum-free media for compatibility with Lipofectamine™ 2000 since some serum-free formulations may inhibit cationic lipid-mediated transfection.

Infusion - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols

Infusion is a method to combine DNA fragments using a combination of PCR and homologous recombination. It can be used as an alternative to ligation with the advantage that it does not require restriction enzyme digests, although specific primers are required for the reaction.
Clonetech page on In-Fusion
PCR amplify the DNA sequence to be recombined into the vector using primers with sequences of the vector on 5' ends (refer to the Clonetech In-Fusion page above).
Linearize the vector using appropriate restriction enzymes.
Mix the purified PCR insert(50-200ng) and linearized vector(100-400ng).
Add 1μl of 5x In-Fusion Reaction Buffer and MilliQ up tp 4.5μl.
Add 0.5μl of In-Fusion Enzyme and mix well.
Incubate at 37ºC for 15 min, followed by 15min ant 50ºC, then place on ice.
Bring the reaction volume up to 25μl with TE buffer(pH8.0) and mix well.
Use 5μl of reaction mixture for transformation.

Gel Purification Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
Cut out volume of agarose gel containing the desired DNA and put in an eppendorf tube
Use the Promega Gel Purification kit according to instructions
Elute DNA with 50μl of MilliQ (or 30μl if yield is low)
Measure concentration using a spectrophotometer and write on tube

Colony PCR Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
Aliquot into PCR tubes 5μl of MilliQ
Pick single colonies with a toothpick and immerse in the above MilliQ to suspend
Put in PCR machine set at 95ºC for 5 min.
Add to each tube 5μl of the following solution and mix by pipetting
0.1μl 100μM 5' primer
0.1μl 100μM 3'primer
0.8μl 2.5mM dNTP
1μl 10x standard buffer
2.92μl MilliQ
0.08μl Ex-Taq
PCR using the following program
95ºC 2 min
95ºC 30 sec
52-55ºC 30 sec （Depending on the Tm of the primers）
72.5ºC 15 sec ×（# of kb of DNA to be amplified）
95ºC 30 sec Repeat 2-4 29 times
25ºC pause

PCR Programs - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
Pfu Standard
95ºC 2 min
95ºC 30 sec
52-55ºC 30 sec （Depending on the Tm of the primers）
72.5ºC 15 sec ×（# of kb of DNA to be amplified）
95ºC 30 sec Repeat 2-4 29 times

25℃ pause

Taq Polymerases - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
Pfu Ultra
Unless otherwise stated, we use this polymerase
0.2µl　100µM 5'primer
0.2µl　100µM 3'primer
1.6µl　2.5mM dNTP
2µl　10 x Pfu Ultra 2 buffer
15.45µl　MilliQ
0.05µl　DNA　(to a final concentration of 5-30ng/20ul)
0.5µl　Pfu Ultra2
Ex Taq
0.2µl　100µM 5'primer
0.2µl　100µM 3'primer
1.6µl　2.5mM dNTP
2µl　10 x Pfu Ultra 2 buffer
Xµl　MilliQ (up to 20ul)
Xµl DNA (to a final concentration of 200ng/20ul)
0.2µl　Ex-Taq
KOD plus
5µl 10 × PCR Buffer for KOD-Plus
5µl 2mM dNTPs
2µl 25mM $MgSO_4$
1.5µl 10µM primer 1
1.5µl 10µM primer 2
>1 µl template DNA
Genomic DNA 10~200ng/50µl
Plasmid DNA 1~50ng/50µl
1 µl KOD-Plus
MilliQ to 50µl

Restriction Enzyme Digest Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
1. Mix the following in a eppendorf tube:
Xµl　plasmid DNA　(to a final concentration of 300ng/30ul)
3µl　10 H　buffer
1.5µl　Restriction Enzyme 1
(1.5µl　Restriction Enzyme 2, if applicable)
MilliQ　up　to　30ul

2. Incubate at 37℃ for 1h or more

Ligation Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols

Mix 2µl of insert DNA with 3µl of vector DNA
Add 5µl Takara Ligation High
Incubate at 17ºC for 30 min.

Transformation Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
From iGEM Plates
Add 15µl TE (Tris-EDTA, pH 8.0) to well containing part and pipette up and down to resuspend
Take 1µl of the above solution and mix with partly thawed competent cells on ice
Leave on ice for 30 min.
Heat Shock for at 42ºC for 45 seconds
Return eppendorf containing cells to ice and leave for 5 min.
Add 500µl LB and culture at 37C for 30 min.
Spread on plate with appropriate antibiotic resistance
Culture plate at 37ºC overnight

After ligation or from miniprep
Take 1µl (if from miniprep)/5µl (if from ligation) of DNA solution and mix with partly thawed competent cells on ice
Leave on ice for 30 min.
Heat Shock for at 42ºC for 45 seconds
Return eppendorf containing cells to ice and leave for 5 min.
Add 500µl LB and culture at 37ºC for 30 min.
Spread on plate with appropriate antibiotic resistance
Culture plate at 37ºC overnight

Sequencing Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
We use the Big Dye Sequencing system with using the following conditions.
Prepare the following solution:
1.8ul 5 × B.D.3.1 buffer
0.4ul B.D.3.1.
6.3ul MilliQ
1ul 0.15ug/ul DNA
0.5ul 3.2pmol/ul primer
Amplify using the following PCR program
96ºC 2min
96ºC 10sec
55ºC 5sec
60ºC 3min
Repeat 2-4 29times
25ºC pause
Add 0.5ul Shrimp Alkaline Phosphatase
Incubate at 37ºC for 1h
Add 1ul of 3M KOAc or NaOAc
Mix and transfer to 1.5ml eppendorf
Add 25ul of 100% EtOH and mix well
Spin for 10min at 20,000g, 4ºC

Discard supernatant and dry pellet
Add 15ul of HiDi Buffer
Transfer to PCR tubes to sequence (use the sequencer in the lab)

Miniprep Protocol - Todai-Tokyo – 2009 http://2009.igem.org/Team:Todai-Tokyo/Protocols
Preparation (previous night)
Pick Single colonies from a transformation plate and culture in 4ml Luria Broth
Culture overnight at 37ºC with vigorous shaking
Miniprep
Aliquot 2ml of the bacterial suspension from above into a 2ml eppendorf
Spin down for 5 min. at max speed to pellet cells
Use the Promega miniprep kit according to instructions
We usually elute the plasmid DNA from the spin column by 50µl MilliQ and measure nucleotide concentration on a spectrophotometer to label on the tube.

Standard biobrick preparation – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Centrifuge the distribution plates for a while so that the dry DNA precipitates onto the bottom of the wells;
Penetrate the aluminum foil with the tip of a pipette (Caution: avoid damaging other wells);
Add 15µl double-distilled water (ddH_2O);
Take 1~2µl to perform following operations.
Transduction – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Set the water bath to 42°C;
Mark and place an EP tube of E.coli competent cells (ca.100µl) in a freezing box;
Add 1~2µl plasmid solution and leave the tube in the freezing box for 30min;
Heat shock the cells in the water bath for an exact time of 90s;
Take out the tube and place it in the ice box for another 5min;
Add 400µl (or 1000µl) of liquid medium into the tube and mark it;
Shaking cultivate at 37℃ for 0.5~2h; meanwhile prepare the culture plates with corresponding antibiotic in the medium;
Inoculate and cultivate the plates upside-down in a 37℃ incubator for 12~14h.
E.coli culture – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Add antibiotics of 0.1% concentration into a culture tube with 4~5ml liquid medium inside;
Pick a single colony with a pipette tip from a culture plate and inoculate it into the liquid medium;
Shaking culture in a 37℃ incubator overnight (ca. 12~16h).
Bacterium strain storage – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Prepare a 1.5ml EP tube and mark it;
Add 400µl of 80% glycerin into the tube;
Add 600µl of cultivated liquid medium into the tube;
Store the tube in a -80C°℃ fridge.
Agarose gel electrophoresis – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Gel preparation:

Ingredients:
(for a smaller piece of gel) 0.3g of agarose + 30ml of TBE + 4μl of EB;
(for a larger piece of gel) 0.8g of agarose + 80ml of TBE + 4μl of EB;
Weigh out the required amount of agarose powder onto a piece of weighing paper;
Add the agarose powder into a glass bottle specific for gel preparation;
Add the required amount of TBE buffer into the bottle;
Microwave the liquid with the bottle lid slightly open until it boils, so that the agarose powder gets well dissolved; meanwhile prepare the gel plates and ensure the right comb is fixed;
Cool the bottle in a sink;
When the solution feels warm but not hot (ca.60℃), add the required amount of EB with a pipette specific for gel preparation into the bottle;
Shake the bottle carefully so that the EB is well mixed;
Pour the liquid onto the gel plate;
Wait for the liquid to be cured.
Spotting:
Place a disposable glove on the experiment table;
Pipette correct amounts of loading buffer (so that the buffer will be diluted to a correct concentration) onto the glove to form a lane of buffer drops;
Pipette correct amounts of samples into the buffer drops and mix them well;
Pipette the correct DNA ladder (or marker) into a gel lane, usually the first or the middle-most one;
Pipette the mixed samples into lanes of the gel; watch out for possible leakage which occurs when the pipette tip gets too deep and penetrates through the gel lane.
Electrophoresis:
Voltage selection:
(for a general resolution) 160V;
(for a high resolution) 120V (and correspondingly longer electrophoresis time);
After spotting the samples, install the lid of electrophoresis chamber and switch the output on;
When the fastest lane runs to the center (or 2/3 the length if follow-up gel purification is intended) of the gel, switch off the output and transfer the gel into the UVP system;
Power on UVP and observe the gel; take photos when necessary;
Switch off the system and discard the gel into a special rubbish bin.
Double digestion – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
System selection:
For identification only, choose the 20μl system;
For following ligation, choose the 100μl system;
Refer to the Takara inventory for the correct buffer. Take EcoRI and PstI for an example:
20μl system:

Plasmid(100ng/μl)	10μl
EcoRI	0.5μl
PstI	0.5μl
1*H	2μl
ddH_2O	7μl

100μl system:

Plasmid(100ng/μl)	30μl
EcoRI	3μl
PstI	3μl
1*H	10μl
ddH_2O	54μl

Prepare the system on a freezing box;
Place the system under 37℃ temperature for 2~3h (for identification) or 9~12h (for ligation).
Tips for digestion
Better limit the volume of restriction enzymes to less than 1/10 of the whole system, for the star activity would be enhanced under high concentration conditions. In most cases it is about 1/20 of the whole system.
To avoid the case in which DNA concentration is too low and hence electrophoresis yields no result, ensure a minimum DNA amount of 50ng.
For identification only, an estimated amount of 0.5μl of enzyme (8~20U) should correspond to 1μg of DNA; For ligation, 1μl of enzyme to 1μg of DNA.
For identification only, a digestion time of 2~3h is enough; for ligation it's better to leave the system overnight, otherwise massive false positive colonies would occur during the following steps of transduction and culture.
Special protocols
SuperScript™ III First-Strand Synthesis System for RT-PCR – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
The following procedure is designed to convert 1 pg to 5 μg of total RN or 1 pg to 500 ng of poly(A)+ RNA into first-strand cDNA.
Mix and briefly centrifuge each component before use.
Combine the following in a 0.2- or 0.5-ml tube:

Component	**Amount**
up to 5 μg total RNA	n μl
Primer: 50 μM oligo(dT)20	1 μl
10 mM dNTP mix	1 μl
DEPC-treated water	to 10 μl

Incubate at 65°C for 5 min, then place on ice for at least 1 min.
Prepare the following cDNA Synthesis Mix, adding each component in the indicated order.

Component	**1 Rxn**	**10 Rxns**
10X RT buffer	2 μl	20 μl
25 mM $MgCl_2$	4 μl	40 μl
0.1 M DTT	2 μl	20 μl
RNaseOUT (40 U/μl)	1 μl	10 μl
SuperScript? III RT (200 U/μl)	1 μl	10 μl

Add 10 μl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and collect by brief centrifugation. Incubate as follows.

Oligo(dT)20 or GSP primed:	50 min at 50°C
Random hexamer primed:	10 min at 25°C, followed by 50 min at 50°C

Terminate the reactions at 85°C for 5 min. Chill on ice.
Collect the reactions by brief centrifugation. Add 1 μl of RNase H to each tube and incubate for 20 min at 37°C.
cDNA synthesis reaction can be stored at -20°C or used for PCR immediately.
RNA extraction – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Get 1ml bacteria and centrifuge 1000rpm for 5mins, discard the pellet, rewash the cells with 1*PBS.
Add in 800ul Trizol. nature at room temp for 5~10 mins. In this process, beat upon the cells with liquid-transfering gun will benefit its homogenization.
The reagent for 12/24-well system

Types of plate	**35-cm**	**12-well**	**24-well**	**Ratio by volume**

Trizol added	1ml	800ul	500ul	Trizol: Chloro = 5:1 Trizol: Isopro = 2:1 Trizol: Ethanol = 1:1
Chloroform added	200ul	160ul	100ul	
Isopropyl-alcohol added	500ul	400ul	250ul	
75% ethanol	1ml	800~ul	500ul	

Add in the corresponding Chloroform, Vortexing 15s, quiescence at room temp for 2~3 mins.
Centrifuge 12000g for 15 mins, recommended at 4~8℃, the liquid is separated in aqueous phase, red phenol-chloroform phase and an interfase. RNA is soluted in aqueous phase, 60% of the Trizol volume
Transfer the aqueous phase carefully into a fresh EP tube. The remaining red phase can be utilized in the following DNA/protein extraction. Add in corresponding volume of Isopropyl-alcohol in accordance with the upper form, after vortexing, quiescence at room temp for 10 mins.
Centrifuge at 12000g for 10 mins, recommended at 4~8 ℃. Now RNA are at the bottom of the tube.
Discard the supernatant carefully, put in corresponding volume of 75% ethanol, vortexing 15s and centrifuge 7500g for 5 mins.
Discard the supernatant carefully, place upside down to airing for 20~30 mins, in case of over airing and harm the RNA solution.
Add in 40~50 ul DEPC to solute RNA, vortexing slightly and centrifuge. Set at 55~65℃ for 10 mins.
Store at –70 ℃.
The long-term storage: 75% ethanol, -20~-70℃ for about a year.

Preparation of reagents

1*PBS
Trizol
Sterilized EP tube (1.5/2.0ml format)
Precooled 75% ethanol
Isopropyl-alcohol
Chloroform
DEPC solution
Western blot – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Prepare gels for SDS-PAGE
heat the sample buffer in heating water bath at 98°C
boil every 15ul sample with 5ul sample buffer at 98 ℃ for 5 mins, marker 4 ul
add in running buffer
run until the marker goes to the end. (100 V constant voltage, 25 min; 200V, 40 min)
SDS-PAGE Transfer Buffer:

Tris-Base	25 mM
Glycin	192 mM
Methanol	20%
SDS	1/1000

For 1 L of buffer mix 3.03 g of Tris-Base, 14.4g of glycin and 200 mL of methanol; Bring to 1L with deionized water. Do not pH.
Set up transfer from the gel to a filter paper in transfer buffer. The PVDF membrane should be as large as the gel. Keep the gel in transfer buffer in case of dry.
Open the transfer chamber, put 2 layers of cushion to absorb water, 1 layer of filter paper from the transfer buffer. Put the gel carefully on the filter paper, and then add the PVDF membrane, add another filter paper and cushion.
Add running buffer, run at 4℃, 350 mA constant current, for 65 mins
Washing buffer:

TBS	500ml
Tween	200ul

Blotting buffer:

milk powder	2.5g
washing buffer	50ml

After electrophoresis, put the PVDF membrane into washing buffer, and then put in blotting buffer at 4 ℃, shaking overnight.
Wash the membrane in washing buffer.
Add primary antibody at proper dilution. Incubate the membrane for 2 hr with shaking at room temp.
Wash the PVDH membrane for 3 times, 5 mins each.
Add secondary antibody at proper dilution. Incubate the membrane for 2 hr with shaking at room temp.
Wash the PVDH membrane for 3 times, 5 mins each.
Detection by glimmering substrate, mix A and B solution by same volume.
Transfer to a dark room.
Put a preservative film on the table, take out the PVDH membrane, absorb the water with absorbant paper. Put the PVDF membrane on the glimmering substrate for 3 mins.
After 3 mins, wrap the PVDF membrane with preservative film, put the film on PVDF membrane in the film cassette. React for 10 mins.
Put in the development solution, wash by water, put in fixing solution, and wash by water again.
PVDF membrane can be regenerated by TBS washing
QuikChange® II XL Site-Directed Mutagenesis Kit & QuikSolution™ Reagent – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Enhanced amplification efficiencies 2.5–3.5µl QuikSolution/50µl reaction, with 3µl being optimal for most targets.
Mutant Strand Synthesis Reaction (Thermal Cycling)
Ensure that the plasmid DNA template is isolated from a dam+ E. coli strain. recommends using thin-walled tubes
Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide primers prior to use in the following steps (see Mutagenic Primer Design).
Prepare the control reaction as indicated below:
5 µl of 10× reaction buffer
2 µl (10 ng) of pWhitescript 4.5-kb control plasmid (5 ng/µl)
1.25 µl (125 ng) of oligonucleotide control primer #1 [34-mer (100 ng/µl)]
1.25 µl (125 ng) of oligonucleotide control primer #2 [34-mer (100 ng/µl)]
1 µl of dNTP mix
3 µl of QuikSolution reagent
36.5 µl of double-distilled water (ddH_2O) to a final volume of 50 µl
Then add 1 µl of PfuUltra HF DNA polymerase (2.5 U/µl)
Prepare the sample reaction(s) as indicated below:
Stratagene recommends setting up an initial sample reaction using 10 ng of dsDNA template.
5 µl of 10× reaction buffer
X µl (10 ng) of dsDNA template
X µl (125 ng) of oligonucleotide primer #1
X µl (125 ng) of oligonucleotide primer #2
1 µl of dNTP mix
3 µl of QuikSolution
ddH_2O to a final volume of 50 µl
Then add 1 µl of PfuUltra HF DNA polymerase (2.5 U/µl)
If the thermal cycler to be used does not have a hot-top assembly, overlay each reaction with ~30 µl of mineral oil.

Cycle each reaction using the cycling parameters outlined in Table I. (For the control reaction, use a 5-minute extension time and run the reaction for 12 cycles.)
Following temperature cycling, place the reaction tubes on ice for 2 minutes to cool the reactions to ≤37°C.

Cycling Parameters for the QuikChange® II XL Method

segment	cycles	temperature	time
1	1	95℃	1 min
2	18	95℃	50 sec
		60℃	50 sec
		68℃	1 minute/kb of plasmid length
3	1	68℃	7 min

Dpn I Digestion of the Amplification Products – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop
Gently thaw the XL10-Gold ultracompetent cells on ice. For each control and sample reaction to be transformed, aliquot 45 μl of the ultracompetent cells to a prechilled Falcon? 2059 polypropylene tube.
Add 2 μl of the β-ME mix provided with the kit to the 45 μl of cells. (Stratagene cannot guarantee highest efficiencies with β-ME from other sources.)
Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
Transfer 2 μl of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the ultracompetent cells.
As an optional control, verify the transformation efficiency of the XL10-Gold ultracompetent cells by adding 1 μl of 0.01 ng/μl pUC18 control plasmid (dilute the control provided 1:10 in high-quality water) to another 45-μl aliquot of cells.
Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes.
Preheat NZY+ broth (see Preparation of Media and Reagents) in a 42°C water bath for use in step 8.
Heat-pulse the tubes in a 42°C water bath for 30 seconds. The duration of the heat pulse is critical for obtaining the highest efficiencies. Do not exceed 42°C.
Incubate the tubes on ice for 2 minutes.
Add 0.5 ml of preheated (42°C) NZY+ broth to each tube, then incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.
Plate the appropriate volume of each transformation reaction, as indicated in the table below, on agar plates containing the appropriate antibiotic for the plasmid vector.
For the mutagenesis and transformation controls, spread cells on LB–ampicillin agar plates containing 80 μg/ml X-gal and 20 mM IPTG (see Preparing the Agar Plates for Color Screening).

Transformation reaction plating volumes

Reaction Type	Volume to Plate
pWhitescript mutagenesis control	250 μl
pUC18 transformation control	5 μl (in 200 μl of NZY+ broth)
Sample mutagenesis	250 μl on each of two plates (entire transformation reaction)

Incubate the transformation plates at 37°C for >16 hours.
Annealing – SJTU-BioX-Shanghai – 2009 - http://2009.igem.org/Team:SJTU-BioX-Shanghai/Protocols#bannerTop

Add 10μl forward, 10μl reverse primer into a tube, together with 80μl anneal buffer. Use PCR to cool down the temperature from 94℃ to 4℃ at the rate of 1℃/min.

DNA purification by phenol/chloroform method

Add 50µl obliges, 150µl ddH$_2$O and 200µl phenol/chloroform into a tube. And spin at the speed of 12000rpm for 10 minutes. Extract the upper layer of the solution, added to a solution containing 400µl NaAc and 800µl absolute ethanol. Spin at the speed of 12000rpm for 10 minutes. Drop the upper layer of the solution. Add 500µl 70% ethanol to it. Spin at the speed of 12000rpm for 5 minutes. Drop the upper layer of the solution. Dry it and add 15µl ddH$_2$O.

Protocol for chemical inducible expression of GFP – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
download PDF version
Materials:
4 groups of induce solution with a concentration gradient of 10^-7, 10^-5, 10^-3, 10^-2;
Overnight bacterial culture or bacterial colonies;
Phosphate Buffered Solution (PBS).
Procedure:
1. Add 20 µl of the overnight bacterial culture or pick a colony to 5ml of LB antibiotic medium, Incubate at 37 degree in a shaker till the OD600 value reaches 0.4-0.6.
2. Add 0.5 mL of the fresh bacterial culture and appropriate volume of inducer solution to prepare induction system with the concentration gradient of 10^-9, 10^-8, 10^-7, 10^-6, 10^-5, 10^-4, 10^-3, 10^-2.
3. Place the induction system at 37 degree for 2 hours.
4. Pellet bacterial cells by 4 min centrifugation at 4000 rpm, discard the supernatant.
5. Resuspend the pelleted cells in 500 µl of PBS.
6. Transfer 100 uL of bacterial resuspention into each well of 96-well plate to test the expression of GFP by flow cytometry or Microplate Reader.

Note:
If desired, time sequential expression of GFP can also be tested, through verifying the incubating time of induction system at 37 degree.

Transformation protocol – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
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Materials:
Plasmid samples or ligation product;
Commercially competent cells;
LB non-antibiotic liquid medium;
LB antibiotic agar plates
Procedure:
1. Get the competent cells from -70 degree, and wait for its fusion. 50 µl of competent E.coli cells for each sample. Put microcentrifuge tubes to chill on ice for at least 2 min.
2. Add 2 - 3 ul of each plasmid sample or all the ligation product into the competent cells in the microcentrifuge tubes. Mix and incubate on ice for 30 min.
3. Heat pulse for 90 sec, at 42 degree. Put back to ice and incubate for 5 min.
4. Add 200 uL LB non-antibiotic liquid medium into each microcentrifuge tube. Shake the microcentrifuge tubes in shaker, at 37 degree, for 30 min to recover.
5. Plate 150 uL of the liquid medium with transformed cells immediately, on pre-warmed LB antibiotic agar plates. Incubate overnight at 37°C.

Tips:
All procedures are performed on ice.
Make sure the cells are not left at ambient temperature for more than 5 min as this will significantly decrease the transformation efficiency.
When got out from the shaker, the competent cells may form pellet in the microcentrifuge tubes. You need to resuspend the cells before plating.
References:
Current protocols in molecular biology.

Site-directed mutagenesis protocol – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
download PDF version
Materials:
Takara MutanBEST Kit with the following content
A pair of primers which juxtapose their 5' ends and have contrary directions of 3' ends to import the mutation;
Template plasmid;
Commercial competent cells.
Procedure:
PCR Reaction
1. Design and synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence. Purify these oligonucleotide "primers" prior to use in the following steps
2. Prepare PCR reaction system which has the following composition(total 50 uL)

10x Pyrobest buffer II	5uL
ddNTP Mixture (2.5 mM for each)	4uL
Primer 1 (20uM)	1uL
Primer 2 (20uM)	1uL
Template plasmid	0.01~1ng
Pyrobest DNA Polymerase(5 U/uL)	0.25uL
ddH2O	up to 50 uL

3. Proceed the reaction at the following reaction conditions
94 degree 30 sec;
55 degree 30 sec;
72 degree 5 min;
Repeat the cycle above.
30 cycles in total
Cycle each reaction using the cycling parameters above (For the control reaction, use a 5-minute extension time).
4. Electrophoresis PCR reaction system in 1% agarose gel.
5. Excise the gel slice and extract the target DNA fragment.
Blunting Kination Reaction
1. Prepare the following reaction system in a microcentrifuge tube.

DNA Fragment	around 1 pmol
10x Blunting Kination Buffer	2 uL
Blunting Kination Enzyme Mix	2 uL
ddH2O	up to 20 uL

2. React for 10 min at 37 degree.
3. React for 10 min at 70 degree.

Ligate Reaction

1. Add about 0.25 pmol (5 uL) of solution marked No.3 into a new microcentrifuge tube.
2. Add 5 uL of ligation solution I, mix gently and thoroughly.
3. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parentalReact for 1 hour at 16 degree.
4. Transfer the whole reacted system into 100 ul of competent cells to transform. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 minutes to be followed by subsequent steps. When the transformation is finished, plate the appropriate volume of each transformation reaction on agar plates containing the appropriate antibiotic for the plasmid vector.

Notes:

1. To maximize temperature-cycling performance, we strongly recommend using thin-walled tubes, which ensure ideal contact with the temperature cycler's heat blocks. The following protocols were optimized using thin-walled tubes.
2. Set up a series of sample reactions using various concentrations of dsDNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of dsDNA template) while keeping the primer concentration constant.

Protocol for preparation of competent cells for transformation – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol

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For two transformations

Materials:

0.1 M Calcium Chloride chilled on the ice;
Overnight bacterial culture or bacterial colonies;

Procedure:

1. Add 20 μl of the overnight bacterial culture or pick a colony to 1 ml of LB antibiotic liquid medium, Incubate at 37 degree in a shaker till the OD600 value reaches 0.4-0.6.
2. Put the tubes on ice to incubate for 5 min.
3. Pellet bacterial cells by 4 min centrifugation at 4000 rpm, discard the supernatant.
4. Resuspend cells in 600 μl of ice-chilled 0.1 M Calcium Chloride solution. Incubate on ice for 30 min.
5. Centrifuge for 4 min at 4000 rpm in a microcentrifuge tube, discard the supernatant.
6. Resuspend the pelleted cells in 100 ul of ice-chilled 0.1 M Calcium Chloride solution. Incubate on ice.
7. Add 50 μl of the prepared cells to each tube containing DNA sample, mix and incubate on ice for 30 min.
8. Transform subsequently as the transformation protocol.

Note:

1. Make sure the cells are not left in the centrifuge at ambient temperature for more than 5 min as this will significantly decrease the transformation efficiency.
2. The rpm at centrifugation is not higher than 4000, as a high rpm may cause the lysis of cells.
3. Competent cells prepared with this protocol are suitable for direct use only. Freezing down and storage

at -70°C is not recommended.
4. The culture can be kept at 4 degree for one week and used for preparation of competent cells, but culture stored longer than 10 ten days is not suitable for competent cells.

References:

Current protocols in molecular biology

Protocol for PCR with EasyPfu DNA Polymerase – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol

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Preparation for Reaction Mixture

Reagent	Final concentration	Volume
10X Taq buffer	1X	2 µl
2.5 mM dNTP mix	0.25 mM	2 µl
Forward Primer	0.2-0.4 µM	1 µl
Reverse Primer	0.2-0.4 µM	1 µl
EasyPfu DNA Polymerase	25 units	1 µl
Template DNA	As required	<0.5 µg
ddH2O	to final volume	

Cycling Conditions

Step	Temperature/℃	Time/min
Initial Denaturation	94	5
Denaturation	94	0.5
Primer Annealing	As required	As required
Extending	72	As required(0.5kb/min)
Final Extending	72	10
Number of Cycles		32-35
Cooling Down	4	10

Protocol for ligation of inserting DNA into plasmid vector DNA – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol

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Materials:

DNA sample(s) in water or TE buffer
10x ligation buffer

T4 DNA Ligase, 5 u/µl
ddwater
Procedure:
1. Test the concentration of the DNA sample(s).
2. Pipet the following into a microfuge tube:

Linearized vector DNA	around 100ng
Insert DNA (at 3:1 molar excess over vector)	variable
10x ligation buffer	1uL
T4 DNA Ligase	1uL
ddwater	Rest of volume
Total volume	10 uL

3. Vortex and spin briefly to collect drops.
4. Incubate the mixture at 16 degree for 60-120 min.
5. Use the ligation mixture for transformation.

Tips:
Thoroughly mix the 10x ligation buffer before use.
The optimal insert/vector molar ratio is 3:1.
To minimize recircularization of the cloning vector, dephosphorylate linearized plasmid DNA with Alkaline Phosphatase(CIAP) prior to ligation. Heats inactivate the phosphatase or remove from the mixture after the dephosphorylation step.
DNA purity is an important factor for successful ligation. Plasmids should be purified using a method that will ensure isolation of high quality DNA. Use only high quality agarose and fresh electrophoresis buffers for gel-purification of DNA fragments.

Protocol for DNA purification from reaction mixture – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
download PDF version
Here is a suggested protocol; this protocol can be used to purify a wide range of DNA fragments with recoveries of >80%. The bolded should be noticed for a nice DNA extraction.
1. Put EB (elution buffer) at 65 degree water bathing.
2. Add a 3:1 volume of Binding Buffer to the reaction mixture (e.g., for every 100 ul of reaction mixture, add 300 ul of Binding Buffer). Mix thoroughly. **Check the color of the solution**. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 ul of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
3. Pour the solution to a fresh adsorption column. Centrifuge at 13000rpm for 1 min. Pour off the liquid in the collection tube. **For critical samples**, repeat the operation above.
4. Add 600 ul washing buffer (WB) before centrifugation at 13000 rpm for 1 min. Pour off the liquid into beaker.
5. **Centrifuge at 13000rpm for 10 min** to spin the ethanol down.
6. Put the column into a fresh EP tube. If necessary air-dry the pellet for 10-15 min to avoid the presence residual ethanol in the purified DNA solution. Residual of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
7. Add 30-50 ul elution buffer (EB) to elute the DNA.
8. Get 5 ul of the eluted sample to identify with electrophoresis.

Note:
1. If a large amount of DNA is purified or if the volume of the binding reaction is greater than 1.5 ml increase the incubation time of the binding step to 15 min.

References:

Current protocols in molecular biology

DNA double digestion protocol – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
download PDF version

Materials
DNA sample(s) in water or TE buffer
10x digestion buffer
Restriction enzymes (EcoRI or SpeI or XbaI or PstI)
DNA loading buffer (if electrophoresis is subsequent)
Agarose gel 0.8% (or different depending on expected band sizes)

Procedure:
1. Test the concentration of the DNA sample(s).
2. Pipet the following into a microfuge tube:

	20uL reaction system	50uL reaction system
DNA	around 1ug	around 2.5ug
10x Digestion buffer	2uL	5uL
1st Enzyme	1-1.5uL	2.5-4uL
2nd Enzyme	1-1.5uL	2.5-4uL
ddWater	Rest of volume	Rest of volume

3. Incubate at recommended temperature (37.0 degrees) for 2 or 4 hours (2h for enzymes of NEB, 4h for enzymes of Takara).
4. Take 2 to 5 uL of the digested sample, add loading buffer, and run it on the agarose gel to check the result, or take the entire sample to run to extract a wanted fragment).

Tips:
1. DNA:
For identification of DNA, use 0.4 ug/uL DNA; (or 2uL from a nice DNA mini prep)
For cloning, 1ug/uL DNA is enough.
2. Buffer: we'd better use the buffer that comes with the enzyme, which means buffers from other company may cause some abnormal results.
3. Enzyme: the maximum volume that an enzyme can be used is 1/10 of the total reaction volume (example: 2 uL for 20 uL reaction system). If you want to do overnight digestion, add less enzyme(example: 1 uL for 20 uL reaction system).
4. Gel: make sure to run the uncut DNA as a control along with the digested DNA sample(s). And, always run a DNA marker!

References:

Current protocols in molecular biology (3.1.1-3.1.2)

DNA Gel extraction protocol – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
download PDF version
Here is a suggested protocol; this protocol can be used to purify a wide range of DNA fragments with recoveries of >80%. The bolded should be noticed for a nice DNA extraction.
1. Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. **Cut as close to the DNA as possible to minimize the gel volume**. Place the gel slice into a pre-weighed 1.5 ml tube and weigh. Record the weight of the gel slice.
2. Put EB (elution buffer) at **65 degree** water bathing.
3. Add a 3:1 volume of Solution Buffer to the gel slice (volume:weight) (e.g., add 300 ul of Binding Buffer for every 100 mg of agarose gel). Incubate the gel mixture at 60 degree for 5 min at least **until the gel slice is completely dissolved**. Mix the tube by inversion every few minutes to facilitate the melting process. **Check the color of the solution**. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 ul of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
4. Pour the solution to a fresh adsorption column. Centrifuge at 13000rpm for 1 min. Pour off the liquid in the collection tube. **For critical samples**, repeat the operation above.
5. Add 600 ul washing buffer (WB) before centrifugation at 13000 rpm for 1 min. Pour off the liquid into beaker.
6. **Centrifuge at 13000rpm for 10 min** to spin the ethanol down.
7. Put the column into a fresh EP tube. If necessary air-dry the pellet for 10-15 min to avoid the presence residual ethanol in the purified DNA solution. **Residual of ethanol in the DNA sample may inhibit downstream enzymatic reactions**.
8. Add 30-50 ul elution buffer (EB) to elute the DNA.
9. Get 5 ul of the eluted sample to identify with electrophoresis.

Notes:
1. Extract the gel as soon as you excise the gel slice.
2. If the purified DNA will be used for cloning, avoid UV damage of the DNA by minimizing the UV exposure to a few seconds or keeping the gel slice on a glass or plastic plate during UV illumination.
3. If a large amount of DNA is purified or if the volume of the binding reaction is greater than 1.5 ml increase the incubation time of the binding step to 15 min.

References:
Current protocols in molecular biology

Miniprep Protocol & Hints – PKU-Beijing – 2009
http://2009.igem.org/Team:PKU_Beijing/Notebook/Protocol
download PDF version
Here is a suggested protocol; the yield of the plasmid should be approximately 0.2-0.3 ug/ul. The bolded should be noticed for a nice miniprep.

Procedures:
1. Inoculate 5ml LB medium (containing antibiotic) with a bacterial clone, culture with vigorous shaking at 37 degree for 12-16 hrs.
2. Put EB (elution buffer) at **65 degree** water bathing.
3. Harvest bacteria by spinning at 13000rpm (~12000g) for 1 min. Aspirate supernatant. Add additional 750 ul culture media, respin and aspirate supernatant for several times.
4. Resuspend bacterial pellet by complete **vortexing** in 250ml resuspension buffer (RB, with 10ul RnaseA in it). The bacteria should be completely resuspended - no clumps should be visible.

5. Add 250ul freshly lysis buffer (LB) and mix **gently** by inverting 5-6 times at room temperature. The mixture should appear translucent and mucous-like. The time of lysis will never be longer than 5 min.
6. Add 350ul neutralization buffer (NB) and mix **gently** by inverting 5-6 times, incubate at room temperature for 3 min. The mixture should contain flocculent white precipitate at this point.
7. Remove bacterial debris by centrifugation at 13000rpm for 10 min; pour supernatant to a fresh adsorption column which can avoid the transfer of precipitate to the new column causing the precipitate is "sticky".
8. Centrifuge at 13000rpm for 1 min. Pour off the liquid in the collection tube. **For critical samples**, repeat the operation above.
9. Add 600 ul washing buffer (WB) before centrifugation at 13000 rpm for 1 min. Pour off the liquid into beaker.
10. **Centrifuge at 13000rpm for 10 min** to spin the ethanol down.
11. Put the column into a fresh EP tube. Air dries DNA for **10 min**.
12. Add 30-50 ul elution buffer (EB) to elute the DNA.

Notes:

1. Typical yield of high-copy-number plasmids, such as PSB1AK3, prepared by this method is about 0.2-0.3 ug of DNA per ul of original bacterial culture, and 0.1 ug of DNA per ul for low-copy-number plasmids such as PSB3T5.
2. To analyze the DNA by cleavage with restriction enzyme(s) remove 2 µl of the DNA solution and add it to fresh microfuge tube that contains 5 µl of water. Add 1 µl of the appropriate 10 x restriction enzyme(s). Incubate the reaction for 2 hr at the appropriate temperature. Store the remainder of the DNA preparation at -20 degree. Analyze the DNA fragments in the restriction digest by gel electrophoresis.
3. For tetracycline, notice its photolysis.
4. Resuspension buffer (RB) should be stored in the refrigerator. RNAse should be in the -20 degree freezer.

References:

Current protocols in molecular biology

PLASMID RESUSPENSION FROM IGEM PAPER SPOTS – NTU-SINGAPORE – 2009

http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols

(*Estimated time: 25 min + 5 min for every part if you use scalpel/tweezers or + 15 min for every part if you use punch tool*)

Materials needed :
Pre-warmed at 42°C TE
Desired spot location information
punch tool
ddH20
99% ethanol
0.5 ml tubes

Protocol
Put 10 µL of pre-warmed TE into a 0.5 ml tube.
Cut paper spots using punch tool, following the instructions provided with the IGEM kit.
Put the cut paper into the 0.5 ml tube.
Clean punch tool with water and ethanol every time after cutting a spot; be careful to dry the punch tool.
Incubate at 42°C for 20 min.
Vortex and spin down.
Pipette 4-6 µl of DNA resuspension for transformation

Side note:
Pick a single colony and inoculate broth (again, with the correct antibiotic) and grow for 18 hours.
Use the resulting culture to miniprep the DNA AND make your own glycerol stock

PLASMID RESUSPENSION FROM WELL – NTU-SINGAPORE – 2009

http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(Estimated time: 25 min)

Materials Needed
DI water

Protocol
With a pipette tip, punch a hole through the foil cover into the corresponding well to the Biobrick™-standard part that you want. Make sure the plate is oriented properly. It is NOT recommended to remove the foil cover, as it could lead to cross contamination between the wells.
Add 15uL of diH2O (deionized water)
Take 2uL DNA to transform your desired competent cells
Side note:
Pick a single colony and inoculate broth (again, with the correct antibiotic) and grow for 18 hours.
Use the resulting culture to miniprep the DNA AND make your own glycerol stock

BIOBRICK DIGESTION WITH RESTRICTION ENZYMES – NTU-SINGAPORE – 2009

http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*estimated time: 3 hours*)

Materials needed: Double Digestion mixture composition (total volume: 50 µL)
DNA 1mg (usually 4~5 µL)
Enzyme-1 1 µL
Enzyme-2 1 µL
NEBBuffer-2 5 µL (1/10 of total volume, check double digestion table for which buffer to use)
BSA 0.5 µL (1/10 of buffer volume)
Mili-Q water 37.5 µL

Protocol
Measure plasmid concentration by Nano-drop
Pipette apropraite amount of vector (usually 4~5 µl for 1 µg)
Take out NEBuffer and BSA to thaw (usually stored at -20 deg C), vortex for well mix
Adding the following components sequentially into 200 µL PCR tube, following the order: Mili-Q water → Enzyme Buffer → BSA (optional) → DNA → Enzyme
Pulse spin the tube and incubate at 37 deg C for 2 hours (2.5hrs)
Do MiniElute PCR purification for all the tubes to remove enzymes and buffers
Load the 10 µL plasmid with 2 µL loading dye for gel running

Notes
check 37 deg C incubator is on, take out buffer, BSA, to thaw and vortex before adding, when adding buffer, enzymes, DNA (not BSA), putting the whole pipette tip into the solution, and then pipette up and down. For adding BSA, just push strongly and fast all the way down.

LB - AGAR PLATE PREPARATION PROTOCOL – NTU-SINGAPORE – 2009

http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 3 hours + 1 hour pouring process*)

Materials needed
LB broth
Agar
Autoclave bottle
Petri dishes

Protocol
Weigh 25g of LB-Agar powder mix (or 15g LB powder + 10g agar) per liter of media desired. One liter makes 40-50 plates
Select an appropriate bottle and dissolve LB-Agar in autoclaved Mili-Q water. Add a stir bar and use a magnetic stirrer to speed dissolve process
Cover the bottle with aluminum foil, and secure the foil with autoclave tape. The foil should be somewhat loose (to avoid building pressure in the bottle while sterilizing and blowing the foil off).
Load the bottle into the autoclave, and sterilize it
Once the autoclave finishes venting (which can take twice as long as the sterilization proper), Unload the hot bottle using the insulated oven gloves
Allow the media to cool until it can be handled without the oven mits.
Once media is cool, add appropriate amount of ampicilin stock (stock 50mg/ml, final 100ug/ml, To achieve final concentrations, add 2mL of stock per 1L of media), use the magnetic stirrer (added before autoclave) to mix.
Pour directly from the bottle into sterile petri plates. Use a inoculation loop to snuff out bubbles that form during pouring. Bubbles can allow cells to access nutrients without being exposed to the plate's antibiotic, and should be blown out immediately before the gel can set.
Allow the plates to stand right side up until the gel sets. Plates should be stored upside down to keep condensation from falling on the media. Store petri plates in the plastic bags they ship in, in the 4°C fridge.

TRANSFORMATION – NTU-SINGAPORE – 2009 http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 3 hours and 30 min + 12-16 hours overnight incubation*)

Materials needed
LB agar plates with proper antibiotic added (Ampicilin)
Thawed Invitrogen TOP10 cells (every tube contains 50 µl of cell suspension)
Resuspended DNA
SOC medium

Protocol
Put 4-6 µl of DNA resuspension into TOP10 tube.
Incubate on ice for 30 min.
Heat shock: 42°C for 1 min.
Put transformed TOP10 tube on ice for 2mins and then add 200 µl SOC medium into the tube.
Incubate for 1 hour at 37°C, 225 rpm.
Plate 200 µl of solution on a proper agar plate (with Ampicilin resistence).
Incubate overnight at 37°C.

INOCULATION FOR OVERNIGHT GROWTH – NTU-SINGAPORE – 2009
http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 10 min + 12-16 hours overnight incubation*)

Materials needed
50mL Falcon tube.
5 mL LB medium
Suitable Antibiotic
Single colonies on a plate

Protocol
Pipet 5uL 1000X antibiotic into culture tube
Add 5mL LB medium with Ampicilin resistance
Select a single colony using a sterile inoculation loop
Place inoculation loop in culture tube and stir
Remove inoculation loop and place culture tube in incubator at 37°C overnight shaking vigorously (250 rpm)

AGAROSE GEL PREPARATION AND ELECTROPHORESIS – NTU-SINGAPORE – 2009
http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 2 hours*)

Materials needed
1X TAE
125 mL flask
Agarose
Gel Pouring Tray
Tape
Gel rig

Protocol
Measure out 40mL of 1X TAE buffer
Transfer buffer to 125 mL flask
Weigh out 0.4g agarose to make a 1% gel
Transfer agarose to 125mL flask
Melt agarose in microwave for 30 seconds,
Take out the flask and shake it gently
Again,put the flask in the microwave for 30 seconds
Assemble the gel pouring apparatus by inserting gate into slots.
Allow gel to cool until flask can be handled comfortably
Add the stain (4 ul for 40 ml TAE) and shake gentely to mix.
Place comb in the gel rig
Pour agarose into gel tray
Allow to solidify. While the gel is solidifying prepare the samples. Mix 10 µl DNA sample with 2 µl 6X loading dye for each slot.
Pour 1X TAE over gel so that gel is covered by a 3-5mm buffer
Load samples into lane (Don't forget to load 5 µl 1kb+ ladder into one of the lanes)
Hook electrodes to gel apparatus
Run the apparatus at 120V for 45 minutes
Visualize the gel and record the results

PREPARATION OF TOP10 CHEMICAL COMPETENT CELLS – NTU-SINGAPORE – 2009
http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 2 hours*)

Materials needed
Top10 *E.coli* cell
50 mL centrifuge tubes
1.5 mL small tubes
LB agar plate
LB medium
Chilled 0.1 M ice-cold MgCl2
Chilled 0.1 M ice-cold CaCl2
Chilled 0.1 M CaCl2 / 15 %glycerol

Protocol
Steak a loopful of Top10 Escherichia coli onto a fresh LB agar plate (without selective antibiotics) and incubated at 37 oC overnight.
Inoculate a single isolated colony of the Top10 E. coli into 5 ml of LB broth (without selective antibiotics) and incubated with shaking at 250 rpm overnight in a 37oC shaking incubator overnight.
Scale up the bacteria culture 100 times into 100 ml of fresh LB and grow to OD 600nm of 0.3 to 0.4 (2-3 hours). The current culture is in the exponential phase
Aliquot the above culture into two 50 ml pre-chilled centrifuge tubes
Incubate the two 50 ml tubes on ice for 10 min.
Centrifuge the two tubes of Top10 cells for 5 min at 5,000 rpm at 4 °C.
Decant the supernatant and re-suspend cells pellet in 30 ml of 0.1 M ice-cold MgCl2 thoroughly for each tube.
Centrifuge the mixture in the two tubes for 5 min at 5,000 rpm in 4 °C.
Decant the supernatant and re-suspend cells pellet in 20 ml of 0.1 M ice-cold CaCl2 for each tube
Incubate on ice for 30 min.
Centrifuge the mixture in the two tubes for 5 min at 5,000 rpm in 4 °C.
Decant the supernatant and re-suspend cells pellet in 1.5 ml of ice-cold 0.1 M CaCl2 / 15 %glycerol for each tube
Aliquot the cell suspension into 60 X 1.5 mL tubes each with 50 µl cell suspension
Store in - 80 °C deep freezer before use.

DNA CONCENTRATION MEASUREMENT USING NANODROP – NTU-SINGAPORE – 2009
http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 10~20 mins*)

Materials needed
DNA suspension in EB
NanoDrop ND-1000 Spectrophotometer

Protocol
Start up software by clicking on shortcut
Chose measurement (Nucleic acid for DNA and RNA samples)
Clean pedestals with tissue with MilliQ water
The software asks for a testmeasurement with MilliQwater: Add 1.8 µl of MilliQ water to front pedestal and click “OK”
Add 1.8 µl of a Blank (DNA: EB) to pedestal and click “Blank”.
Add 1.8 µl of sample, click “Measure”, note results and type in a sample name in the software window
Several different values can be read:
DNA or RNA conc. in ng/microliter at 260 nm
Protein conc. at 230 nm

Quality at 260:280 (should be around 1.9)
Clean pedestals with tissue before measuring next sample
Close software by clicking "Exit" (twice)
Retrieve data by clicking on shortcut to "nanodrop data"

SDS PAGE FOR PROTEIN – NTU-SINGAPORE – 2009 http://2009.igem.org/Team:NTU-Singapore/Notebook/Protocols
(*Estimated time: 2~3hrs*)

Materials needed
SDS page kit (invitrogen)
Overnight cell culture

Protocol
Inoculate interested colony in LB medium at 37°C overnight (250 rpm)
Dilute the above cell culture to 100 times in flask with LB (0.5mL in 50mL LB)
Incubate the flask until the culture OD ~= 0.8
Divide the above cell culture into two flask each with ~25mL
One flask is for control while the other one for experiment
Incubate until OD=1.6
Pipette 1mL cell culture from the above two flask and centrifuge at 12000rpm for 10 mins
Re-suspend the cell in 150 μL TE buffer
Pipette 15 μL cell sample into two fresh 1.5mL tubes
Add 15 μL sample buffer into the above two tubes
Let the tubes stands in boiling water for 10 mins
Take the gel out of fridge for it to thaw
Spin down the vapor and Load 15 μL sample into each slot
Gel run at 200V for 30mins
Remove the cover and extract out the gel
Wash and stain for 1~2hrs
Wash and de-stain for overnight
check gel band by densitometer

Notes
Sample buffer lyses the cell and bind to the protein
After staining, the entire gel show blue color, the de-stain procedure is to remove the straining, however, the site where protein resides will remain blue since stain bind to the protein

Ultracompetent Cell Preparation – ITT Madras – 2009
http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Materials/Buffers
SOB SOLUTION FOR COMPETENT CELL PREPARATION
0.5% yeast Extract
2% Tryptone
10mM NaCl
2.5mM KCl
10mM MgCl2
10mM MgSO4.

Dissolve all in nanopure water and autoclave
TRANSFORMATION BUFFER FOR COMPETENT CELL PREPARATION
10mM PIPES
15mM $CaCl_2$
250mM KCl
Dissolve in nanopure water and adjust pH to 6.7 with KOH or HCl. The add $MnCl_2$ to 55mM and adjust final volume. Sterilize by filtration with 0.45 µm filter. Store at 4C
Cells were cultured on 2xYT agar plate overnight at 37C.
10-12 colonies were cultured in 250ml SOB medium.
It was incubated at 37C for 1hour. Then the flasks were transferred to 19C. It was incubated till the OD600 reached 0.5
Flask was placed in ice for 10min.
The cells were pelleted by spinning at 4000rpm for 10min at 4C.
Cells were resuspended in 80ml ice cold TB(Transformation Buffer) and stored on ice for 10min.
It was centrifuged again at 4000rpm for 10min at 4C.
Pellet was resuspended in 20ml of TB with 1.5ml DMSO.
Final volume was aliquoted into microcentrifuge tubes (100-500µl) and stored at -80C
CAUTION!
Caution: The whole procedure after the cells are pelleted out needs to be carried out in ice.
Caution: TB buffer is heat sensitive, never take it out of ice.

Transformation – ITT Madras – 2009 http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
100µl competent cells were thawed on ice
2 µl Plasmid DNA added to the tube and shaken gently.
Mixture left on ice for 30 min.
Heat shock given at 42C for 2min.
Incubated on ice for 3-5 min.
800 µl of 2xYT broth added.
Flasks were shaken at 37C for 1hr.
They were centrifuged at 3000rpm for 5min and the pellet was resuspended into 100ul of the supernatant.
The 100 µl of the transformation mix was plated on 2xYT agar plates.
Plates were incubated at 37C overnight.
Variant
The amount of cells used can be varied greatly from 30ul to100ul.
Caution: The amount of DNA added should not exceed 10% of the total volume (it generally doesn't work, don't flood the cells with DNA)
The heat shock step greatly varies from one lab to another (anywhere from 30s to 2 mins). For us 2 mins worked fine.
The cooling step after heat shock can also vary from 2-5 mins.
Spinning the cells down and resuspending them in a small volume to plate out everything reduces the chances of losing transformed cells.

Miniprep – ITT Madras – 2009 http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Overnight cultures were harvested (2-3ml broth cultures).
They were centrifuged at 13000rpm for 1min.
The pellet was resuspended in 250 µl of HP1 solution.
The cells were lysed by adding 250 µl of lysis solution i.e. HP2. Tubes were inverted 5-6 times.

350 µl of neutralization solution i.e. HN3 was added. Tbes were inverted 5-6 times to mix the solutions.
They were centrifuged at 13000rpm for 10 mins to get a white pellet.
The supernatant was carefully transferred to a HiElute Miniprep spin column.
It was centrifuged at 13000rpm for 1 min. Flow through was discarded.
500 µl of wash solution i.e. HPB was added to the column.
It was centrifuged at 13000rpm for 1 min. Flow through was discarded.
700 µl of wash solution i.e. HPE was added to the column.
It was centrifuged at 13000rpm for 1 min. Flow through was discarded.
It was centrifuged at 13000rpm for 1 min.
The column was transferred to a fresh tube.
50 µl of elution buffer was added carefully to the center of the column.
Incubate for 1 min
It was centrifuged at 13000rpm for 1 min by placing it in a fresh tube.

Restriction Digest (3A as well as Standard assembly) – ITT Madras – 2009
http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Mix contains:
5 µl NEB buffer 2.
0.5 µl BSA
1 µl Forward Enzyme
1 µl Backward Enzyme.
5 µl DNA part
37.5 µl MilliQ water(DNAase free)
Reaction mix is incubated at 37C for 30min
It is then heat inactivated at 80C for 20 min.
Variant
For the digestions, enzymes from NEB were used.
The digestion time can be varied from 15-45 mins depending on the biobrick part. Some parts seem to require more than the others for complete digestion.
The amount of DNA added will alter the amount of water that needs to be added up to make the total reaction volume to 50ul. As much as 42.5ul of DNA can be digested in one reaction (the rest 7.5ul are enzymes and buffers).
In case of the PCR assembly procedure, only a single digest is required (instead of the regular double digest for standard and 3A assembly).
Tip: Before proceeding to the ligation step, chopping off the phosphate ends of the loose ends with alkaline phosphatise in one of the 2 or 3 pieces in the ligation would help in preventing ligation of unneccesary fragments.
Tip: Gel eluting the required product can increase the yield of the ligation product.
Tip: Double digests with S,P or E,X followed by purifying the reaction mix using a PCR column can help in eliminating the tiny fragments that are released into the solution which helps reduce unwanted ligation products during the ligation step.
Warning: Do not digest for too long, else the enzymes might show star activity.

Ligation (3A Assembly) – ITT Madras – 2009
http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Mix contains:
9 µl MilliQ water.

3 µl Upstream Digest.
3 µl Downstream Digest.
2 µl Plasmid backbone.
2 µl Ligase Buffer.
1 µl Ligase.
Mix in incubated at Room Temperature for 15 min.
It is then heat inactivated at 65C for 20 min.
Variant
For the ligations, NEB T4 ligase was used
The incubation time can vary from 10 mins to 1 hour. The reaction went to completion in about 20 mins.
There is not much difference with a 20 min ligation and an overnight 16C ligation.
The amount of water can be reduced to accommodate more DNA in the reaction mix.
Heat inactivation is required as the intact ligase tends to reduce the transformation efficiency.

Ligation (Standard Assembly) – ITT Madras – 2009

http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Mix contains:
12 µl MilliQ water.
3 µl Insert.
2 µl Plasmid backbone.
2 µl Ligase Buffer.
1 µl Ligase.
Mix in incubated at Room Temperature for 15 min.
It is then heat inactivated at 65C for 20 min.
Variant
For the ligations, NEB T4 ligase was used
The incubation time can vary from 10 mins to 1 hour. The reaction went to completion in about 20 mins.
There is not much difference with a 20 min ligation and an overnight 16C ligation.
The amount of water can be reduced to accommodate more DNA in the reaction mix.
Heat inactivation is required as the intact ligase tends to reduce the transformation efficiency.

PCR with Deep Vent Polymerase – ITT Madras – 2009

http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Mix contains:
1 µl Deep Vent polymerase.
5 µl Bffer (10X).
2 µl dNTPs.
1 µl forward Primer.
1 µl backward Primer.
2 µl Template.
38 µl MilliQ water.
Program used:
96C for 2 min.
96C for 30sec.
(Tm-5)C for 30sec.
72C for 'x'min (1min per kb).
GOTO 2 30 times.
72C for 30min.
4C for storage.

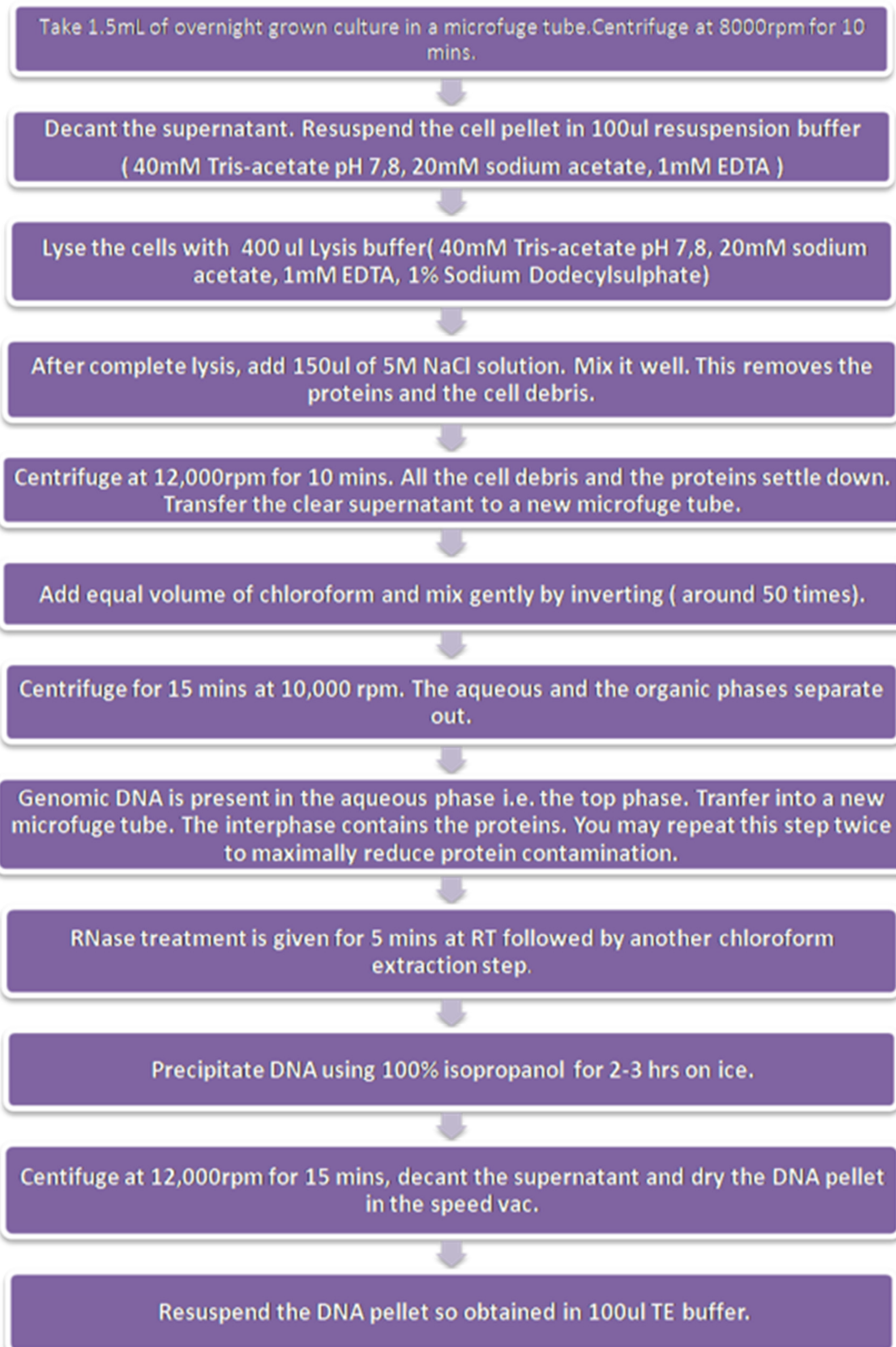
DpnI Digestion – ITT Madras – 2009 http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol

1 µl of DpnI was added to 1 tube of PCR product (<50 µl).
Incubated at 37C for 2hrs.
Heated at 80C for 15-20mins.

PCR with Phusion® Polymerase – ITT Madras – 2009
http://2009.igem.org/Team:IIT_Madras/Notebook/Protocols
Protocol
Mix contains:
10 µl Phusion® HF Buffer (5X).
1 µl 10mM dNTPs.
0.25 µl forward primer (0.5 µM final conc).
0.25 µl reverse primer (0.5 µM final conc).
x µl template (add as needed).
0.5 µl Phusion® DNA Polymerase.
Add MilliQ water to make p 50 µl volume.
Program used:
98C for 2min.
98C for 30sec.
(Tm+3)C for 30sec.
72C for x sec(15sec per kb).
GOTO 2 30 times.
72C for 30 min.
4C for storage.

Maintenance of microbial cultures – IBB Pune – 2009 http://2009.igem.org/Team:IBB_Pune/Protocols
Standard cultures used in our project are
1) *E.coli DH5a*
2) *E.coli JM101*
3) *Acinetobacter baylyi BD413*
4) *Streptococcus pneumoniae R6*
Glycerol stocks of all the strains are maintained at -80oC.
Strains are maintained on working plates for daily use, subcultured every week. Working plates used are Luria Bertani Agar for *E.coli* and *Streptococcus* strains and CLED (Cysteine Lactose Electrolyte Deficient) Agar for *Acinetobacter*sp.

Extraction of Genomic DNA – IBB Pune – 2009 http://2009.igem.org/Team:IBB_Pune/Protocols
Extraction of genomic DNA was done by the Chen and Kuo method with some modifications.



Extraction of Plasmid DNA – IBB Pune – 2009 http://2009.igem.org/Team:IBB_Pune/Protocols

Extraction of plasmid DNA was done by the Birnboim and Doly (1979)method.The principle of the method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed circular DNA remains double-stranded. Adequate pH control is accomplished without using a pH meter. Upon neutralization, chromosomal DNA renatures to form an insoluble clot, leaving plasmid DNA in the supernatant. Large and small plasmid DNAs have been extracted by this method.

Take 1.5mL of overnight grown culture in a microfuge tube.Centrifuge at 8000rpm for 10 mins.

Decant the supernatant. Resuspend the cell pellet gently in 100ul solution I (2 mg/ml lysozyme, 50 mM glucose, 10 mM CDTA, 25 mM Tris-HC1 pH 8.0)

Add 300 ul solution II (0.2 N NaOH, 1% sodium dodecyl sulfate,freshly made). Keep for 5 mins till the suspension becomes clear.

Neutralize with 250ul of solution III (3 M sodium acetate pH 4.8). Mix it gently by inverting.

Centifuge for 10 mins, 10,000rpm.

Add equal volume of chloroform and mix gently by inverting (around 50 times).

Take the supernatant into a new microfuge tube.

Add equal volume phenol:chloroform:isoamyl alcohol (25:24:1) an dmix by inverting gently(around 50 times).

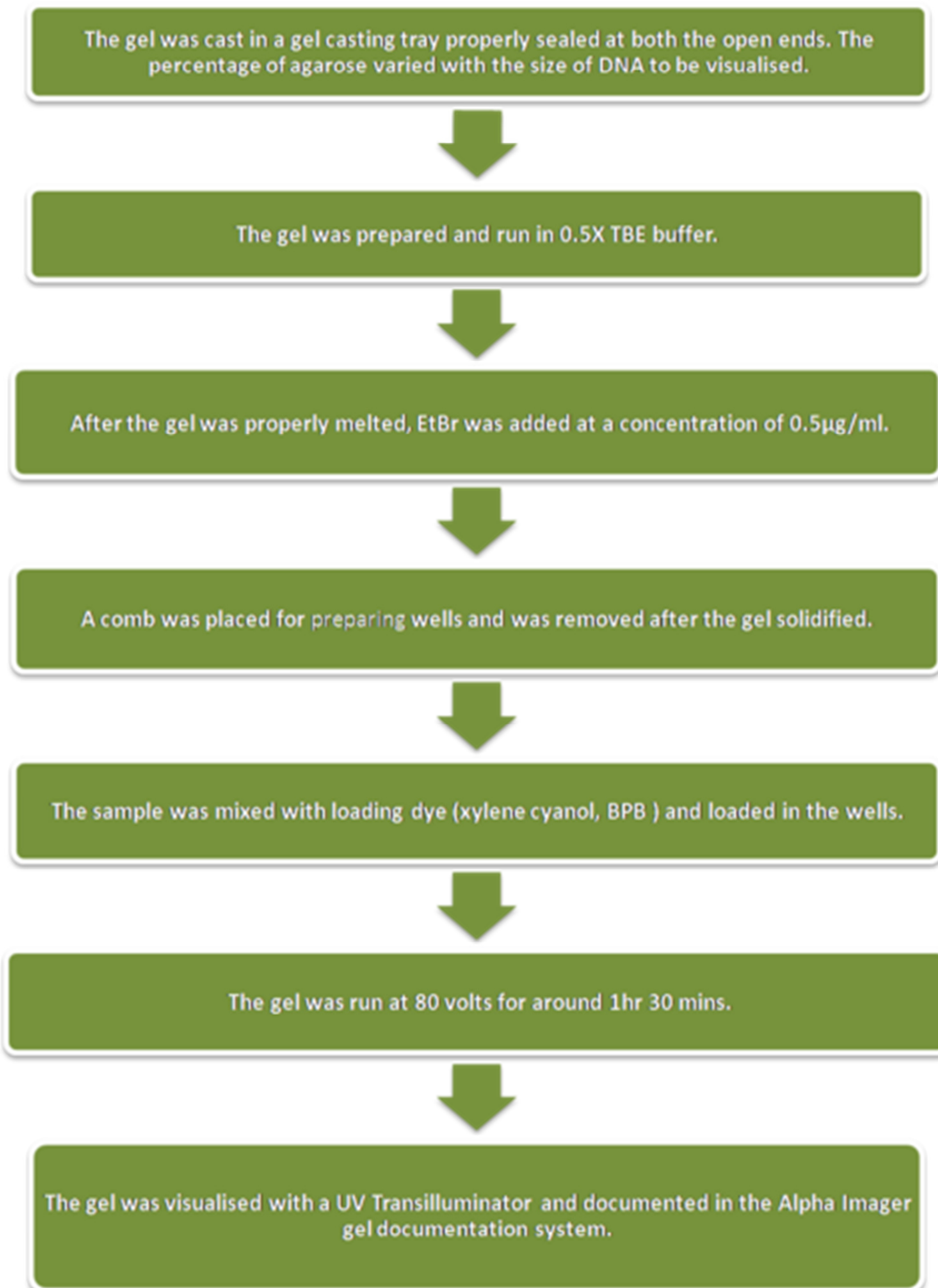
Centrifuge at 12,000rpm for 10 mins. The organic and the aqueous phase separate out. Remove the top aqueous phase into a new mcrofuge tube.

Add equal volume 100% isopropanol for precipitation. Keep for 2-3 hrs on ice.

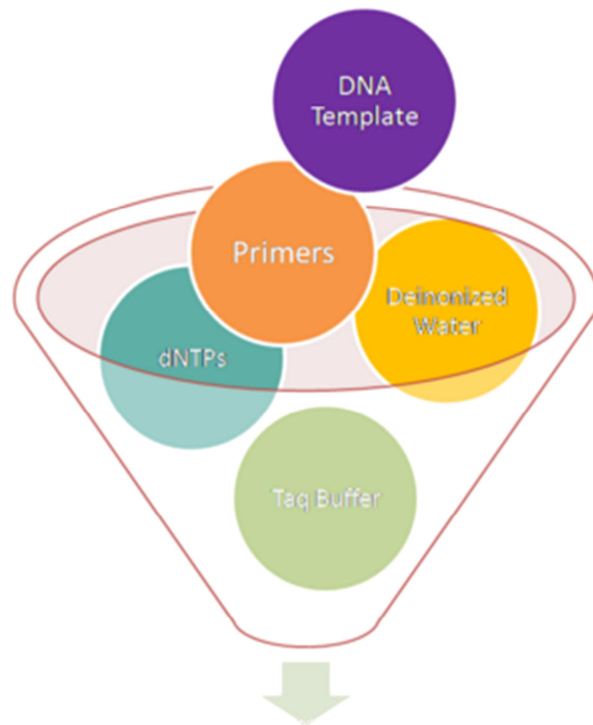
Centrifuge at 12,000rpm for 10 mins and decant the supernatant. Dry the DNA pellet obtained in a speed-vac.

Resuspend the plasmid DNA pellet in 50ul TE buffer.

Agarose Gel Electrophoresis – IBB Pune – 2009 http://2009.igem.org/Team:IBB_Pune/Protocols
Agarose Gel Electrophoresis was performed on a horizontal gel apparatus for visualising DNA. Ethidium Bromide was used as the fluorescing dye. EtBr intercalates with the DNA strands and fluoresces under UV light thereby indicating the position of the band



***in vitro* Gene Amplification – IBB Pune – 2009** http://2009.igem.org/Team:IBB_Pune/Protocols
Genes to be amplified and identified by Polymerase Chain Reaction (PCR).



Ingredients		Test Volume
DNA template		4ul
Taq Polymerase Buffer (10X, with 15mM MgCl2)		2.5ul
Mixed Nucleotides (10mM,2.5mM each)		2ul
Primers	Forward	2ul
	Reverse	2ul
Taq polymerase		1 unit
Deionised water		10.5

Fluorescence microscopy visualization – HKUST – 2009
http://2009.igem.org/Team:HKUST/Protocols/GFP_testing
Procedure:
1. Grow yeast transformed cells containing appropriate plasmids in 5 ml SCM minus His & glucose at 30ºC overnight.
2. Add galactose (to a conc.of 2%) at a cell density corresponding to an OD600 of a 10-fold dilution reaching 0.8.
3. Continue growing cells at 30ºC for 30min to 1.5hr.

4. At time 30min, 60min, 90min, use 1ml of cells, spin down and resuspend cells in 10µl ddH2O.
5. Use 2µl cells and visualize the cells in a fluorescency microscopy.

Western Blotting – HKUST – 2009 http://2009.igem.org/Team:HKUST/Protocols/Western_blotting
Procedure:
1. Yeast transformed cells containing appropriate plasmids were grown in SCM with appropriate amino acids deleted and harvested at a cell density corresponding to an OD600 of a 10-fold dilution reaching 0.2.
2. 2× Laemmli's buffer (20% v/v glycerol, 0.1M Tris-HCl pH 6.9, 4% SDS, 0.2% bromophenol blue, 10% mercaptoethanol) was added and yeast crude extracts were prepared by glass bead lysis and separated on 10% SDS-PAGE.
3. The 10% resolving gel and the 5% stacking gel were cast according to the method described in Molecular Cloning (Sambrook et al., 1989).Separation was performed on a Mini Protean 3 gel and transblot system (Biorad). Proteins were stacked at 60-80 volts in the stacking gel and resolved at 120-150 volts in the resolving gel.
4. The proteins were then transferred onto a nitrocellulose membrane (pore size 0.45 µm, Schleicher &Schuell) in electrophoretic blotting system (C.B.S. Scientific) by applying a constant current of 0.5 mA for 1.5 hours.
5. After transferring, the membrane was taken out and socked in Ponceau S solution (0.5% Ponceau S in 1% HAc) for 1 min. After rinsed with ddH2O and labeled with protein marker bands.
6. The membrane was destained with TBST buffer (10 mM Tris-HCl pH8.0, 150 mM NaCl, 0.05% v/v Tween-20) and blocked with 5% milk in TBST for 30 min.
7. Then the membrane was incubated with the primary antibody (mouse monoclonal antibody against Flag) for 1 hour at room temperature followed by 3 × 15 min washing with TBST.
8. The membrane was then incubated with secondary antibody (goat anti-mouse antibody conjugated with horseradish peroxidase) for 1 hour at room temperature followed by 3 × 15 min washing with TBST.
9. After that, the membrane was incubated in SuperSignal chemiluminescence substrate (Pierce) for 5 min and exposed to light-sensitive films (Fuji). In the end, the film was developed in a film-processing machine (Eastman Kodak).

Yeast genomic DNA extraction – HKUST – 2009
http://2009.igem.org/Team:HKUST/Protocols/Yeast_genomic_DNA_extraction
Purpose: To extract yeast genomic DNA from yeast strain (strain YPH501, in our case).
Materials: yeast strain, lysis buffer, phenol, chloroform, 3M NaAc, 100% ethanol, 70% ethanol, ddH2O, glass beads.

Procedure:
a.Add 200µL lysis buffer.
b.Pick a whole patch of colony and add it in the tube.
c.Add 100µL phenol, and mix it well.
d.Add 100µL chloroform, and a few glass beads.
e.Vortex for 4-5mins and centrifuge for 5mins (13,000 rpm).
f.Put the supernatant (~160µL) to another tube.
g.Add 200µL phenol/chloroform (1:1 in volume)
h.Vortex for 4-5mins and centrifuge for 5mins (13,000 rpm).
i.Put the supernatant (~120µL) to another tube.
j.Add 0.1 volume NaAc(pH5.2), approximately 12µL.
k.Add 2 volume of 100% ethanol, approximately 240µL.

l.Store it at -20 °C for 20mins.
m.Centrifuge for 10mins (13,000 rpm) and remove the supernatant.
n.Add 2 volume of 70% ethanol.
o.Centrifuge for 5mins (13,000 rpm) and remove the supernatant.
p.Stand the tube without closing it for a while to let the ethanol evaporate.
q.Add 20µL ddH2O to resuspend it.
r.Store it at -20 °C.

Safety tips:
Phenol is a strong toxin, make sure to wear gloves and be careful with this chemical.

Cell lysis – HKUST – 2009 http://2009.igem.org/Team:HKUST/Protocols/Cell_lysis
Purpose: to lyse E.coli cell to get linearized ligated plasmid.
Materials: cultured transformed E.coli colony, phenol, chloroform, ddH2O

Procedure:
a.Add 40µL ddH2O in a 1.5mL microcentrigfuge tube.
b.Use a toothpick to pick a colony and dissolve it in the water.
c.Add 20µL phenol and 20µL chloroform.
d.Scrape the tube to mix the solution. A well mixed white solution can be seen after this step.
e.Centrifuge it for 10mins (13,000 rpm).
f.Place the supernatant in another tube.
g.Load 10µL to test the result by gel electrophoresis.

Tips:
Ignite the fire for sterile environment when transforming the colony.

Safety tips:
1. Phenol is a strong toxin, make sure to wear gloves and be careful with this chemical.
2. Be careful with the fire, and extinguish it after use.

Transformation to E.coli – HKUST - 2009
http://2009.igem.org/Team:HKUST/Protocols/Transformation_to_yeast
Purpose: To transform desired plasmid DNA in to E.coli in order to amplify it.
Materials: E.coli competent cell, desired plasmid DNA, LB media, agar plate with desired selection antibiotic (ampicillin)

Procedure:
a.Perform ligation or any other manipulation of DNA to yield circularized plasmids.
b.Remove bacterial aliquots (usually the DH5 strain of E.coli) from -70°C freezer and thaw on ice for 10-15 min; briefly flick the tube to resuspend the bacterial cells, but minimize any time the cells are not on ice.
c.Add desired amount of plasmid DNA (usually 1-5µL of a standard ligation or 1.0ng of purified plasmid) to the bacteria at the bottom of the tube, mix it with pipette and incubate 5min on ice.
d.Remove the tube from ice and immediately begin heat shock in a water bath at 42°C for 45 sec.

e.After heat shock, immediately add 500µL of room temperature LB media to the bacterial cells, then incubate in a 37 °C shaker incubator for 45mins.
f.Plate 50µL of the bacterial culture to a LB/agar plate with desired selection antibiotic (ampicillin) using a sterile bacterial streaking rod, and allow the plate to absorb the extra liquid for 15 min on the benchtop at room temperature.
g.Invert the plates and place in the 37°C bacterial incubator overnight.

Tips:
A. The competent cell is quite fragile, so make sure that it does not leave the ice before heat shocking.
B. LB is easily contaminated, make sure to sterile it with fire each time when opening or closing it. Add it quickly to minimize the time of leave it open.
C. Ignite the fire for sterile environment when adding LB.

Safety tips:
Be careful with the fire, and extinguish it after use.

Transformation to yeast – HKUST – 2009
http://2009.igem.org/Team:HKUST/Protocols/Transformation_to_Ecoli
Purpose: To transform desired constructed plamid DNA to yeast to test its effect.
Materials: LiOAc solution, PEG4000 solution, DMSO (dimethyl sulfoxide), yeast strain, desired plasmid, YEPD, SD media with specific selection marker absent (-Leu, -His, or -Ura), ddH2O

Procedure:
a.A stationary culture of yeast grown in YEPD is used to inoculate 10mL of YEPD in a 100mL Pyrex flask.
b.Cells are grown at 30 °C with shaking (200 rpm) until a density of 1-4x107 is reached (OD660=0.4).
c.Yeast is transformed into 4 sterile 1.5mL tubes and are centrifuged for 2mins (4,000 rpm).
d.Damp off media and wash the pellet with 100µL ddH2O (to culture 1:1). Resuspend the cells by gently shake it or flip it.
e.Centrifuge it again for 2mins (4,000 rpm). Remove the supernatant.
f.Wash the pellet with 1mL LiOAc solution and pour it into a single tube. Resuspend the cell by gently shake it or flip it.
g.Repeat step e and f once.
h.Add 100µL of yeast suspension to a 1.5mL microcentrifuge tube, and add 10µL of DNA to be transformed. Mix them gently and stand the tube at room temperature for 5mins.
i.Add 280µL PEG4000 solution. Mix it gently by inverting 4-6 times, then the tube is stored at 30 °C for 45mins without shaking.
j.Add 43µL DMSO to give an approximate 10% (volume percentage) DMSO solution. Mix the solution gently by inverting.
k.Heat shock at 42°C for 5mins.
l.Centrifuge it for time long enough to get pellet (usually 5 sec) at 12,000rpm.
m.Remove the supernatant and wash it with ddH2O.
n.Centrifuge it again for 50sec (13,000 rpm). Remove the supernatant.
o.Resuspend the cell with 1mL ddH2O.
p.Plate the solution on SD media with specific selection marker absent.

Tips:

Ignite the fire for sterile environment when transforming the yeast cell, since there is no any antibiotic in the culture.br>
Safety tips:
Be careful with the fire, and extinguish it after use.

Ligation – HKUST – 2009 http://2009.igem.org/Team:HKUST/Protocols/Ligation
Purpose: To ligate desired insert DNA fragment into vector with specific restriction site.
Materials: digested insert DNA and plasmid DNA with the same restriction site, T4 DNA ligase, 10X T4 Ligation Buffer, CIP (Calf Intestinal Alkaline Phosphatase), ddH2O

Procedure:
a.Add sufficient water to bring to a total volume of 10 µL or 20 µL, depending on the volume of the two DNA pieces.
b.Add 50ng of vector DNA.
c.Add enough amount of insert DNA, which can be calculated by ligation calculator online.
d.Add 2µL ligation buffer and 0.5µL ligase.
e.Incubate at 16 °C overnight or at room temperature for 8 hours.
Tips:
A. Ligase blank control group is recommended.
B. The plasmid DNA and insert DNA for ligation must be added with CIP when enzyme digestion.

Ethanol precipitation – HKUST – 2009
http://2009.igem.org/Team:HKUST/Protocols/Ethanol_precipitation
Purpose: To concentrate DNA product.
Materials: 100% ethanol, 75% ethanol, 3M NaAc, ddH2O, DNA product

Procedure:
a.Add 2 volume of 100% ethanol.
b.Add 0.1 volume of 3M NaAc.
c.Put the tube in fridge at -20 °C for 10 minutes.
d.Put it out and centrifuge it for 10-15 minutes (13,000 rpm). Remove the supernatant.
e.Wash with 100µL 75% ethanol.
f.Centrifuge for 5 minutes (13,000 rpm). Remove all the supernatant.
g.Add 10µL ddH2O to resuspend DNA.

Tips:
A.If the volume of DNA product is too low, make it to higher volume with ddH2O. The recommended lowest volume is 50µL. Combine several tubes of DNA product is also suggested for higher DNA product concentration.
B. In step f, make sure to remove all the supernatant without touching the pellet. If it is too hard to do so, open the tube and leave it at room temperature for a while to make the ethanol evaporate.

gel_extraction – HKUST – 2009 http://2009.igem.org/Team:HKUST/Protocols/gel_extraction
Gel extraction
Purpose: To clean up the gel after band cutting from the gel and leave only the desired DNA fragment.

Materials: GEX Buffer, WN Buffer, WS Buffer, Elution Buffer (all provided in FavorPrepTM Gel/PCR DNA Clean-Up Kit)

Procedure:
a.Add 0.5ml GEX buffer into the tube with gel fragment in it.
b.Incubate at 60°C for 5-10mins until the gel is completely dissolved. Invert the tube every 2 mins during the incubation. Stop incubation after the gel is completely dissolved. Let the gel mixture to cool down to room temperature.
c.Place a GP column onto a collection tube. Load no more than 0.7ml gel mixture into each column. Centrifuge for 30s-60s (9000rpm). Discard the flow through.
d.Repeat step c for the excessive gel mixture.
e.Wash the column once with 0.5ml WN buffer by centrifuging for 30s-60s (9000rpm). Discard the flow through.
f.Wash the column once with 0.5ml WS buffer by centrifuging for 30s-60s (9000rpm).
g.Centrifuge for another 3 mins (13200rpm) to remove all the ethanol
h.Place the column onto a new 1.5ml centrifuge tube. Add 15μL -30μL elution buffer onto the center of the membrane. (Preheated the Elution buffer to 60°C).
i.Stand the column for another 3mins and then centrifuge at full speed for 1-2 mins to elute DNA.

Tips:
The gel extraction has a low recovery rate of about 10%. Stand the column longer in step i may give relatively higher recovery rate.

gel_cut – HKUST – 2009 http://2009.igem.org/Team:HKUST/Protocols/gel_cut
Cutting bands on the gel
Purpose: To extract specific DNA fragment.
Procedure:
a.Visualize the bands under UV light. Use long-wavelength UV to minimize damage to the DNA.
b.Cut the band with a clean razor blade.
c.Turn the gel slice on its side to trim off extra agarose. Place the gel in a microcentrifuge tube.
Tips:
EB staining is needed for higher resolution under UV light.

Safety tips:
1. ermission is needed from technician before doing this! Follow the safety instruction in the room.

2.When using UV light, protect your skin by wearing safety goggles or a face shield, gloves, and a lab coat.

plasmid_extraction – HKUST - 2009 http://2009.igem.org/Team:HKUST/Protocols/plasmid_extraction
Plamid DNA extraction
Purpose: To extract plasmid DNA from E.coli.
Materials: MX1 Buffer, MX2 Buffer, MX3 Buffer, WN Buffer, WS Buffer, Elution Buffer (all provided in Mini Plus Plasmid DNA Extraction System)

Procedure:

a.Grow 1 to 5 ml plasmid-containing bacterial cells in LB medium with appropriate anlibiotic overnight (12-16 hours) with vigorous agitation.
b. Pellet the cells by centrifuging for 1-2 minutes. Decant the supernatant and remove all medium residues by pipette.
c.Add 200 µL of MX1 Buffer 10 the pellet, and resuspend the cells completely by vortexing or pipetting.
d.Add 250 µL of MX2 Buffer and gently mix well (invert the tube 6-10 times) to lyse the cells until the lysate becomes d ear. Incubate at room temperature for 2-5 minutes.
e.Add 350 µL of MX3 Buffer to neutralize the lysate, then immediately and gently mix the solution well. A white precipitate should be formed.
f.Centrifuge at 10,000 x g (13,000rpm) for 5-10 minutes, meanwhile place a Mini Plus™ Column onto a Collection Tube. Transfer the supernatant carefully into the column.
g.Centrifuge at 7,000x g (9,000rpm) for 30-60 seconds. Discard the flow-through.
h.Wash the column once with 0.5 ml of WN Buffer by centrifuging at 7,000 x g (9,000rpm) for 30-60 seconds. Discard the flow-through.
i.Wash the column once with 0.7 ml of WS Buffer by centrifuging at 7,000 x g (9,000rpm) for 30-60 seconds. Discard the flow-through.
j.Centrifuge the column at 10,000 x g (13,000rpm) for another 3 minutes to remove residual ethanol.
k.Place the column into a new 1.5-ml centrifuge tube. Add 50 µL of Elution Buffer onto the center of the membrane.
l.Stand the column for 2-3 minutes, and centrifuge at 10,000 x g (13,000rpm) for 2-3 minutes to elute DNA. Store plasmid DNA at 4 °C or -20 °C.
Tips:
A. In step c, no clump should be visible after resuspension. Clumped cells lead to bad plasmid yield and quality.
B. Do not vortex in step d, otherwise genomic DNA will be sheared and contaminate the sample, which can be observed as serious foaming.
C. In step d, the lysate should become clear and viscous. Insufficient cell-lysis leads to low plasmid yield and quality.
D. MX1 Buffer must be stored at 4 °C.

Enzyme_digestion - HKUST - 2009 http://2009.igem.org/Team:HKUST/Protocols/Enzyme_digestion
Enzyme digestion (vector, insert)
Purpose: To cut the sequence with specific restriction enzyme
Materials: substrate DNA, restriction enzymes, corresponding 10X buffer, ddH2O, CIP (Calf Intestinal Alkaline Phosphatase, for vector digestion and DNA to be ligated)

Procedure:
a.Add sufficient water to make it a 20 µL reaction.
b.Add 1 µg DNA, 2 µL buffer in total, 0.5 µL each restriction enzyme.
c.Vortex and then spin down.
d.Keep it in 37 °C incubator for one hour. (If needed, incubate for 1.5-2hours)
e.If the substrate DNA is vector or pieces to be ligated, add 0.5 ul (for 20ul reaction) CIP after incubated for 1.5-2 hours, and then incubate at 37 °C for another 30mins.
f.Check the result by gel electrophoresis.

Tips:
A. Restriction enzymes are easy to be denatured, so do not leave it at room temperature.
B. If two restriction enzymes must be added with different buffer, digest the DNA with respective restriction enzyme sequentially. Incubate for one hour after each enzyme added.

PCR_cleanup – HKUST - 2009 http://2009.igem.org/Team:HKUST/Protocols/PCR_cleanup
PCR product clean-up
Purpose: To clean up primers, dNTPs, enzymes, short-failed PCR products in the PCR reaction.
Materials: FAPC Buffer, Wash Buffer, Elution Buffer, PCR Product (all provided in FavorPrepTM PCR Clean-Up Kit)

Procedure:
a.Transfer 30µL of PCR product and add 5 volumes of FAPC buffer to a 1.5mL microcentrifuge tube, then mix well by vortexing.
b.Place a FAPC Column into a Collection Tube and transfer the sample mixture to FAPC Column.
c.Centrifuge at 6,000 rpm for1 min then discard the flow-through.
d.Add 750 µl of Wash Buffer (ethanol added) to FAPC Column. Centrifuge at 6,000 rpm for 1 min, then discard the flow-through.
e.Centrifuge at 14,000 rpm for an additional 3 min to dry the column.
f.Place FAPC Column into an Elution Tube.
g.Add 40 µl of Elution Buffer or ddH2O (pH 7.0~8.5) to the membrane center of FAPC Column. Stand FAPC Column for 2 minutes.
h.Centrifuge at 14,000 rpm for 1 min to elute the DNA.

Tips:
In step f, for effective elution, make sure that the elution solution is dispensed onto the membrane center and is absorbed completely.

PCR – HKUST – 2009 http://2009.igem.org/Team:HKUST/Protocols/PCR
Purpose: To amplify a specific piece of DNA out from the whole.
Materials: ddH2O, 10X ThermoPol Buffer, 1mM dNTP, Forward primer, Reverse Primer, Taq or Vent DNA polymerase, DNA template Choice of DNA polymerase: Taq gives higher rate of mutation compared with Vent. Taq gives sticky ends after PCR, while Vent gives blunt ends.

Procedure:
1. Add 10 µL water to make it a 20 µL reaction.
2. Add 2 µL buffer, 4 µL dNTP, 1 µL forward primer (5 µM), 1 µL reverse primer (5 µM), 1 µL DNA template, 1 µL DNA polymerase
3. Vortex and then spin down.
4. Put it into the PCR machine and set the program.
Program
(1) Initial denaturation 95 °C 4 mins
(2) Run 25-30 cycles of:
Denaturation 95 °C 30 secs
Annealing 30 secs
Temperature is depended on melting temperature of primer.
Lower Tm-5°C, for Taq; Lower Tm +3°C for Vent
Extension 72 °C 30 secs per 500bp PCR product length
(3) Final extension 72 °C 3~5 mins

Tips:
The DNA polymerase is easy to be denatured, so do not leave it at room temperature. It is highly recommended to use an ice holder or do the adding in the fridge.

Agarose gel preparation and gel electrophoresis – HKUST – 2009
http://2009.igem.org/Team:HKUST/Protocols/Agarose_gel
Purpose: To check the result
Materials: Agarose, 0.5X TBE, SYBR safe DNA gel stain, DNA loading dye, DNA ladder/marker

Choice of ladders: VC100bp Plus DNA ladder (highest band 3000bp); Lamda DNA BstEII marker (highest band 8400bp, 1% argarose)

Procedure:
1. To make a 0.8% agarose gel, use 0.16 g agarose per 20 mL 0.5x TBE.
2. Cover the flask with plastic wrap to prevent boiling over, then microwave the solution for 1-2 minutes to dissolve the agarose.
3. Let agarose solution cool for 5 minutes, then add SYBR safe DNA gel stain to a final concentration of 0.5 μg/mL.
4. Pour the agarose solution into a casting tray with well comb in place. Allow 20-30 minutes to completely solidify.
5. Place the agarose gel into the gel box and fill the box with 0.5X TBE until the gel is immersed.
6. Load an appropriate molecular weight ladder into the first lane of the gel. If needed, an additional ladder with different molecular weight could be loaded into the last lane.
7. Add 1 μL loading dye per 5 μL of sample.
8. Load the samples from left to right.
9. Cover the gel box and plug in the electrodes in a way that the samples will run towards the positive, red electrode.
10. Run the gel at 100V until the dye line is approximately 50-75% of the way down the gel. Approximately 20-30 minutes.
11. Carefully remove the gel from the gel box and check the result under UV exposure.

Tips:
A. Higher concentration of agarose solution makes better resolution for less molecular weight expected band.
B. Let bottom of the flask be immersed in a cup of cold water for faster cooling.
C. In order for clearer band to cut specific band on the gel, immerse the gel in Ethidium bromide for 10-15 minutes after step k.

Safety tips:
1. Be sure to wear a glove before treating the hot flask.

2. Ethidium bromide is a strong mutagen. Wear a lab coat, eye protection, and gloves when working with this chemical.

Bacteria Lysis – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols
Harvest the culture by centrifuge (13krpm*1.5min) followed by washing with 1ml PBS twice. Later steps should be done on the ice.
Re-suspend the pellet with PBS (five times volume of the pellet) and protease inhibitor cocktail.
Sonication for (5.5seconds+1second pulse)*10 minutes and protein solutions are obtained.
BCA Quantification Analysis – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols

Prepare a working solution of BCA reagent just prior to use by adding BCA Reagent A and BCA Reagent B with a ratio of 50:1. Mix the two solutions until a clear green solution forms. Prepare the BCA working reagent fresh daily. 100uL is required for each sample.
For each protein determination, add 2~5uL protein sample and 100uL BCA mixture into each well. Usually each sample will be loaded into 3 wells to reduce errors.
Put the 96-well plate in the warm room for about 30 minutes.
Read the wells in a suitable plate reader (e.g. Molecular Devices) at the wavelength of 562 nm.

Competent Cell Preparation for Electro Transformation – HKU-HKBU – 2009
http://2009.igem.org/Team:HKU-HKBU/Protocols

Materials
Media: LB (Both liquid media and media containing agar. Add certain antibiotic if necessary.)
Buffers and Solutions: 10% Glycerol.
Special Equipments : EP tubes (1.5mL), micropipette tips, centrifugation bottles (polypropylene tubes, 50mL), graduated flask (250mL*2, 5mL*1), plates and test tubes.
Steps
Sterilization
Including all materials in materials section.
Caution (Remind): use some special marks to distinguish the sterilized materials from the unsterilized ones.
Preparation after sterilization
Chill the 10%Glycerin to 4°C.
Decant LB containing agar into the plates.(If antibiotics are necessary, be sure that they are added when the media temperature is below 60°C.)
Streak the prepared strains onto the agar plates. Then incubate it at 37°C for 10-16 hours.
Pick up one single colony from the plate and pre-culture it overnight
Take 0.5mL overnight culture to 50mL LB bottle.
37 centigrade degree shaking 100~120 min to O.D. (wavelength 600) 0.45~0.6
On ice for 30min
4000rpm, 7min at 4 centigrade degree
Add origin volume 10% glycerol, suspend softly.
Repeat the step 7,8
Add origin volume 1/10 10% glycerol, suspend softly.
4000rpm, 7 min at 4 centigrade degree
Add origin volume 1/100 10% glycerol, suspend softly, store at -80°C.

Electro Transformation – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols

Hold competent cells (from -80 centigrade degree refrigerator) on ice.
Gently mix ligation product (1-5 µL) with cells.
Transfer the cell/DNA mix into an electroporation cuvette. (Note: the gene pulser should already be set properly)
Time constant = 4.5 - 5.0 ms
Resistance = 200 W
Capacitance = 25 mFD for 0.1 cm gap cuvettes, set the volts to 1.8 kV
Pulse the cells once; the voltage display blinks, and the gene pulser beeps
Quickly transfer 37°C SOC to cuvette, mix by gently pipetting up and down, and transfer SOC/cells back to culture tube.
Bath in 37°C for 30~60 min.
Separate cells on petri-dishes, and cultivate them in 37°C for 12-16 hours.

Ligation – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols

England Biolabs T4 DNA ligases are used here.
Choose reaction volume: 5-10 µL
Mix proper proportion (usually 3~5:1) of DNA fragment and vector.

Add 10 × ligase buffers.
Add 0.5 µL ligase per 10 µL final volume.
Bath in 16 centigrade degree water for 12 hour,
Begin transformation.

Membrane Biotinylation – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols

Activate the membrane with methanol for 10-30seconds
Balance the membrane in PBS for 5 minutes
Place the membrane on a piece of filter paper.
Drop the protein-biotin complex onto the membrane.
Air-dry for 5 minutes.
Soak in methanol for 1minutes.
Place the membrane on a piece of filter paper.
Air dry for 15 minutes.

Pre-culture

One single colony is picked up from the agar plate and transferred to a tube.
Add 3~5mL LB broth to the tube and the specific resistance.
Culture overnight at 32 centigrade degree or 37°C.

Photolithography – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols

The motors are fabricated by using a general surface manufacturing process for Si and SiO_2. The motor bodies are made by reactive ion etching of thermal SiO_2 layer on a Si substrate with a mask. Then the Si under the motors is anisotropically etched by wet etching, so that the motors are tethered to the Si base by two thin bridges designed to break upon sonication.

Photolithography is an optical means for transferring patterns onto a substrate. It is essentially the same process that is used in lithographic printing. Patterns are first transferred to an imagable photoresist layer. Photoresist is a liquid film that can be spread out onto a substrate, exposed with a desired pattern, and developed into a selectively placed layer for subsequent processing.

Overview of the photolithography process

Surface Preparation
Typical contaminants that must be removed prior to photoresist coating:
dust from scribing or cleaving (minimized by laser scribing)
atmospheric dust (minimized by good clean room practice)
abrasive particles (from lapping or CMP)
lint from wipers (minimized by using lint-free wipers)
photoresist residue from previous photolithography (minimized by performing oxygen plasma ashing)
bacteria (minimized by good DI water system)
films from other sources:
solvent residue
H_2O residue
photoresist or developer residue
oil

silicone
Wafer priming: Adhesion promoters are used to assist resist coating.
Resist adhesion factors:
moisture content on surface
wetting characteristics of resist
type of primer
delay in exposure and prebake
resist chemistry
surface smoothness
stress from coating process
surface contamination
Wafer primers
Used for silicon:
primers form bonds with surface and produce a polar (electrostatic) surface
most are based upon siloxane linkages (Si-O-Si)
1,1,1,3,3,3-hexamethyldisilazane (HMDS), $(CH_3)_3SiNHSi(CH_3)_3$
trichlorophenylsilane (TCPS), $C_6H_5SiCl_3$
bistrimethylsilylacetamide (BSA), $(CH_3)_3SiNCH_3COSi(CH_3)_3$
Used for gallium arsenide:
GaAs already has a polar surface
monazoline C
trichlorobenzene
xylene
Coating (Spin Casting)
Wafer is held on a spinner chuck by vacuum and resist is coated to uniform thickness by spin coating. Resist thickness is set by primarily resist viscosity and secondarily spinner rotational speed. Most resist thicknesses are 1-2 mm for commercial Si processes.

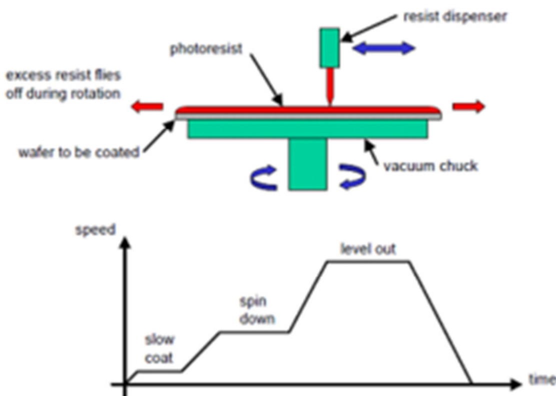


Fig 1 Photoresist Spin Coating

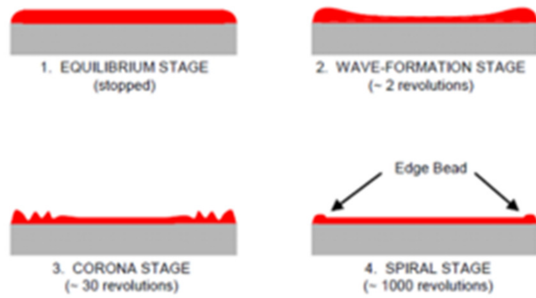


Fig 2 Stages of Resist Coating

Pre-Bake (Soft Bake)

Pre-bake is used to evaporate the coating solvent and to densify the resist after spin coating. Commercially, microwave heating or IR lamps are also used in production lines. Hot plating the resist is usually faster, more controllable, and does not trap solvent like convection oven baking.

Fig 3 Soft Bake

Alignment

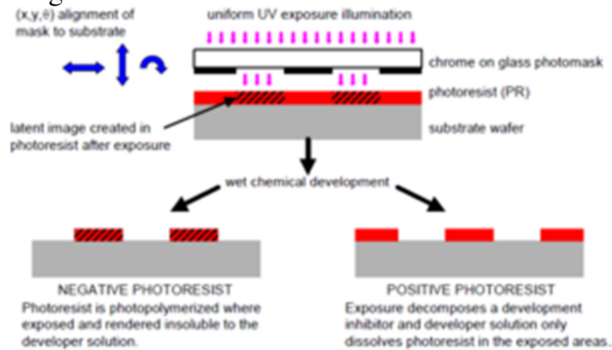


Fig 4 Overview of Align/Expose/Develop Steps

Exposure

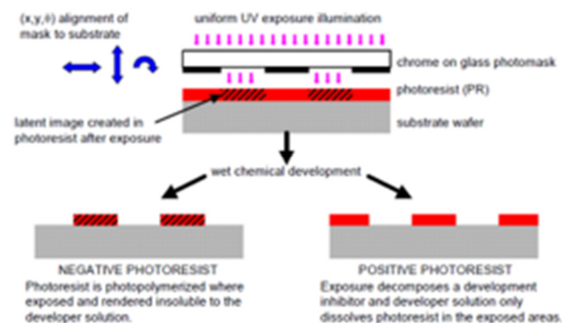


Fig 5 Exposure ("printing") systems

For simple contact, proximity, and projection systems, the mask is the same size and scale as the printed wafer pattern. I.e. the reproduction ratio is 1:1.

Projection systems give the ability to change the reproduction ratio. Going to 10:1 reduction allows larger size patterns on the mask, which is more robust to mask defects.
Mask size can get unwieldy for large wafers.
Most wafers contain an array of the same pattern, so only one cell of the array is needed on the mask. This system is called Direct Step on Wafer (DSW). These machines are also called "Steppers".
Development
Types:
photographic emulsion on soda lime glass (cheapest)
Fe_2O_3 on soda lime glass
Cr on soda lime glass
Cr on quartz glass (most expensive, needed for deep UV litho)
Dimensions:
4" x 4" x 0.060" for 3-inch wafers
5" x 5" x 0.060" for 4-inch wafers
Polarity:
"light-field" = mostly clear, drawn feature = opaque
"dark-field" = mostly opaque, drawn feature = clear
Normally, this process requires at least two alignment mark sets on opposite sides of wafer or stepped region.
Post-Bake (Hard Bake)
Post bake is used to stabilize and harden the developed photoresist prior to processing steps that the resist will mask. The main parameter of this process is the plastic flow or glass transition temperature. It can remove any remaining traces of the coating solvent or developer. Also, it eliminates the solvent burst effects in vacuum processing. Post-bake introduces some stress into the photoresist and some shrinkage of the photoresist may occur. Longer or hotter post-bake makes resist removal much more difficult.
Processing Using the Photoresist as a Masking Film
Photoresist will undergo plastic flow with sufficient time and/or temperature.
Stripping
This process is to remove the photoresist and any of its residues. Simple solvents are generally sufficient for non-postbaked photoresists. There are two kinds of photoresist, i.e. positive photoresist and negative photoresist. Besides, plasma etching with O_2 (ashing) is also effective for removing organic polymer debris.
Post Processing Cleaning (Ashing)

Light Sources

Ultraviolet light from gas-discharge lamps
"deep ultraviolet", produced by excimer lasers
Immersion lithography with numerical apertures
High-index immersion lithography (193nm)

Basics of Photolithography for Processing

Additive -> deposition
Subtractive -> etching
Modifying -> doping, annealing, or curing

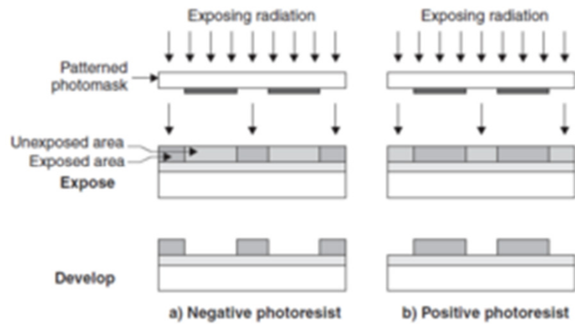


Fig 6 Microfabrication Processes

Two primary techniques for patterning additive and subtractive processes

Etch-back:

photoresist is applied overtop of the layer to be patterned

unwanted material is etched away

Lift-off:

patterned layer is deposited over top of the photoresist

unwanted material is lifted off when resist is removed

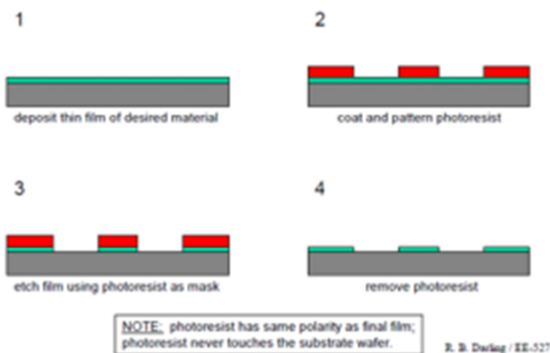


Fig 7 Etch-back

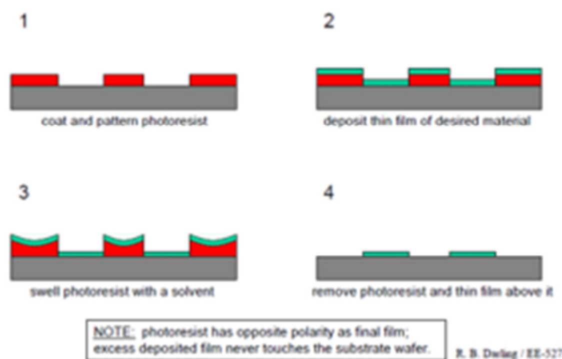


Fig 8 Lift-off

Recombineering – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols

Overnight cultures: 5mL medium (containing antibiotic where applicable) from single colonies grown at 32°C for 18 h.

Get 500uL from overnight cultures expanded into 50 ml of L medium in a 250 ml Erlenmeyer flask, and incubated at 32°C for 2h (until OD600 of ca. 0.4–0.6).

Flasks were transferred to a shaking water bath at 42°C and incubated for 14–15 min, before cooling to 0°C as rapidly as possible in iced water.
After 15–20 min, cells were harvested by centrifugation at 0°C (4000 g, 9 min).
Cell pellets were carefully washed three times with sterilized ice-cold water (2 × 50 ml, then 1 × 1.5 ml) then re-suspended in 100–200 µl of ice-cold water.
Competent cells (50 µl) were transformed with 50–200 ng of (gel purified) linear dsDNA targeting cassette using a BioRad electroporator (1.8 kV, 25 mF, 200 W).
The LB medium (1 ml) was added to the transformed cell mixture, which was incubated at 32°C, for 2 h.
Cells were collected by centrifugation, ca. 900 µl of supernatant media was discarded, and then the re-suspended cells were plated onto LB agar containing the appropriate antibiotic to select for resistant colonies. Reference to Watt et al [1].
SDS-PAGE and Western Blotting – HKU-HKBU – 2009 http://2009.igem.org/Team:HKU-HKBU/Protocols
Buffer preparation
Rususpension buffer: 1ml stock + 0.1ml protease inhibitor (10x)
Loading buffer: 1ml stock + 0.2 ml DTT + Phenol Blue
Sample treatment
Centrifuge the culture into pellet (13k rpm 10min)
Resuspend the pellet with 5xVolume(pellet) Resuspension buffer
Add 5xVolume(pellet) loading buffer
Boil the resuspend for 10 minutes at 100'C
Centrifuge for 1 min at 13krpm
SDS-PAGE
15% separation gel
5% stacking gel
Load the sample and run under the constant voltage of 100V.
Visualize your proteins using Coomassie Brilliant Blue, Silver stain, or any of the other protein stains or blot the gel for western blotting.
Western blotting
Transfer from gel to membrane
Assemble "sandwich" Transblot.
Prewet the sponges, filter papers (slightly bigger than gel) in 1x Blotting buffer.
Transfer for 1 hr at 1 amp at 4°C on a stir plate. Bigger proteins might take longer to transfer. For the Mini-Transblot, it's 100 V for 1 hr with the cold pack and prechilled buffer. When finished, immerse membrane in Blocking buffer and block overnight.
Hybridization with antibodies
Incubate with primary antibody diluted in Blocking buffer for 60 min at room temp.
Wash 3 x 10 min with 0.05% Tween 20 in PBS.
Incubate with secondary antibody diluted in PBS for 45 min at room temp.
Wash 3 x 10 min with 0.05% Tween 20 in PBS.
Detect with SUPER SIGNAL WEST PICO Kit (1ml luminol solution + 1ml stable peroxide solution
Result analysis
The size of the tagged protein can be determined by the marker (protein ladder)
The amount of the protein can be estimated by the brightness of the band, or accurately analyzed by the software.
Trouble shooting
Unspecific binding : 1st antibody-membrane and irrelevant protein to 1st antibody
Film over-exposure or lack of exposure

Transformation – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Day 1 morning
100 ml SOB medium in 1L or 500 mL flask and sterilize
E.coli culture grown in 2 mL of fresh LB medium.
Day 1 night
Inoculate preculture (100 µL-1 mL) to sterile SOB medium.Shake culture vigoruoussly at 20-25 °C until OD is 0.4-0.6.
Day 2
Transfer the culture to ice 10min.
Prepare Wash buffer and Competent buffer by adding 3 mL Dilution buffer to 3 mL of Wash buffer(x2) and to 2.5 mL of Competent buffer(2x), respectively.(on ice)
Pellet the cells by centrifugarion at 2500rpm for 6 min.
Remove supernatant and genlly resupend the cells in 6 mL ice-cold Wash buffer(1x).
Pellet the cells by centrifugarion at 2500rpm for 6 min.
Completely remove the supernatant and gently resuspend the cells in 6 mL ice-cold Competent buffer(1x).
Aliquot (on ice) 100µL of cell suspension into sterile 1.5 mL microtube and store in deep freezer.

DNA Purification – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Sigma prep
Zymo DNA Cleam&Concentrator Kit
Add 2 volume of DNA binding buffer to each volume of DNA sample, Use voltex to mix.
Load mixture silica column and place column into a 2 ml collection tube
Centrifuge at full speed for 30 sec. Discard the flow-through.
Add 200µL of wash buffer and spin 30 sec.
Place siliva volumn into a new 1.5 ml tube. Add water directly to the column matrix and spin to elute the DNA.

Agarose gel electrophoresis – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Agalose Gel casting
Measure out the appropriate mass of agarose into glass bottle with the appropriate volume of TAE buffer
Microwave until the agarose is fully melted
Pour the agarose solution into the gelbox and let it cool for about 30 minutes, until the gel is solid
Remove comb

Running agalose gel
Load 5 µL prepared 1kbp ladder
Mix DNA solution with loading dye(6x) and water
Load it into agalose gel
Run the gel at ~100 volts for 35 mins.

Visualizing agarose gels
Remove gel from gel box
Soak the gel in ethidium bromide solution
Let it 30 min.
Place the gel in Trans-Illuminator and turn on UV light after make sure the door closing.
Print the picture.
Remove gel and throw in trash
Wipe down Trans-Illuminator if necessary.

Digestion – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol

Mix (in a PCR tube)
plasmid DNA
buffer
Restriction Enzyme
NFW
Incubate at 37℃ for 3h
PCR – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Resuspend primer in Nuculease free water to 100 µM
PCR mix
DNA template 1µL
Fwd primer 10µL (final con. 10 pM)
Rev primer 10µL
10x thermo pol buffer 10µL
dNTP mix 10µL
DNA pol. 1µL
dH20 58µL

100µL

PCR cycle
Start: 94 °C for 5 min. (melt)
cycle: melt: 1 min.
anneal : 30 sec.
cycle end: extension:72 °C for 3.5 min.
25 cycles
72 °C for 10 min
store: keep at 6 °C forever

Gel extract – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Agalose Gel casting
Measure out the appropriate mass of agarose into glass bottle with the appropriate volume of TAE buffer
Microwave until the agarose is fully melted
Pour the agarose solution into the gelbox and let it cool for about 30 minutes, until the gel is solid
Remove comb

Running agalose gel
Load 5 µL prepared 1kbp ladder
Mix DNA solution with loading dye(6x) and water
Load it into agalose gel
Run the gel at ~100 volts for 35 mins.

Visualizing agarose gels
Remove gel from gel box
Soak the gel in ethidium bromide solution
Let it 30 min.
Place the gel in Trans-Illuminator and turn on UV light after make sure the door closing.
Print the picture.
Remove gel and throw in trash
Wipe down Trans-Illuminator if necessary.

Extract
Cut the agarose target band
The chip of the gel into 2mL ADB buffer
Let it in 37 degree 30 min to solve the agarose gel.
Purify the DNA with Zymo DNA Cleam&Concentrator Kit
Dephosphorylation of DNA – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
SAP: Alkaline Phosphatase (Shrimp)
Mix
DNA fragment 1~10 pmol
shrimp Alkaline Phosphatase (1~5 μ l) 1~5 U
10X SAP Buffer 5 μ l
Sterilized distilled water up to 50 μ l
Incubate at 37°C for 15~30 min.
Incubate at 65°C for 15 min. (for inactivation by heat treatment)
Purify the DNA with Zymo DNA Cleam&Concentrator Kit

Ligation – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Mix insert DNA with vector DNA.
Add 1u Invitrogen Ligase and Ligase-Buffer(x5).
Store RT for 3h.
Time Delay Test – Chiba – 2009 http://2009.igem.org/Team:Chiba/Notebook/protocol
Transformed sender and receiver into E coli strains.
Inoculated them independently in liquid media. Incubated at 37°C 12h.
Inoculated again in Fresh liquid media upto about OD600=2 at 37°C
Washed sender and receiver.
Mixed them. (Sender:Receiver=1000μL:1000μL)
Incubated at 25°C, 30°C or 37°C.
Measured intensity of green fluorescence at regular time intervals.(Fluoroskan Ascent^R FL&Fluoroskan Ascent^R Thermo ELECTRON CORPORATION)

2008

Bacterial Transformation – 2008 - Utah State

http://2008.igem.org/Team:Utah_State/Protocols

Once the target DNA has been successfully ligated into the plasmid vector, the plasmid must be transferred into the host cell for replication and cloning. In order to do this, the bacterial cells must first be made "competent." The term "competent" is to describe a cell state in which there exist gaps or openings in the cell wall which will allow the plasmid containing the target genes to enter into the cell. Several methods to make bacterial cells competent exist, such as the calcium chloride method and electroporation. Competent cells may also be purchased commercially. The team at USU has purchased competent cells for all experiments. The following is the method used by the USU team to insert the plasmids containing the PHB biobricks into the cells. *Trent Mortensen is shown in the right image inspecting agar plates containing transforming cells*

Method

1. Ensure the necessary antibiotic agar plates have been prepared or begin their preparation now. Four plates per transformation will be necessary (two today, then two tomorrow for streaking). Also ensure that 10 ml liquid media is made up per transformation (also for tomorrow).
2. Clean punchout tool by dipping in 10% chlorox, deionized water, deionized water again, then 80%aq ethanol. Let dry for 2 minutes and repeat cleaning procedure between punchouts.
3. Punch out gene of choice with a twisting motion, allowing the metal to cut the paper. Use the center part of the punchout tool to dislodge the paper into a 2.5 ml microcentrifuge tube.
4. Add 5 µl TE buffer, place in a 42˚C water bath, and allow plasmids in the paper to elute for 20 minutes.
5. Centrifuge tube(s) at 15K RPM for 3 minutes. Remove SOC media from the -20˚C freezer and leave out to thaw.
6. Take competent cells from the -80˚C freezer and place on an ice bath.
7. Pipette contents of tube up and down a few times then take 2 µl of the DNA solution and add to the competent cells. Ensure the pipetting is done directly into the cell solution. Let cells incubate in the ice bath for 30 minutes. Heat water bath to 42˚C.
8. Heat shock cells in the 42˚C water bath for 30 seconds. Remove and place back in the ice bath for 2 minutes.
9. In the hood, add 250 µl SOC media to each tube, bringing the total cell solution to 300 µl. Incubate at 37˚C for 1 hour.
10. Get out the antibiotic agar plates. In the hood, add 200 µl of each transformed cell solution to the appropriate antibiotic plate. Use the Bunsen burner to create a "hockey stick" out of a glass pipette tip by holding over the flame until it bends. Allow to cool. Spread cell solution uniformly over the agar plate using the "hockey stick," then before discarding, spread residual solution on the "stick" over a second plate to get more a more sparse colony distribution.
11. Parafilm all plates and place in 37˚C incubator 12-14 hours, or overnight if that is not possible.

Streak Plates and Liquid Cultures from Transformed Colonies – 2008 - Utah State
http://2008.igem.org/Team:Utah_State/Protocols

After bacterial cells have been transformed, successfully transformed cells must be selected. Because 100% of the cells do not receive the desired plasmid and target gene, it is essential to select for cells that do have the target genes. Several methods are used to accomplish this, such as incorporation of antibiotic resistance and also the lac operon. The USU team has used antibiotic resistance to select for successful transformations. To do this, an antibiotic resistance gene is also added to the plasmid vector that contains the target genes. By doing so, it is possible to know that a cell was successfully transformed based on its ability to grow on an agar plate with antibiotics added. Because the cell is able to grow, the antibiotic resistance gene must be present as well as the target gene. From the agar plates containing the antibiotics, a colony is picked off and transferred into a liquid culture for further analysis and cellular production. The following is the method used by USU to clone the DNA and select for the successful transformation of the PHB biobrick into the cells.

Method

1. Prepare two 12 ml tubes per transformation, each with 5 ml media containing the appropriate antibiotic (if felt necessary). Get out antibiotic agar plates. Inspect plates from yesterday for colonies. At least two colonies are preferred, but one will do. Select two colonies and label them.
2. Use a pipette tip to extract half of each colony and inoculate one agar plate per colony. Using a pipette with a tip, extract the other half of each colony and inoculate one liquid media tube per colony. Label all tubes and plates and place in the 37˚C incubator until tomorrow morning.

Preparation for DNA Separation – 2008 - Utah State
http://2008.igem.org/Team:Utah_State/Protocols

Following successful bacterial cloning and isolation, it is important to verify that the target gene is in the cell and that the plasmid is functional. To do this, it is a common practice to sequence the DNA plasmid. To obtain enough DNA for sequencing, the bacterial clones are grown in a liquid culture. The cells are harvested by centrifugation and then prepared for DNA plasmid extraction. DNA plasmid extraction can be done several ways, and the overall purpose is to lyse the cells and separate the plasmid DNA from all other cellular proteins, DNA, and debris. The following is the method used by the USU team to isolate the plasmid DNA containing the PHB biobricks. *An Eppendorf Microcentrifuge was used in these experiments and is shown on the right.*

Method

1. Prepare two water baths, one boiling and the other 68˚C.
2. Centrifuge the 12 ml tubes containing the 5 ml cultures in the large centrifuge at 8K RPM for 20 min. Discard supernatant.
3. Resuspend cells in 200 µl of STET buffer.
4. Add 10 µl (if older preparation) Lysozyme (50 mg/ml) and incubate at room temperature for 5 min.
5. Boil for 45 sec and centrifuge for 20 min at 13K RPM (or until pellet gets tight).
6. Use a pipette tip to remove the pellet.
7. Add 5 µl RNase A (10 mg/ml) and incubate at 68˚C for 10 minutes.
8. Add 10 µl of 5% CTAB and incubate at room temperature for 3 min.
9. Centrifuge for 5 min at 13K RPM, discard supernatant, and resuspend in 300 µl of 1.2 M NaCl using a vortex mixer.
10. Add 750 µl of ethanol and centrifuge for 5 min at 13K RPM.
11. Discard supernatant, rinse pellet (cannot see) in 80% ethanol, and let tubes dry upside down with caps open.

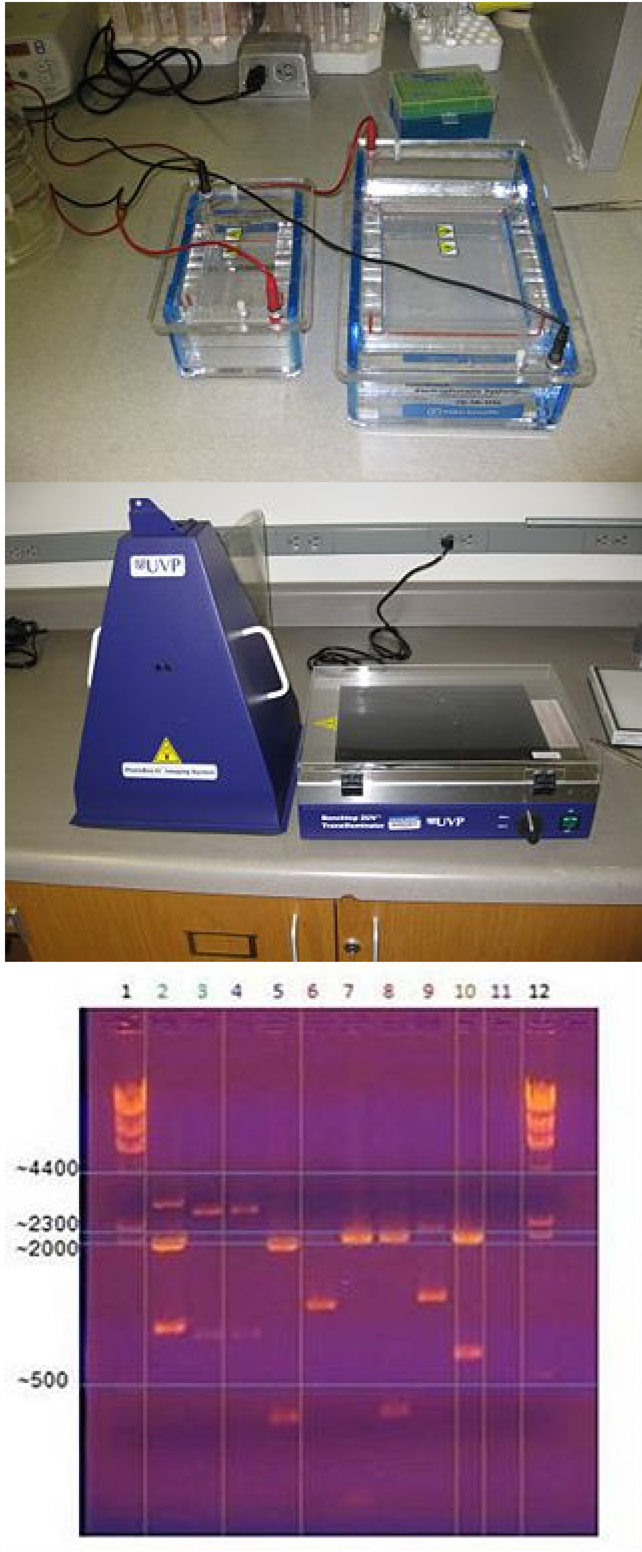
Restriction Enzyme Digestion and Electrophoresis – 2008 - Utah State
http://2008.igem.org/Team:Utah_State/Protocols

Restriction enzyme digestion is the process by which an insert DNA sequence is separated from the rest of the DNA molecule. Specific knowledge of the DNA insert is needed to determine which enzyme and conditions to use during the digestion reaction. Once the DNA sequence is known and the correct enzymes have been selected, the DNA may be digested. Listed below is the procedure used by USU to digest the cellular DNA. After enzyme digestion, electrophoresis is used to separate the plasmid from the insert. A gel is prepared and the respective reaction mixes are loaded into the gel. Using a DNA ladder, and knowing the size of the insert, the corresponding band can be seen and cut out of the gel. The insert may then be removed and isolated from the gel, thus yielding the desired DNA. The DNA from this may then be used in PCR reactions, sequencing, ligations for further experimentation, and many more. Listed below are the protocols used by the USU team to run the electrophoresis reaction.

Method

1. Resuspend DNA in 40 µl water, vortex, and do a brief centrifuge to get solution to the bottom of the tube.
2. Add components to the digestion solution in the following order: DNA (23 µl), 10X Tango buffer (3 µl), Xba1 (2 µl), and Pst1 (2 µl). The volume and restriction enzymes can be varied, but it should be ensured that the total volume is 10X the amount of Tango buffer added. Tap tubes periodically and allow to digest while preparing electrophoresis gel.
3. Prepare electrophoresis gel by adding 2 g agarose to 200 ml TAE (1% solution). This is best done in an Erlenmeyer flask of adequate volume as swirling will need to be done. Place in the microwave and microwave on high for 20 seconds at a time, pulling it out and swirling until solution is homogeneous again, then repeating (BE CAREFUL to watch the solution closely when swirling – it superheats and can boil over and cause severe burns). Continue until solution is seen boiling in the microwave then gently swirl again.
4. Add 20 µl ethidium bromide to solution and swirl until dissolved evenly.
5. Add 6 µl of 6X loading dye to each tube of digested DNA solution.
6. Prepare the electrophoresis unit by orienting the basin sideways with rubber gaskets firmly against the side. Place desired well template in the basin.
7. When the agarose solution is cool enough to comfortably touch the flask, pour into the basin until the solution is about ¾ of the way to the top of the well template.
8. When the gel is solidified (should look somewhat cloudy), remove the well template and change basin orientation to have the wells closest to the negative pole (as the DNA will flow towards the positive pole). Pour 1X TAE buffer into both sides of the electrophoresis unit until it just covers the gel and fills the wells.
9. By inserting the pipette tip below the TAE liquid and into the well, add 10 µl of DNA ladder solution to first (and last if desired) well, skip one well, then begin adding the digested DNA solutions to the wells by adding about 2 µl less than the total volume in the tubes to prevent air bubbles in the wells.
10. Place the cover on the electrophoresis unit, plug into the power source, and turn on voltage to 70 V (this can be as high as 100 V if time is an issue), and press the start button. Separation should take two to three hours. The yellow dye shows the location of the smaller nucleotide lengths and the blue dye shows the location of the larger nucleotide lengths. DNA separation can be observed as time goes on by turning off the power supply then gently removing the basin from the electrophoresis unit (be careful not to let the gel slip out of the basin) and placing on the UV transilluminator to see DNA bands. The basin can then be placed back in the electrophoresis unit for further separation if desired. Take care to not have the power supply on without the lid to the unit in place.
11. When the desired level of separation is attained, the basin can be placed on the transilluminator for picture taking. Place the cone-shaped cover over the transilluminator and place the digital camera in the top hole for pictures.

The electrophoresis units and UV transilluminator used in this project are shown in the left and center images below. An electrophoresis image is given on the right.



Media Preparation – 2008 - Utah State
http://2008.igem.org/Team:Utah_State/Protocols

For all experimentation involving the need for bacterial biomass and experimentation, proper media is needed to grow the cells. The following is the media composition and methods used by USU to prepare the media.
1. Add 5 g yeast extract, 10 g NaCl, 10 g Bacto Tryptone, and 15 g agar (if desired) to a 2 L Erlenmeyer flask and bring the volume up to 1 L with ddH20. Mix by swirling. Cover top with foil.
2. Autoclave for 45 minutes (liquid setting, 0 minutes drying time). It will take an additional half hour for the autoclave to finish cooling then an additional 20 to 30 minutes until the media is cool enough to pour.
1X TAE Preparation – 2008 - Utah State
http://2008.igem.org/Team:Utah_State/Protocols

1. Add 40 ml 50X TAE solution to a 2 L flask and bring level up to 2 L with ddH20.
Polymerase Chain Reaction (PCR) – 2008 - Utah State
http://2008.igem.org/Team:Utah_State/Protocols

PCR is used to amplify a desired DNA sequence. The reaction is first set up by designing primers that will bind only to the desired regions of the DNA sequence. Once the primer and polymerase have been selected, the reaction parameters of time and temperature must be optimized. When the reaction works properly only the target DNA will be amplified into large quantities that may then be isolated and used for further experimentation. The following is the procedure used by USU for PCR reactions to amplify the PHB synthesis and promotion genes.
An Eppendorf Thermocycle was used in these experiments, as shown in the image on the right.
Method
1. Obtain the following reagents from the freezer: DNA template (cells or DNA), 10X Taq buffer (+KCl, -Mg/Cl_2), $MgCl_2$, 10 mM dNTP Mix, Taq polymerase (take out of freezer only immediately when needed and put back), and sterile distilled H_2O. Place all reagents on ice. Also obtain PCR (either 0.2 or 0.5 ml) tubes.
2. Add the following reagents to a tube (50 µl reaction) in the following volumes and order:
• 32 µl sterile H_2O,
• 5 µl 10X buffer,
• 2 µl dNTP Mix,
• 6 µl $MgCl_2$

• 3 µl cells/DNA,
• 0.25 µl Taq Polymerase
• 1 µl primer 1
• 1 µl primer 2

MgCl2 volume can be varied (lower to increase specificity – just ensure total volume is 50 ul with H2O). If many reactions are to be constructed, a master mix can be made up to cut down on time and pipette tip usage (if this is done, ensure primers are added to the appropriate reaction, i.e. perhaps not to the master mix). Tap or vortex tubes and take to the thermocyler. Place all reagents back in the -20˚C freezer.

3. Choose thermocycler temperatures. The Eppendorf Mastercycler will cycle between three temperatures: typical temperatures are 94˚C for denaturing, 50-60˚C for primer annealing, and 72˚C for polymerase extending. Lowering the annealing temperature decreases DNA specificity; 55˚C is a good temperature to begin if no trials have been made with the sample.

4. Turn on thermocycler with the switch in the back of the unit and open the lid. The placement of the tubes depends on the size of the tube (0.2 or 0.5 ml) and whether or not a temperature gradient is to be used.

If no gradient will be used, tubes can be placed anywhere on the unit in the appropriately-sized hole. Select "Files" and press enter. Select "Load" and then "Standard." If cells will be used in the reaction, include a 1-minute lysing step at the beginning (step 1); this will be followed by a 1-minute DNA denaturing step (step 2). If purified DNA will be used, set step 1 to 1 second. Set an annealing temperature for step 3. Ensure the lid temperature is 105˚C and the extending temperature is 72˚C. Press exit. If prompted to save, save by pressing enter three times. Press exit to return to the main menu. Choose "Start" on the main menu and select "Standard." The program should begin.

If a gradient is to be used, temperature will vary according to column. A 20˚C range is the maximum range that can be used (+/- 10˚C). The range is made by setting a temperature for the middle column and then setting a +/- range. To see what the temperatures will be if a gradient is used, select "OPTIONS" on the main menu, then select "Gradient." Select the size tube that is being used by pressing "Sel," then press enter. Choose a temperature for the center column, press enter, then select a +/- range and press enter. The column number along with the corresponding temperature is shown. Decide tube placement based on this information. Press exit twice to return on the main menu. Select "Files" then "Load," then "Gradient." If cells are being used, set the cell lysing step (step 1) to 1 minute (1:00); if purified DNA is being used, set this time to 1 second (0:01). Step 2 should be 94C, Step 4 should be 72˚C, and the lid temperature should be 105˚C. Go to step 3 and set an annealing temperature for the center column. Leave the next two lines as they are, and change the gradient setting ("G") to the +/- the center temperature amount. Press exit. If prompted to save, press enter three times; if not prompted to save, press enter once. Press exit to get back to the main menu. To begin cycle, select "Start," then select "Gradient." The program should begin.

5. The thermocycler is set to store the completed reaction tubes at 4˚C when finished.

Ligation – 2008 - Utah State

http://2008.igem.org/Team:Utah_State/Protocols

Ligation is the process by which the insert (target DNA gene) is inserted into a plasmid. Both the plasmid and insert have been digested and have the proper "sticky" or blunt ends which are compatible for joining the two DNA pieces together into one molecule. These two DNA pieces are placed in a reaction tube and the proper DNA ligase, buffer, and cofactors are added for the reaction to take place. When done properly, the ligation will result in a successful combination of the insert and plasmid into one plasmid. This newly formed plasmid may then be isolated using gel electrophoresis and then used for bacterial transformation or other experimentation. The following is the procedure used by USU to ligate PHB genes into the biobrick plasmids.

Method

1. Obtain the following reagents, some of which are in the -20˚C freezer: DNA vector, DNA insert, 10X ligation buffer, T4 DNA ligase (take out only when needed, then return immediately to freezer), and sterile distilled water.

2. Ideally, it is desirable to have the concentration of insert ends (or moles of insert) be two to three times

the concentration of vector ends (or moles of vector), with a total DNA concentration of 50-400 ng/µl in the reaction. If determining the DNA concentration is not possible, place two to three times the volume of vector as the volume of insert in the reaction. As this is often the case, place the following reagents in a thin-walled PCR tube in the following volumes:

- 10 µl insert DNA
- 3 µl vector DNA
- 2 µl 10X ligation buffer
- 4 µl H_2O
- 1 µl T4 DNA ligase

= 20 µl total

This could also be done in different volumes depending on DNA concentration/total volume desired.

3. Gently mix the tube, and place the tube in the PCR thermocyler, turn on the machine, select "Start," from the main menu, select "22" and press "Start." The thermocycler will keep the reaction at 22˚C.
4. Incubate for 60 minutes. Heat-inactivate by placing tubes in 68C water bath for 10 minutes. Place in the freezer if storing for later use.

Glycerol Stocks – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

For the long-term storage of microbial cultures

Make sure that you do not contaminate glycerol stocks

1. Grow overnight culture (1 mL) of your cells in selective media

2. Mix 750 uL of your culture with 750 uL of 40% glycerol. Immediately place at -80 C (glycerol is toxic to cells and prolonged exposure to it kills them at RT).

If you need to take from the stocks, do not thaw - just scrape some ice off of the top with a sterile loop and smear across a plate.

Double antibiotic plate

·add 20ul of the secondary antibiotic to a plate with the first antibiotic, and spread with a glass rod or inoculation loop.

Electrocompetent Cells – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

One Sample
This is for making one/two aliquots of electrocompetent cells

1. Grow overnight culture of your cells

2. Back-dilute 1:50 into 3 mLs (so 60 uL), put on rotator in 37 degree incubator for 3 hours (put water on ice during this time)

3. Pellet in epi tube, top speed for ~20-30 sec

4. Remove supernatant. Wash cells 3X in cold water 1 mL each.

5. Resuspend in 40 uL cold water, add DNA, electroporate

Electrocompetent DY331 for Lambda Red Recombineering
modified from the preceding protocol 07-25

1. O.N. culture in yeast incubator at 30 C

2. Back-dilute 1:50 into several 4 mL TSY broth cultures (so 80 uL), put in rotator in yeast incubator at 30 C for at least 3 hrs. Chill sterile dH20 on ice and allow the ice to melt to a slurry during the incubation.

3. OPTIONAL: Confirm OD between 0.4-0.6 before proceeding.

4. Transfer tubes to a 42 C water bath and incubate for 15 minutes. For the north water bath in the lab, this is approximately 4.2 on the temperature control knob. The increased temperature will induce Lambda red genes. Use a rotator if possible, otherwise, shake by hand every few minutes.

5. Chill and swirl in slurry for 15 minutes.

6. Divide each broth culture into three epis (or cryo-tubes for glycerol stocks). Pellet at top speed for ~20-30 sec. Wash cells 3X with 1 mL volumes of ice-cold sterile dH20.

7. Resuspend in 40 uL dH20 for immediate transformation. Alternatively, resuspend in 28 uL dH20 + 12 uL 40% glycerol and freeze -80 C immediately.

Lambda Red Recombination – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

This is for site-directed mutagenesis of chromosomes or large plasmids

1. Amplify antibiotic resistance cassette with primers that are homologous to cassette and your region of interest.

For Kan or Cm:
Primer F: 5' 40 bp your sequence + GTGTAGGCTGGAGCTGCTTC - 3' (Tm = 64C)
Primer R: 5' 40 bp your sequence + CATATGAATATCCTCCTTAG - 3' (Tm = 54C)
The Kan Cassette is approx. ? bp
The Cm Cassette is approx. 900-1000 bp

For Tet:
Primer F: 5' 40 bp your sequence + TTAAGAACCCACTTTCACA - 3'
Primer R: 5' 40 bp your sequence + CTAAGCACTTGTCTCCTG - 3'
The Tet Cassette is approx. ? bp

Set up your PCR as follows:
-34.5 uL dH20 for taq

-5 uL of 10X polymerase buffer

-2.5 uL of 10 uM primer F (1:10 dilution of reconstituted stock)

-2.5 uL of 10 uM primer R (1:10 dilution of reconstituted stock)

-2 uL of 50 mM MgCl2 (2 mM final)

-1 uL of 10 mM dNTPs (0.2 mM final)

-0.5 uL polymerase (Invitrogen taq)

-2 uL plasmid template (or colony lysate if doing colony PCR)

Segment		Cycles	Temperature	Time
1	1	95°C	1 minute	
2	30	95°C	1 minute	
45°C	1 minute			
72°C	2 minutes			
3	1	72°C	10 minutes	
4	1	4°C	HOLD	

2. Run small aliquot on gel to verify.

3. Qiagen PCR purification kit, elute with water.

4. Transform 15 uL into cells that express lambda red machinery

If these cells have machinery on pKD46 plasmid: Grow O/N culture at 30C, Sub 1:100 in LB Amp100 + 0.2% arabinose, grow at 30C until OD600 is around 0.6 to 0.8 (25mLs). Wash 2X in cold water (25 mLs, spin 7' at 7K RPM), resuspend in water (100 uL) at density high enough for electroporation.
5. Recover with SOC, plate all on selective media.

6. Streak for isolation

7. Confirm by PCR

Mini Preps – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

Qiagen Kit
1. 250µL P1 resuspend pellet
2. 250µL P2
3. 350µL N3
4. Centrifuge full speed 10 min.
5. Decant supernatant into filter cartridge
6. Spin 1 min.
·empty collection tube
7. Add 750µL PE wash buffer
8. Spin 1 min.
·empty collection tube
9. Dry spin 2 min.
10. Put filter cartridge in clean eppendorf tube
11. Add 50µL EB
·Centrifuge 2 min. @ 9000 rpm

QuikChange Mutagenesis – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

Reference: Stratagene QuikChange® XL Site-Directed Mutagenesis Kit Instruction Manual

Prep:
- Design the sequences
- Design primers(>= 40% GC, boiling point >= 78C, length ~25-45 bp, mutation between 10-15 correct bases of sequence, terminate in G or C)

1. Add followings to the thin-walled tube for thermocycling
- 5 µl 10× reaction buffer
- X µl (10 ng) dsDNA template
- X µl (125 ng) oligonucleotide primer #1
- X µl (125 ng) oligonucleotide primer #2
- 1 µl dNTP mix
- 3 µl DMSO
2. Add ddH2O to a final volume of 50 µl
3. Add 1 µl of PfuTurbo DNA polymerase (2.5 U/µl)
4. Do temperature cycling.

Segment		Cycles	Temperature	Time
1	1	95°C	1 minute	
2	18	95°C	50 seconds	
60°C	50 seconds			
68°C	1 minute/kb of plasmid length			
3	1	68°C	7 minutes	

5. Cool on ice ~2 mins
6. Add 1 µl Dpn1(10 U/µl)
7. Mix solution using a pipet several times, Spin in a Microcentrifuge for 1 min
8. Incubate the reactions at 37°C for 1 hour

Post:
- PRC purification
- Transformation

Sequencing – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

From medium- or high-copy miniprepped plasmid DNA obtained via Qiagen kit

1. Dilute primer to 1 pmole/uL (= 1 uM or a 1:10 dilution of your PCR primers)

2. Aliquot 8 uL of primer (=8 pmole) per epi tube for each sequencing reaction (1 primer/tube, so if you are doing forward and reverse reaction, two tubes). Make sure that you use the correct primers for your reaction.

3. Add 4 uL Qiagen-miniprepped plasmid DNA

4. Fill out form on UW DNA Sequencing Facility website. Make sure that you are connected to a printer for this. Use budget number: 75-1064, Box number: 355761. If you do not already have an account, you will have to make one. Use your own phone number, and Herbert's info for PI information (Box 355061, William H. Foege Building, Room N210E). Select "Plasmid DNA", give short description to each tube. Write down the number they give you (usually your initials and then

some number) on the top of the epindorf tube. At the bottom, select "Rxn and analysis" and "BDSF's Choice", and NO for printing.

5. Print out two copies of the form, one on colored paper (or on white paper that you can then scribble around the edges with a highlighter or a Sharpie). Give the other copy to Ingrid.

6. Submit to sequencing on the 2nd floor of the Hitchcock building. Turn in the colored form where it says "Drop off". Place your samples in the fridge that says "Rxn and analysis", anywhere in a box that says "Template and primer mix".

Conjugation – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

Method #1
Bac-Bac 1. Grow overnight cultures of donor and recipient cells
2. Back dilute 1:100 in 2mL of media and grow to an OD of about .1
3. Plate out 1:1E6 dilution of donor/recipient culture on a selection plate.

Bac-Yeast

1. Grow overnight cultures of donor and recipient cells
2. Back dilute 1:100 in 2mL of media and grow to an OD of about .1
3. Plate out 1:1E4 dilution of donor/recipient culture on a selection plate.

Method #2
(This is taken from the Bates/Cashmore/Wilkins paper)
Bac-Bac

1. Grow donor and recipient bacteria at 37°C for three mass doublings to about 2 × 10^8 organisms per ml.
2. Mix 0.5ml of each culture and collect on a cellulose-acetate filter (Sartorius; 0.45-µm pore size; 25-mm diameter)
3. Incubate for 1 h at 37°C on prewarmed nutrient agar.
4. Resuspend cells by vigorously agitating and plate at appropriate dilutions on media selective for transconjugants.

Bac-Yeast

(This is taken from the Bates/Cashmore/Wilkins paper)
1. Grow donor bacteria and recipient yeast at 30°C for three mass doublings resuspend at a dilution of about 2 × 10^8 organisms per ml.
2. Mix 0.5ml of each culture and collect on a cellulose-acetate filter (Sartorius; 0.45-µm pore size; 25-mm diameter)
3. Incubate for 1 h at 30°C on prewarmed nutrient agar.
4. Resuspend cells by vigorously agitating and plate at appropriate dilutions on media selective for transconjugants.

Double Restriction Digest for AHL Cloning – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

Modified from Endy protocol specifically for AHL cloning 07-26

37.5 ul of sterile dH2O
5 ul of Buffer 2
0.5 ul of BSA
5 ul of DNA (ideally, 0.2-1 ug of mini-prep or PCR product)
1 ul of XbaI
1 ul of SpeI
1. Incubate reaction at 37°C in a microtube rack in the bacterial incubator for 2 hours

2. Heat kill enzymes at 80C for 20 min.

3. Store at -20°C.

4. Run on gel. Controls: intact plasmid, single digest nicked plasmid, reaction without enzyme to identify any non-specific nuclease activity.

Running Gel – 2008 – University of Washington
http://2008.igem.org/Team:University_of_Washington/Protocols

NEB 1kb Ladder
NEB 1kb Ladder.jpg

SDS-PAGE – 2008 – University of Chicago
http://2008.igem.org/Team:_University_of_Chicago/Notebook/SDS-PAGE
SDS-PAGE Protocol
Materials
polyacrylamide gel
1X SDS buffer (6.05g Tris, 28.83g glycine, 2g SDS per liter)
Benchmark Protein Ladder
Denatured protein samples
Commassie blue (50% methanol, 10% acetic acid, 0.2% commassie)
Destain solution (10% methanol, 10% acetic acid)
Procedures
1. Take the comb out of the prepared polyacrylamide gel. Place the gel in a holder and attach the holder to the PAGE apparatus.
2. Insert the apparatus in the tank and pour 1X SDS buffer in the tank and inside the apparatus. 3. Pipette 10ul of Benchmark ladder in one lane of the gel
4. Pipette desired amounts of protein samples in other lanes of the gel.
5. Put the lid on and connect the cables to the power supply
6. Set the power supply to 80V. Run for 20 minutes or until the dye front passes the stack.
7. Increase the voltage to 120V and continue running until the dye front reaches the end of the gel.
8. Cut off the power supply and remove gel from the apparatus.
9. Stain gel in commasie blue stain for 1h
10. Destain in destain solution for 30min.
<<Protocol acquired from http://2007.igem.org/Melbourne/SDS_PAGE>>

DNA mini-prep – 2008 – University of Chicago
http://2008.igem.org/Team:University_of_Chicago/Notebook/DNA_mini-prep
Zyppy mini-prep

The following procedure is performed at room temperature. Ensure that RNase A has been added to the Neutralization Buffer, ethanol has been added to the Zyppy™ Wash Buffer, and that the 7X Lysis Buffer has not precipitated during shipping (to completely resuspend the buffer, incubate the bottle at 30 – 37 ºC for 30 minutes and mix by inversion).
Add 600 µl of bacterial culture grown in LB medium to a 1.5 ml microcentrifuge tube
The Zyppy™ Plasmid Miniprep Kit may also be used with the classical centrifuge- based procedure for processing up to 3 ml of bacterial culture. The procedure should be modified as follows: **1A)** Centrifuge 1.5 ml of bacterial culture for 30 seconds at maximum speed. **1B)** Discard the supernatant. **1C)** Repeat steps 1A and 1B as needed. **1D)** Add 600 µl of TE or water to the bacterial cell pellet and resuspend completely.
Add 100 µl of 7X Lysis Buffer (Blue)1 and mix by inverting the tube 4-6 times. Proceed to step 3 within 2 minutes. After addition of 7X Lysis Buffer the solution should change from opaque to clear blue, indicating complete lysis.
Add 350 µl of cold Neutralization Buffer (Yellow)2 and mix thoroughly. The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form. Invert the sample an additional 2-3 times to ensure complete neutralization.
Centrifuge at 11,000 – 16,000 x g for 2-4 minutes.
Transfer the supernatant (~900 µl) into the provided Zymo-Spin™ IIN column.**Avoid disturbing the cell debris pellet.**
Place the column into a Collection Tube and centrifuge for 15 seconds.
Discard the flow-through and place the column back into the same Collection Tube.
Add 200 µl of Endo-Wash Buffer to the column. Centrifuge for 15 seconds. It is not necessary to empty the collection tube.
Add 400 µl of Zyppy™ Wash Buffer2 to the column. Centrifuge for 30 seconds.
Transfer the column into a clean 1.5 ml microcentrifuge tube then add 30 µl of Zyppy™ Elution Buffer3 directly to the column matrix and let stand for one minute at room temperature.
Centrifuge for 15 seconds to elute the plasmid DNA.
Notes
Excessive lysis can result in denatured plasmid DNA. If processing a large number of samples, we recommend working with groups of ten or less at a time. Continue with the next set of ten samples after the first set has been
neutralized and mixed thoroughly.
Ensure that RNase A has been added to the Neutralization Buffer and that ethanol has been added to the concentrated Zyppy™ Wash

The Zyppy Elution Buffer cProxy-Connection: keep-alive
Cache-Control: max-age=0
tains 10 mM Tris-HCl, pH 8.5 and 0.1 mM EDTA. If required, pure water (neutral pH) can also be used to elute the DNA.

Glycerol stocks – 2008 – University of Chicago
http://2008.igem.org/Team:University_of_Chicago/Notebook/Glycerol_stocks
Glycerol Stocks
Materials
40% glycerol solution
Cryogenic vials
Method
Add 1 ml of 40% glycerol in H_2O to a cryogenic vial.
Add 1 ml sample from the culture of bacteria to be stored.
Gently vortex the cryogenic vial to ensure the culture and glycerol is well-mixed.

Alternatively, pipet to mix.
Use a tough spot to put the name of the strain or some useful identifier on the top of the vial.
On the side of the vial list all relevant information - part, vector, strain, date, researcher, etc.
Store in a freezer box in a -80C freezer. Remember to record where the vial is stored for fast retrieval later.
Notes
While it is possible to make a long term stock from cells in stationary phase, ideally your culture should be in logarithmic growth phase. Prof. Schonbaum's version
Grow a 3 ml culture overnight
Add 200 µl 100% glycerol to 1 ml cells in a cryotube (final concentration should be 15-20% glycerol different concentrations from different protocols
Mix by vortexing and let sit for a little bit. Then put the tubes in the -80°C freezer.

Transformations – *2008 – University of Chicago*
http://2008.igem.org/Team:University_of_Chicago/Notebook/Transformations
Chemocompetent cells
General transformation
Transform 50 _l of cells with 1 _l of standard pUC19 plasmid (Invitrogen)
This is at 10 pg/microliter or 10-5 _g/_l
This can be made by diluting 1 _l of NEB pUC19 plasmid (1 _g/_l, NEB part number N3401S) into 100 ml of TE
Hold on ice 0.5 hours
Heat shock 60 sec at 42C
Add 250 _l SOC
Incubate at 37 C for 1 hour in 2 ml centrifuge tubes rotated
using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
For our plasmids (pSB1AC3, pSPAT3) which are chloramphicol and tetracycline resistant, we find growing for 2 hours yields many more colonies
Ampicillin and kanamycin appear to do fine with 1 hour growth
Plate 20 _l on AMP plates using sterile 3.5 mm glass beads
Good cells should yield around 100 - 400 colonies
Transformation efficiency is (dilution factor=15) x colony count x 105/µgDNA
We expect that the transformation efficiency should be between 5x108 and 5x109 cfu/µgDNA
iGEM transformation (using paper spots)
Materials needed
Spots soaked in 5microliters warm TE for 20 minutes
2.0 conical bottom tubes
Ice
Competent cells
42C water bath/37C incubator
SOC--freshly made!
Petri plates with appropriate antibiotic
Transformaiton control DNA, like pGreen
Procedure
Soak the spots in 5microliters of the warmed TE for 20 minutes. This allows the maximum concentration of DNA in solution. Start thawing the competent cells on wet crushed ice
Chill lageled 2mL conical bottom tubes on wet ice. Add 2 microliters of DNA in TE into 50microliters thawed TOP10 cells in 2mL tubes. 2mL tubes allow better liquid movement durin incubation.

Make sure to do a control transformation! iGEM uses 1microliter of pUC19 plasmid DNA from invitrogen; we'll use pGreen from Dr. Schonbaum
Hold the DNA and competent cells on ice for 30 mintues.
Heat shock cells by immersion in a pre-heated water bath at 42C for 60 seconds. A water bath is important to improve heat transfer to the cells.
Incubate cell son ice for 2 minutes
Add 200microliters of SOC broth. This should have NO antibiotics.
Incubate cells at 37C for 2 hours while tubes are rotating/shaking. 2 hours growth helps in transfomation efficiency, especially for plasmids with resistance other than ampicillin.
LAbel an LB agar plate w/appropriate antibiotic(s) with the part number, plasmid, and resistance. Plate 250 microliters of the cell culture on the plate.
Incubate plate at 37C ofr 12-14 hours, making sure agar side is up. If incubated for too long, antibiotics, **especially ampicillin** to break down and untransformed cells will grow.

DH5-alpha competent cells – *2008 – University of Chicago*
http://2008.igem.org/Team:University_of_Chicago/Notebook/DH5-alpha_competent_cells
Materials
TB Solution
10mM PIPES
15mM CaCl2
250mM KCl
Dissolve in nanopure water and adjust pH 6.7 with KOH or HCL (solutes will dissolve as you do this) and then add
55mM MnCl2. Adjust to final volume. Sterilize by filtration with 0.45um filter and store at 4°C
LB Media
(per 1L batch)
30 g LB mix
Dissolve in ddH2O, filling to 1L.
for 20mM MgSO4 FIRST add 16mL 1M MgSO4 solution THEN fill to 1L

Procedure
Inoculate a 3ml overnight of E.coli in LB+20 mM MgSO4.
Next morning, inoculate 250 ml LB+20 mM Mg++ in a 2L flask with about 2ml
overnight culture. Grow at room temp (23°C) with good aeration (250rpm) to an A600 of 0.4-0.6.
Note: TEMPERATURE IS IMPORTANT! At 37C cells will grow up to proper OD in ~3 hours. A faster growing time, however, compromises efficiency, so choose temperature accordingly.
Place cells 10 min on ice. Transfer to a sterile bottle and spin 3K, 10', 4°C.
Resuspend pellet in 80 ml cold TB (swirl cells in bottle). Leave 10'/ice.
Spin cells 3K, 10', 4°C.
Resuspend cells in 20 ml cold TB then add 1.5 ml DMSO. Leave 10'/ice.
Dispense into 220 ul and 525 ul aliquots (in cold sterile tubes) and
freeze in dry ice/EtOH bath. Store -70°C. Typically, competency about 5X 106 cfu/ug DNA. Note, improves after freezing. Cells good for a year and counting.

TOP10 Competent Cells – *2008 – University of Chicago*
http://2008.igem.org/Team:University_of_Chicago/Notebook/TOP10_competent_cells
Prechill plasticware and glassware
Preparing seed stocks

Streak TOP10 cells on an SOB plate and grow for single colonies at 23°C
room temperature works well
Pick single colonies into 2 ml of SOB medium and shake overnight at 23°C
room temperature works well
Add glycerol to 15%
Aliquot 1 ml samples to Nunc cryotubes
Place tubes into a zip lock bag, immerse bag into a dry ice/ethanol bath for 5 minutes
This step may not be necessary
Place in -80°C freezer indefinitely.
Preparing competent cells
Inoculate 250 ml of SOB medium with 1 ml vial of seed stock and grow at 20°C to an OD600nm of 0.3
This takes approximately 16 hours.
Controlling the temperature makes this a more reproducible process, but is not essential.
Room temperature will work. You can adjust this temperature somewhat to fit your schedule
Aim for lower, not higher OD if you can't hit this mark
Centrifuge at 3000g at 4°C for 10 minutes in a flat bottom centrifuge bottle.
Flat bottom centrifuge tubes make the fragile cells much easier to resuspend
It is often easier to resuspend pellets by mixing before adding large amounts of buffer
Gently resuspend in 80 ml of ice cold CCMB80 buffer
sometimes this is less than completely gentle. It still works.
Incubate on ice 20 minutes
Centrifuge again at 4°C and resuspend in 10 ml of ice cold CCMB80 buffer.
Test OD of a mixture of 200 _l SOC and 50 _l of the resuspended cells.
Add chilled CCMB80 to yield a final OD of 1.0-1.5 in this test.
Incubate on ice for 20 minutes
Aliquot to chilled screw top 2 ml vials or 50 _l into chilled microtiter plates
Store at -80°C indefinitely.
Flash freezing does not appear to be necessary
Test competence (see below)
Thawing and refreezing partially used cell aliquots dramatically reduces transformation efficiency by about 3x the first time, and about 6x total after several freeze/thaw cycles.
Measurement of competence
Transform 50 _l of cells with 1 _l of standard pUC19 plasmid (Invitrogen)
This is at 10 pg/_l or 10-5 _g/_l
This can be made by diluting 1 _l of NEB pUC19 plasmid (1 _g/_l, NEB part number N3401S) into 100 ml of TE
Hold on ice 0.5 hours
Heat shock 60 sec at 42C
Add 250 _l SOC
Incubate at 37 C for 1 hour in 2 ml centrifuge tubes rotated
using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
For our plasmids (pSB1AC3, pSPAT3) which are chloramphicoProxy-Connection: keep-alive
Cache-Control: max-age=0
and tetracycline resistant, we find growing for 2 hours yields many more colonies

Ampicillin and kanamycin appear to do fine with 1 hour growth
Plate 20 _l on AMP plates using sterile 3.5 mm glass beads
Good cells should yield around 100 - 400 colonies
Transformation efficiency is (dilution factor=15) x colony count x 105/µgDNA
We expect that the transformation efficiency should be between 5x108 and 5x109 cfu/µgDNA

PCR – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols
PCR (25ul)

Mastermix: 20.375 ul water, 0.5ul 10mM dNTP, 2.5 ul 10x Buffer 2, 0.375 ul Expand Polymerase 1. *For reactions with DMSO, use 2.5uL of DMSO and 17.875uL of water.*
Aliquot 23.75 ul into each tube.
Add 0.5ul Oligo 1 (10uM), 0.5ul Oligo 2 (10uM), 0.25 ul template DNA.
Load into thermocycler. *Note: small holes are for 0.2 ul tube and big holes for 0.5 ul to maximize contact*
Select the program (55/45 if under 1kb, 2K55/2K45 for 1kb to 2kb, 4K55/45 for 2kb to 4kb, 8K55/45 if over 4kb)
Wobble PCR (50ul)

Mastermix: 40 ul water, 1.5 ul MgCl2 (50mM), 5 ul 10x buffer (Taq), 1 ul 10mM dNTP, 0.5 ul Taq Polymerase
Aliquot 48 ul into each tube
Add 1 ul 100 uM Oligo 1, 1 ul 100 uM Oligo 2.
Load into thermocycler.
Select the program WOBBLE55 or WOBBLE45 and run.
Colony PCR (20ul)

Mastermix: 15.95 ul water, 0.6 ul MgCl2 (50mM), 2 ul 10x buffer (Taq), 0.4 ul 10mM dNTP, 0.25 ul Taq Polymerase, 0.4 ul ca998 oligo (10uM), 0.4 ul g00101 oligo (10uM)
Aliquot 20 ul into each tube
Pick the desired colony with a 10 ul pipette tip and swirl around in the PCR mix before putting into culture tube with appropriate antibiotic.
Load into thermocycler.
Select the program COLONY and run.
Purification and Visualization of PCR Product – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols

Label new tubes with the names of your samples for isolation.
Open a 0.8% CloneWell E-Gel. *Only do this unless you are planning on running it in less than 15 min or it will dry out!*
Place E-gel in apparatus (don't remove combs yet!) and do a PreRun for 2 minutes.
Remove combs and add about 25 ul of water to all cloning wells, 10 ul of ladder (GeneRuler, 1kb DNA Ladder) to the M lane, and 25 ul of your PCR products to each well. *If you have fewer than 8 samples, try not to use the two outer lanes (1 and 8) since they tend to dry out/distort the DNA, and the water in these clone wells evaporated extremely quickly. Also, fill any unused lanes with 25 ul of water.*
Select Run CloneWell and hit Go. After about 1 minute, turn on the blue light and view the gel through the orange shield; you should see small, bright bands under your sample lanes. Every couple minutes, check to make sure the clone wells are still filled with water.
Allow the bands to run until they reach the tiny horizontal scratches just above the clone wells. At this point, hit Go to stop the gel from running. Completely fill all clone wells with water and prepare for extraction.
Hit Go to start again and watch until the band has mostly entered the clone well and water. Hit Go again to stop the gel.

Extract the water/sample from the clone well with a pipette and transfer to appropriately-labeled new tube. *If you miss your sample and it has continued on past your clone well, you can stop the program and select a reverse run to get it back, but avoid doing this if you can*
Go ahead and freeze your samples if you don't plan on doing a digestion immediately.

Digestion (w/o DpnI) – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols

Combine 5 ul NEB2, x ul of your DNA samples from the CloneWell, 1 ul EcoRI, 1 ul BamHI, (43-x) ul water. *It's usually safe to assume you'll have at least 15 ul of any given sample extracted from the E-gel, so if you have many samples make a master mix in which there are 15 ul of DNA sample and 28 ul of water and multiply accordingly depending on the number of samples you have.*
Incubate at 37 C for one hour.

Clean-up with Zymo Columns – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols
Products Greater Than 300 bp

Label each Zymo column and fresh Eppendorf tubes with the appropriate names of your samples.
Add 200ul ADB buffer to each of the digestion samples, mix and pipette into zymo columns. Spin for 30 sec at full speed to pass the liquid through the column.
Empty collection tubes and put back onto the columns. Wash with 200 ul wash buffer and again spin for 30 sec at full speed. *This will dissolve any extra guanidinium chloride and salts sitting on the membrane*
Repeat the wash buffer step 3. *Now nothing is present on the membrane but the DNA and a little ethanol and water*
Now spin the column for 90 sec at full speed to remove all traces of water/ethanol.
Empty and discard the collection tube. Replace each collection tube with the appropriately-labeled fresh Eppendorf tubes.
Add 6 ul of water directly to each of the membranes of the Zymo columns, and spin for 45 sec to elute the DNA into your fresh Eppendorf tubes.
Products Between 20 and 300 bp

Add 1 volume equivalent of Zymo ADB buffer to each reaction. Vortex to mix.
Add 5 volumes of 95% ethanol (under fume hood, labeled flammable). Vortex to mix. *The remaining steps are just like a normal Zymo clean-up reaction*
Label each Zymo column and fresh Eppendorf tubes with the appropriate names of your samples.
Transfer your reaction mixtures into Zymo columns. Spin for 30 sec at full speed to pass the liquid through the column.
Empty collection tubes and put back onto the columns. Wash with 200 ul wash buffer and again spin for 30 sec at full speed. *This will dissolve any extra guanidinium chloride and salts sitting on the membrane*
Repeat the wash buffer step 3. *Now nothing is present on the membrane but the DNA and a little ethanol and water*
Now spin the column for 90 sec at full speed to remove all traces of water/ethanol.
Empty and discard the collection tube. Replace each collection tube with the appropriately-labeled fresh Eppendorf tubes.
Add 6 ul of water directly to each of the membranes of the Zymo columns, and spin for 45 sec to elute the DNA into your fresh Eppendorf tubes.

Ligation – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols

Mastermix: 6.5 ul water, 1 ul ligation buffer, 0.5 T4 DNA ligase, 1 ul pBca1256 digested with EcoRI and BamHI.
Aliquot 9 ul into fresh tubes.
Add 1 ul of your insert.
Cover with foil and incubate for 30 min at room temperature.

Transformation – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols
Remember to pre-warm your plates at 37 degrees C!!
Transformation from Ligation Reaction

Retrieve your competent cells from the -80 (on the third shelf) and thaw on ice. *There are 220 ul of competent cells in each tube*
Add 30 ul cold KCM and 20 ul cold water to each tube of competent cells. Invert ~2x to mix.
Take the top of an empty pipette tip box, add ice and water. Cut out the appropriate amount of tubes from the sets of attached tubes and float them in this ice bath. *Do NOT let any water get into the tubes or else you will have to start over due to possible contamination!*
Combine 45 ul of your cells into 10 ul of your ligation rxn while remembering to keep all tubes on ice.
Swirl and pipette up and down once to mix.
Foil all tubes and incubate 10 min in ice-water.
Heat shock your cells by placing them in a 42 C water bath for 90 sec.
Remove and incubate on ice for 2 min.
Rub ethanol/flame top of foil to sterilize. Add 50 ul of LB 2YT to each tube by poking holes through the foil with your pipette tips.
Re-cover all tubes with foil and incubate at 37 C for 1 hour.
Plate using the sterilized glass-bead method or with standard spreaders.
Transformation from Mini-prep

Retrieve your competent cells from the -80 (on the third shelf) and thaw on ice. *There are 220 ul of competent cells in each tube*
Add 30 ul cold KCM and 50 ul cold water to each tube of competent cells. Invert ~2x to mix. *This will give you a total of 300 ul of competent cells*
Take the top of an empty pipette tip box, add ice and water. Cut out the appropriate amount of tubes from the sets of attached tubes and float them in this ice bath. *Do NOT let any water get into the tubes or else you will have to start over due to possible contamination!*
Combine 30 ul of your cells into 1 ul of your plasmid prep while remembering to keep all tubes on ice.
Swirl and pipette up and down once to mix.
Foil all tubes and incubate 10 min in ice-water.
Heat shock your cells by placing them in a 42 C water bath for 90 sec.
Remove and incubate on ice for 2 min.
Rub ethanol/flame top of foil to sterilize. Add 50 ul of 2YT to each tube by poking holes through the foil with your pipette tips.
Re-cover all tubes with foil and incubate at 37 C for 1 hour.
Plate using the sterilized glass-bead method or with standard spreaders.
Transformation into Cell Culture

Grow cells in ~4 mL LB until cloudy (OD600=0.5)
Put on ice
Transfer 1mL into an eppendorf tube on ice, let cool
Centrifuge full speed for 30 sec, toss out supernatant
Resuspend in 90uL of TSS solution

Add 10uL KCM
Add 1uL plasmid DNA
Let sit on ice for 10min, heat shock 90 sec at 42, incubate and/or plate

MiniPrep (1-5 mL) – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols
QIAPrep Spin Miniprep Kit

Pellet 1.5 mL saturated culture by spinning full speed for 30 sec.
Dump supernatant, repeat to pellet another 1.5 mL (for a total of 3 mL)
Add 250 ul of P1 buffer into each tube. Vortex well to resuspend cells. *You should not see anything solid at the bottom of the tube*
Add 250 ul of P2 buffer (a base that denature everything and causes cells to lyse). Gently invert four times until uniformly light blue.
Add 350 ul of N3 buffer (an acid of pH ~5 that cuases cell junk, including protein and chromosomal DNA, to precipitate and leaves plasmids and other small molecules in solution). Slowly invert six times.
Spin in centrifuge at top speed for 5 min.
Label blue columns with an alcohol-resistant lab pen.
Pour liquid into columns, and place the columns into the centrifuge. Spin at max speed for 30 sec.
Dump liquid out of the collection tubes into waste container. *The DNA should be stuck to the white resin now*
Wash each column with 500 ul of PB buffer.
Spin in centrifuge at max speed for approximately 15 sec, then flick out the liquid again.
Wash with 750 ul of PE buffer. *This washes the salts off the resins*
Spin in centrifuge at max speed for approximately 15 sec, then flick out the liquid again.
Spin one more time at max speed for 90 sec to dry out resin.
Label new tubes and put columns in them. Discard collection tubes.
Elute them by squirting 50 ul of water directly onto the resin in each column. *Get as close to the resin with your pipette tip as possible without touching it*
Spin in centrifuge at top speed for 30 sec.
Take out columns and cap the tubes. *Check to make sure there is something in the tube! It's hard to keep track of which columns you added water to when working with several samples. If there is nothing in the tube, just add your water and spin again. (Don't make Jin dig through the garbage for your column!)*
Clean-up. Note: *the P1 buffer is stored at 4 C and all the rest are stored at room temp*
Agencourt Miniprep – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols

Pellet 800 ul 2YT saturated cultures
Resuspend in 100 ul RE1
Add 100 ul L2 to alkaline lyse
Add 100 ul N3
Wait 10 min for precipitate to float out, or spit it out
Transfer 110 ul of lysate to round bottom microtiter plate
Add 10 ul of PUR4 beads
Add 80 ul of isopropanol
Pipette 10x to mix
Allow plate to separate on magnet for 15 min
Dump supernatant
Add 200 ul of 70% ethanol while still on magnet
Let incubate for 30 sec, then discard
Do the above 70% ethanol wash 3x

Let beads dry for 30 min
Add 40 ul RE1 and shake to elute
Aspirate over liquid
High-throughput miniprep using magnetic beads
1. Inoculate each well of a 96 well block with your desired plasma containing bacterium. The wells should have 1mL of 2YT with appropriate antibiotic complement 2. Culture on shaker for 24 hours 3. Spin 5 min on balanced centrifuge at max speed (~5000 rpm) to pellet the cells. 4. Toss liquid media supernatant in one wrist-driven swoop of elegance. Avoid cross drip. Remove ALL LIQUID- to ensure the success of subsequent steps. 5. Add 100ul of P1 buffer to each well. Try to use one set of tips if you can, but be mindful of splatter. 6. Vortex periodically over 5 mins to resuspend cells such that no pellet remains. You may use foil to avoid splashing of the wells if nec. The buffer should be completely homogeneous. 7. Add 100ul P2 lysis buffer to each well. Squirt forcefully to facilitate mixing of buffer with cells. The wells should adopt a uniform blue color when properly mixed 8. Shake gently to mix. Ensure that all wells are uniformly mixed by observing color. DO NOT VORTEX, as shearing of denatured genomic DNA will evolve fragments which will remain in solution, rather than pelleting out with cell constituents as it should. 9. Add 100ul of N3 neutralizing buffer. Again, pipet forcefully to facilitate mixing and observe color to verify neutralization. Shake gently until blue is gone. 10. Add foil to top and invert brick 10 times 11. Spin on balanced centrifuge at 5000rpm (~full speed) for 10 mins. Stand by centrifuge until it reaches speed 12. Load 10uL beads into Co-Star brand 96-well plate. Work rapidly to ensure the beads remain in suspension. Put back any remainder, because they're expensive (at least for now). 13. When centrifugation is done, pipet out 340uL supernatant from each well and pipet it into each bead-containing well of the Co-Star plate. The genomic DNA should be left as part of the pellet; the plasmid will be in the supernatant. Take care not to slurp pellet back into pipet tips. 14. Pipet up and down 10x to thoroughly mix beads with DNA solution 15. Put Block on magnet. Wait until the beads have cozied up to the magnet before removing supernatant. 16. Calibrate pipet to 500ul (a volume greater than the qty in the wells) and slurp out as much of the supernatant as possible- but if you don't get all the supernatant, you'll do another wash so don't worry too much, 17. Remove plate from magnetic platform 18. Using the multichannel p200 Add 250uL of PB to each well. Try to rinse down the sides as you add; pipette up and down 10X to mix Retain tips if possible. 19. Replace plate onto magnetic platform and wait for beads to precipitate. 20. Calibrate pipette to 275uL and slurp off supernatant. Your DNA is bound to the beads. 21. Remove supernatant. Take 2 pipet lifts to complete this if necessary. If you take up beads inadvertently, pipet back, wait for the magnet, and try again. 22. Remove plate from magnet. 23. Pipet 350uL of PE wash buffer into each well Pipet up and down 10x to ensure mixing. 24. When pipetting during steps ___-24, do not push pipettor plunger past first stop, or aspiriation could cause splatter. 25. Replace plate onto magnet 26. Over two lifts remove supernatant. 27. Remove plate from magnet. 28. Repeat PE wash with another 350uL 29. Dry wells for 5 mins in 37C incubator. 30. Add 50 uL EB elution buffer to each well and pipet to mix. 31. Wait 2 mins before placing plate back on magnet 32. During wait, prepare 96 well PCR plate for receiving samples. 33. Transfer samples from Co-Star plate to PCR plate. Though the intention is to remove DNA and not beads, a few beads should not ruin your samples.

Transfer of Basic Parts into Assembly Vectors Using Eco/Bam – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols
Digesting Basic Part in Entry Vector

Set up the digest - make a master mix of 21 uL water, 3 uL NEB2 buffer, .5 uL EcoRI, and .5 uL BamHI per reaction. Aliquot 25 uL, then add 5 uL miniprepped plasmid. Incubate for 45 min. at 37 degrees C.
Cleanup the Digest
Remember to make fresh 70% ethanol before starting

Add 70 uL RE1, 5 uL SPRI beads, and 75 uL isopropanol. Pipette up and down 10 times **slowly** and **carefully**. Transfer to the magnet plate and wait for 5 minutes. Pipette out the liquid,

being careful to stick pipette tips straight down through the ring of magnetic beads. Fill wells with 70% ethanol, wait 30 seconds, pipette out the ethanol. Repeat once. Let beads dry for 30 minutes.
Setting up the Ligation

Make a ligation master mix #1 with 1 uL predigested assembly vector, 1.5 uL ligase buffer, and 12.5 uL water. Add 15 uL to each tube, scraping the beads down with your pipette tip. Then make ligation master mix #2 with .3 uL ligase, .5 uL ligase buffer, and 4.2 uL water. Add 5 uL to each tube, pipetting up and down to mix. Cover with foil, vortex, and incubate at room temperature for 30 minutes.
Periplasmic Prep – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols

Make TES (0.5 M sucrose, 0.1 M Tris-HCl pH 8.2 and 1 mM EDTA) and place TES and water on ice
Chill cells on ice
Spin out media
Resuspend cells in 1/10 culture volume of cold TES
Let sit on ice for 5 minutes
Spin out cells and dump supernatant
Resuspend cells in 1/20 culture volume of ice cold ddH20
Spin out cells and save supernatant
Alkaline Phosphatase Assay – 2008 – UC Berkeley
http://2008.igem.org/Team:UC_Berkeley/Protocols
SOLUTIONS NEEDED:
Tris Buffer: 100 mM Tris, pH 8.0
SDS solution: 0.1% SDS
Deoxycholic Acid Na Salt 10 mg/mL
PNP solution: Freshly dissolve a 5 mg Sigma S0942 PNP tablet in 5 mL Tris Buffer
Add 100 uL of culture to 900 uL of Tris buffer.
Add 10 uL of Deoxycholic Acid, 10 uL of SDS solution
Vortex for 10 sec.
Add 100 uL of PNP solution
Incubate at 37 about 10 min., or until yellow color emerges from positive control

Midiprep/ Maxiprep Growth Protocol – 2008 – Princeton
http://2008.igem.org/Extract_DNA_(Maxiprep_or_Midiprep)
1. Aliquot 400 ml (Maxiprep)or 50ml (Midiprep) of TB into a clean (autoclaved) 1L flask (Maxiprep) or 250-500ml flask (Midiprep). LB can be used if TB is not available.
2. Add 400 µl of appropriate antibiotic, i.e. a 1:1000 dilution to the above solution (LB/TB). The correct antibiotic should be added (same as the plate from which the colonies are picked.)
3. Use a sterile wooden applicator (autoclaved) or pipette tip to carefully pick an individual colony and dip the colony end of the applicator / tip into the flask.
4. Place the flask securely in the 37C shaker at 280-300 rpm and grow the cells for 14-18 hours. The tubes should be murky after the overnight growth.

Midiprep Protocol (QIAGEN kit) Midiprep/ Maxiprep Growth Protocol – 2008 – Princeton
http://2008.igem.org/Extract_DNA_(Maxiprep_or_Midiprep)
1. Transfer the overnight culture of plasmid cells into 2ml microcentrifuge collection tubes (1 per try). Pellet for 3 min at >8000 rpm in a conventional table-top microcentrifuge at room temperature. Decant all the liquid.
2. Add 250ul of ice cold resuspension Buffer P1 (make sure RNase is already added and mixed. Buffer P1 is stored at 4C)

3. Add 250 uL of Buffer P2 and mix thoroughly by inverting the tube 4-6 times for exactly 5 min: no more, no less. Mix gently. DO NOT vortex. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after the addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If there are still clumps, keep mixing until the homogeneous suspension is achieved.
4. Add 350 uL of Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. The solution should become cloudy. If LyseBlue has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless.
5. Centrifuge for 10 min at 13,000rpm (17900 g) in a table-top microcentrifuge. A compact white pellet will form.
6. Setup the QIAprep spin column
7. Transfer the supernatants from step 5 to the QIAprep spin column by decanting or pipetting.
8. Centrifuge for 1 min at 13,000 rpm. Discard the flow through.
9. Wash the QIAprep spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 min at 13,000 rpm. Discard flow-through.
10. Wash QIAprep spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 min at 13,000rpm
11. Transfer the filters to a new clean and autoclaved 2ml eppendorf tube.
12. Label the tubes.
13. Add 50 µl of EB per column and wait 3 min.
14. Spin for 5 mins.
15. Measure the concentration of DNA.
16. Digest the tries which seem to have the correct supercoil size with an appropriate restriction enzyme to verify plasmid construction. Make sure to create a gel map on vector for the restriction digest. Also remember to digest the parents with the same enzyme. You need atleast 100ng of DNA per band to see it on the gel. The DNA for each band is proportional to its size : for example if you expect to see a 500 bp band and a 4.5 kb out of a 5 kb plasmid after your digest, you need to digest atleast 1 µg as they will be divided as 900ng of the 4.5kb band and 100ng of the 500 bp band.
17. Run on the agarose gel for as long as required to obtain maximum resolution.

Maxiprep Protocol (QIAGEN kit) Midiprep/ Maxiprep Growth Protocol – 2008 – Princeton
http://2008.igem.org/Extract_DNA_(Maxiprep_or_Midiprep)
All centrifugation steps are done at 4000 g, 4C unless otherwise specified.

1. Transfer 400ml of the overnight culture of plasmid cells into two clean, autoclaved 200ml centrifuge bottles (1 per plasmid). Pellet for 15 min at 6000 g, 4C. Decant all the liquid. Make sure not to mix up the plasmids.
2. Add 10ml of Resuspension Buffer P1 to the cell pellet and vortex the bottle. No cell clumps should be observed.
3. Add 10ml of Lysis solution Buffer P2. Do not vortex. Mix gently by swirling the bottle. If LyseBlue was added to Buffer P1, the cell suspension will turn blue after the addition of Buffer P2. Mixing should result in a homogeneously colored suspension.
4. Add 10ml of chilled Neutralization solution Buffer P3. Do not vortex. Mix gently by swirling the bottle. A white precipitate will appear. If LyseBlue was used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless.
5. Centrifuge the cells for 30 mins at ≥20,000 g, 4C. Remove supernatant containing plasmid DNA promptly. Centrifugation should be performed in non-glass tubes. After centrifugation the supernatant should be clear.
6. Centrifuge the supernatant again for 15 mins at ≥20,000 g, 4C. Remove supernatant containing plasmid DNA promptly. Second centrifugation should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material can clog the QIAGEN-tip and reduce or eliminate gravity flow.
7. Add 10ml of the matrix per bottle. Swirl and mix for 30 sec. Centrifuge for 5 min. The matrix binds the DNA and settles at the bottom.

8. Throw out the supernatant. Resuspend the matrix in 25 ml of wash buffer. Centrifuge for 5 min. Make sure that the wash buffer bottle is marked ‘ethanol added’. If not, add 100% ethanol to fill the wash buffer bottle.
9. Assemble the filters. Place each filter provided in the kit in a 50ml centrifuge tube (blue cap, also provided in the kit). Label the tubes.
10. Throw out the supernatant. Resuspend the wash buffer in 15 ml of the wash buffer and mix thoroughly. Transfer the solution to the corresponding filter/tube from the above step.
11. Centrifuge for 5 min.
12. Remove the filter and throw out the solution. Place the filter back in the same tube. Add 10 ml of wash buffer and centrifuge for 5 min.
13. Remove the filter and place it in a new 50 ml centrifuge tube (provided in the kit). Label the tube and add 4 ml of EB (elution buffer) to the filter. Let it stand for 3 min at RT.
14. Centrifuge for 5 min.
15. Discard the filters from each tube. Add 222 µl of 5M NaCl to each tube. Mix well by swirling gently. Add 8ml of ice cold 100% ethanol to each tube. Centrifuge at speeds > 10000g for 15 min.
16. You will see a translucent white DNA precipitate at the bottom. Carefully throw away the supernatant without disturbing the pellet.
17. Add 10 ml of 70% ethanol to each tube. Centrifuge at speeds > 10000g for 15 min.
18. Once again, you will see a translucent white DNA precipitate at the bottom. Carefully throw away the supernatant without disturbing the pellet.
19. Air dry the pellet (leave it on your bench for 10-15 min.) Don’t leave the pellet for a longer duration as it might be difficult to resuspend.
20. Resuspend the pellet in 1.5 ml of EB.
21. Measure the concentration and aliquot into labeled 2ml Eppendorf tubes.
22. Store the DNA at -20C.

Transformation Protocol – 2008 – Princeton
http://2008.igem.org/Re-transform_with_selected_plasmid
Transformations are used to insert the designed DNA into bacteria, which is then grown to multiply the plasmid DNA.

Heat Transformation Protocol

1. Make sure that the incubator (30/37C) and water bath (42C) are ON
2. Make sure required antibiotic plates are present. Check the antibiotic resistance on the plasmid map.
3. Take the DNA out of –20 frig, let it thaw
4. Thaw the competent cells on ice for 7-8 min.
5. Add 1.0 µl of DNA (about 10ng) into the liquid (Don’t vortex). Tap the sides of the tube to mix. For Ligation add 1-5 ul
6. Incubate the cells on ice for 30 min
7. Heat shock the cells for EXACTLY 30 sec at 42 C water bath.
8. Place on ice for 2 min.
9. Add 0.9ml of 37° C S.O.C medium to each tube (S.O.C is made by dissolving 0.5 ml of 20% glucose in 25 ml of SOB. Make sure that the SOC is clear and not cloudy/ contaminated.)
10. Shake the tubes at 37 C, 280 rpm for 60 min or 30 C for 90 min
11. Spin down the cells at 6000 rpm for 1 minute (should see white clumps at bottom).
12. Take out 0.85 ml of S.O.C. (so there’s only 50 ul of SOC left inside)
13. Resuspend the cells.
14. Plate all 50 ul.
15. Incubate upright for 20 min., then upside down overnight (12-14 h) at 37 C or 16-18h at 30C.

Can leave the cells in the incubator for up to 18 hours but no more

Restriction Map Design – 2008 – Princeton
http://2008.igem.org/Restriction_Map/_Restriction_Digest
Restriction maps are designed through Vector NTI plasmid sequences. Restriction sites with compatible enzymes (similar temperatures and buffers needed) are identified, and those enzymes are then used to digest the vector plasmid. The restriction site must also be in the proper area of the plasmid for insertion of other necessary isolated DNA. The inserted DNA must also have the cut sites, so areas of DNA will overlap when the plasmid is ligated.

Restriction Digest Protocol – 2008 – Princeton
http://2008.igem.org/Restriction_Map/_Restriction_Digest

Calculations
Divide the OD of the DNA by a suitable number, depending on concentration. If it is a high concentration, divide by 1000. If not, then something such as 750 will also work. Do not use concentrations that are too low, however, because there will not be enough DNA.

Judging from this number, choose a round number (increasing by 5s usually works best) for your total volume that will allow the next steps to be completed succesfully, without an excessive amount of water being added.

The amount of enzyme and buffer should be the same, at 10% of the TOTAL value.
IF BSE is added, that should be 10% of the amount of enzyme.

Use autoclaved water to add the rest of the volume until the total amount is reached.

Digestion and Running on Gel
Place in machine on "Digestion" Cycle for 3hrs.
Run on gel, analyze for correct bands based on restriction map

Miniprep Protocol (QIAGEN Miniprep kit) – 2008 – Princeton
http://2008.igem.org/Extract_DNA_(Miniprep)
Minipreps are used to extract the DNA from the grown bacteria.

Miniprep Growth Protocol

1. Calculate the approx. S/N ratio of the transformation.
2. Decide on number of tries to be setup accordingly. Pick 12 tries if the S/N ratio is good (5:1), more otherwise.
3. Aliquot X ml (X = Y number of tries * 3.5, where Y = number of tries.) of TB into a clean (autoclaved) beaker or flask. Lb can be used if TB is not available.
4. Add X µl of appropriate antibiotic, i.e. a 1:1000 dilution to the above solution. The correct antibiotic should be added (same as the plate from which the colonies are picked.)
5. Take Y clean and autoclaved 14 ml polystyrene tubes (ones with thw white cap) and place them on a rack. Label them clearly. Aliquot 3.5 ml of TB/LB into each 14 ml tube.
6. Use a sterile wooden applicator (autoclaved) or pipette tip to carefully pick an individual colony and dip the colony end of the applicator / tip into a 14 ml tube. Repeat this Y times using a fresh applicator each time. Make sure to pick a single colony per try.

7. Place the tubes in the 37C shaker at 280-300 rpm and grow the cells for 12-14 hours. The tubes should be murky after the overnight growth.

Miniprep Extract DNA Protocol
All centrifugation steps are done at 13000 rpm, after step 2, in a table-top microcentrifuge

1. Transfer the overnight culture of plasmid cells into 2ml microcentrifuge collection tubes (1 per try). Pellet for 3 min at >8000 rpm in a conventional table-top microcentrifuge at room temperature. Decant all the liquid.
2. Add 250ul of ice cold resuspension Buffer P1 (make sure RNase is already added and mixed. Buffer P1 is stored at 4C)
3. Add 250 uL of Buffer P2 and mix thoroughly by inverting the tube 4-6 times for exactly 5 min: no more, no less. Mix gently. DO NOT vortex. If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after the addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If there are still clumps, keep mixing until the homogeneous suspension is achieved.
4. Add 350 uL of Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. The solution should become cloudy. If LyseBlue has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless.
5. Centrifuge for 10 min at 13,000rpm (17900 g) in a table-top microcentrifuge. A compact white pellet will form.
6. Setup the QIAprep spin column
7. Transfer the supernatants from step 5 to the QIAprep spin column by decanting or pipetting.
8. Centrifuge for 1 min at 13,000 rpm. Discard the flow through.
9. Wash the QIAprep spin column by adding 0.5 mL Buffer PB. Centrifuge for 1 min at 13,000 rpm. Discard flow-through.
10. Wash QIAprep spin column by adding 0.75 mL Buffer PE. Centrifuge for 1 min at 13,000rpm
11. Transfer the filters to a new clean and autoclaved 2ml eppendorf tube.
12. Label the tubes.
13. Add 50 µl of EB per column and wait 3 min.
14. Spin for 5 mins.
15. Measure the concentration of DNA.
16. Digest the tries which seem to have the correct supercoil size with an appropriate restriction enzyme to verify plasmid construction. Make sure to create a gel map on vector for the restriction digest. Also remember to digest the parents with the same enzyme. You need atleast 100ng of DNA per band to see it on the gel. The DNA for each band is proportional to its size : for example if you expect to see a 500 bp band and a 4.5 kb out of a 5 kb plasmid after your digest, you need to digest atleast 1 µg as they will be divided as 900ng of the 4.5kb band and 100ng of the 500 bp band.
17. Run on the agarose gel for as long as required to obtain maximum resolution.

Transformation Protocol – 2008 – Princeton
http://2008.igem.org/Transformation_and_Plating
Transformations are used to insert the designed DNA into bacteria, which is then grown to multiply the plasmid DNA.

Heat transformation Protocol

1. Make sure that the incubator (30/37C) and water bath (42C) are ON
2. Make sure required antibiotic plates are present. Check the antibiotic resistance on the plasmid map.
3. Take the DNA out of –20 frig, let it thaw
4. Thaw the competent cells on ice for 7-8 min.

5. Add 1.0 µl of DNA (about 10ng) into the liquid (Don't vortex). Tap the sides of the tube to mix. For Ligation add 1-5 ul
6. Incubate the cells on ice for 30 min
7. Heat shock the cells for EXACTLY 30 sec at 42 C water bath.
8. Place on ice for 2 min.
9. Add 0.9ml of 37° C S.O.C medium to each tube (S.O.C is made by dissolving 0.5 ml of 20% glucose in 25 ml of SOB. Make sure that the SOC is clear and not cloudy/ contaminated.)
10. Shake the tubes at 37 C, 280 rpm for 60 min or 30 C for 90 min
11. Spin down the cells at 6000 rpm for 1 minute (should see white clumps at bottom).
12. Take out 0.85 ml of S.O.C. (so there's only 50 ul of SOC left inside)
13. Resuspend the cells.
14. Plate all 50 ul.
15. Incubate upright for 20 min., then upside down overnight (12-14 h) at 37 C or 16-18h at 30C.
Can leave the cells in the incubator for up to 18 hours but no more

Ligation Protocol – 2008 – Princeton
http://2008.igem.org/Ligate_DNA
Ligations attach two pieces of DNA at the cut site of the previous digestion, utilizing overlapping DNA sequences.

(For our lab)

1. Enter the names of the plasmids from which the vector and insert is obtained in the Excel worksheet (created with calculations listed below). Also enter the lengths of the vector and insert fragments.
2. The spreadsheet fills out the other columns to do a ligation with 100 ng of the vector fragment and a 1:3 molar ratio of vector to insert DNA.
3. All ligations are typically done at RT for 2 hours, however difficult ligations or ligations with low concentration of vector or inserts (<10 ng/µl) can be done for 16 hours at 16C.
4. Keep the buffer on ice at all times when out of -20C. Keep the enzyme in coolers at all times when out of the -20C.

Specific Ligation Calculations
1. Make a chart of the ODs and concentrations of your backbone and your insert. Add a column for Molecular weight.
Concentration = ng/ul
2. Calculate molecular weights by using this formula:
(# nucleotides)/2628 x (1.75*10^6) = g/mole (or ng/nmole)
3. Calculate nmole/ul (concentration/MW) for each
4. Calculate the number of moles in 100 ng of backbone.
5. Multiply that number by 3,4, or 5 (I don't know what molar ratio iGEM typically uses....I use either 3 or 5 molar ratio. The ratio is this: 5:1 insert:backbone (in moles).
6. Now you have the number of moles you need for the inserts. Convert that number to the number of ul you need. Also figure out number of ul of backbone.
7. Now you know the relative volumes for backbone and insert. From that, you can figure out how large your ligation reaction needs to be. You will have a 10x buffer and the T4 ligase in addition to your insert and backbone and you will also bring the volume up to the proper amount (if necessary) with sterile water.
8. Mix gently and leave at room temp (labeled well!) for 2 hours.

PCR Purification – 2008 – Princeton
http://2008.igem.org/PCR_Purification
PCR Purification allows for a buffer exchange and the ability to get rid of unwanted enzymes still surrounding the plasmid DNA.

Using QIAquick kit

1. Add 5 volumes of Buffer PBI to 1 volume of sample.
2. Pipette into a QIAquick spin column(max 770 µl) and centrifuge for 60 sec at 10,000g
3. Discard flow-through.
4. Wash: add 0.75ml Buffer PE(make sure that the buffer has ethanol added to it) to column and centrifuge for 1 min
5. Discard flow-through & centrifuge for 1 min
6. Place column into clean Eppendorf tube
7. Add 50ul Buffer EB or water to center of membrane
8. Let stand at RT for 3 min
9. Centrifuge for 5 min.

Measure the concentration using the UV spectrophotometer

CIP Treatment Protocol – 2008 – Princeton
http://2008.igem.org/CIP_Treatment
CIP treatment is done to phosphatase the vector used for plasmid ligations. This is done to reduce the self ligation of a vector digested with enzyme(s) creating compatible sticky ends and hence enhancing the Signal/Noise ratio of transformations.
1. Use 10 units of CIP per 1µg of DNA (over digesting by factor of X)
2. Calculate volumes

DNA µg = DNA volume * concentration
Enzyme volume = Enzyme unit/µl* # units = X [µl]
Buffer is dilution factor x dilution of the total volume.
[i.e. for 10X over digest - buffer is 10%, 3x - 30% of total volume]

3. Order of filling
• DNA
• Water
• Buffer
• CIP
4. Incubate for 3 hours at the specified temperature for the enzyme (37C).
5. Keep the buffer on ice and the CIP in the benchtop coolers when on the bench.

Digestion Protocol – 2008 – Princeton
http://2008.igem.org/Digestion
A Digestion is used to cut a plasmid at a specific site.

1. Use X units of enzymes per 1ug of DNA (over digesting by factor of X)

Usually we use over digest factor of 10 unless otherwise specified in the enzyme tech sheet. If over digestion results in star activity use 3X.

Total volume must be greater than or equal to 10X volume of enzyme.

3. Calculate the volume required for each
DNA µg = DNA volume * concentration
Enzyme volume = Enzyme unit/µl* # units = X [µl]
Buffer is dilution factor x dilution of the total volume.
[i.e. for 10X over digest - buffer is 10%, 3x - 30% of total volume]

6. Order of filling
• DNA
• Water
• Buffer
• Enzyme

7. Incubate for 3 hours at the specified temperature for the enzyme and deactivate at the appropriate temperature for 20 mins.

8. Please keep the buffer on ice at all times when out of -20C. Keep the enzyme in the benchtop coolers at all times when out of the -20C.

PCR SOEing Protocol – 2008 – Princeton
http://2008.igem.org/PCR_SOEing
PCR SOEing (Polymerase Chain Reaction - Splicing by Overlapping Extension) is a technique we've begun using in our lab for the first time this year. Two or more linear DNA fragments can be attached together without numerous steps of restriction digestion and ligation. 3' primers are created for each fragment which match the overlap 3' regions on the next downstream fragment, and 5' primers are created for each fragment which match the overlap 5' region on the previous upstream fragment.
For the process to work, equal amounts of molecules of all fragments are required. The calculations we used for finding these amounts are as follow :

[concentration of fragment #1]*[length of fragment #1 in bp]*[volume of fragment #1] = [concentration of fragment #2]*[length of fragment #2 in bp]*[volume of fragment #2]

Equal amounts of the oligos are then mixed with the DNA fragments along with PCR Supermix and placed into a Thermocycler under the following protocol:

Step1: 95C for 5 min.
Step2: 95C for 15 sec.
Step3: [lowest annealing temperature of all the primers] for 60 sec.
Step4: 68C for [1 minute plus 1 minute for every 1kb of the total PCR product].
Repeat Step 2: 35 times.
Step6: Hold at 4C.

At the end of the process, the resulting DNA must be run through gel electrophoresis to ensure the correct products were formed.

Run Gel Protocol – 2008 – Princeton
http://2008.igem.org/Run_Gel/_Gel_extraction

Gel extractions are used to extract the DNA from the correct bands on a gel.

Preparing the Gel
Dissolve UltraPure agarose to a final concentration of 1% in TAE buffer in a glass bottle.
Heat the solution in the microwave with frequent stirring to dissolve the agarose homogenously.
Place the solution in a 55C water bath for 15 mins.
Add 1 µl EtBr (Ethidium Bromide) per 50 ml of the solution and mix well.
Pour 50ml of solution per small gel tray. (the gel trays and combs should be pre-cleaned with water and wiped dry).
Wait for the gels to solidify. Label and store at 4C.
Use 120ml per large gel tray. Insert one large and one small comb in each small gel tray.

Running the Gel
There are two kinds of wells that fit different volumes:
Small - 15 ul
Big - 40 ul

Appropriate Hyperladder to be used for PCR product which is linear. Usually Hyperladder I will be used.
1. Add gel loading buffer (Orange G 6X), add 1X to 5X of DNA (it helps DNA sink into the bottom of the well) to DNA.
2. Make sure there is enough 1xTAE in the plate holder.
3. Load 5ul of appropriate hyperladder to one of the lanes.
4. Load appropriate amount of DNA (mixed with the buffer) in each well.
5. Add 10ul Ethidium Bromide to 400 ml Buffer at the positive end (Approx. 1 ul EtBr per 50 ml Buffer).
6. Set the timer and voltage to 120V and 60 min.

Gel Extraction Protocol using QIAquick Gel Extraction Kit:
All centrifugation steps are done at speeds > 13000 rpm
1. Look at the gel under low wavelength UV (high wavelengths will denature DNA). Quickly take a polaroid image, cut out relevant bands and shut OFF the UV.
2. Place the cut bands in 2ml Eppendorf tubes; Weigh slices; No more than 400mg per tube
3. Add 3 volumes of Buffer QG to 1 volume of gel (100mg ~ 100ul)
4. Incubate at 50C for 10min or until gel is dissolved; vortex every 2-3 min
5. Confirm that color of mixture is yellow (if not, add 10ul of 3M NaAc, pH 5.0)
6. Add 1 gel volume of isopropanol (not necessary for DNA fragments between 500-4000bp)
7. Add max of 770ul to QIAquick column and centrifuge for 1 min (max speed, ~13,000rpm, RT)
8. Discard flow-through and place column back in tube.
9. If needed, add rest of mixture to same tube (up to additional 770ul), spin, and discard flow-through
10. Wash: add 0.75ml Buffer PE(make sure that the buffer has ethanol added to it) to column and centrifuge for 1 min
11. Discard flow-through & centrifuge for 1 min
12. Place column into clean Eppendorf tube
13. Add 50ul Buffer EB or water to center of membrane
14. Let stand at RT for 3 min
15. Centrifuge for 5 min
16. Measure the concentration using the UV spectrophotometer.

PCR Protocol – 2008 – Princeton
http://2008.igem.org/PCR_Amplification
Materials:
Primers (FWD, RVS)

PCR Supermix
Parent plasmid

Methods:
1. Measure the concentration of the primers and the parent plasmid as follow: Do a dilution of 50:1 with 1EB buffer [do 98ul tris into 2 ul of DNA] Use the below formula to find the concentrations
Conc. (ng/ul) = OD reading * dilution factor * DNA factor = OD reading * 50 * 30
2. Mix 90ul of PCR supermix with 80ng of each DNA, 200nM of each primer
3. Put 90µl in 0.5ml tubes.
4. Find the length of the PCR product from Vector NTI
5. Run the PCR program

Thermocycler program:
Step1: 95C for 5 min.
Step2: 95C for 15 sec.
Step3: [lowest primer annealing temperature] for 60 sec.
Step4: 68C for [1 minute + 1 minute per 1 kb).
Go to Step 2: 35 times.
Step6: Hold at 4C.

Plasmid Extraction – 2008 – Prairie View
http://2008.igem.org/Protocols_PlasmidExtraction
Overnight Cultures

Flask with 25mL - 50mL media with corresponding antibiotic
Pick colony with pipette tip/loop and swirl in media
37*C Shake overnight

Pellet Cells
Transfer overnight cultures to 15mL centrifuge tube (blue cap)
Centrifuge 3000rpm for 6 min

Miniprep
1 Resuspend pellet in 250ul Buffer P1. Transfer to 1.5 mL microcentrifuge tube (by pipeting)
Be sure to vortex (with blue cap tube) thoroughly and that RNAse A was added to Buffer P1

2 Add 250ul Buffer P2 and mix thoroughly by inverting the tube gently 4-6 times
Do not vortex. Maximum reaction time: 5 minutes

3 Add 350ul Buffer N3 and mix immediately and thoroughly by inverting tube 4-6 times
4 Centrifuge for 10 minutes at 13,000 rpm (or 14,000) in a table-top microcentrifuge
5a Prepare the vacuum manifold and spin columns (blue)
5b Apply the supernatant from step 4 to the spin columns by decanting or pipetting
6 Vacuum
7 Wash the spin columns by adding 0.5mL Buffer PB. Vacuum
8a Wash the spin columns by adding 0.75mL Buffer PE. Vacuum
8b Repeat step 8a
9 Transfer the spin columns to a microcentrifuge tube. Centrifuge for 3 minutes
10 Place the column in a clean 1.5 mL microcentrifuge tube

To elute DNA, add 50ul 2mM Tris-Cl or d.s. H2O to the center of the spin column, let stand for 1 minute, and centrifuge for 1 minute

E.coli Transformation – 2008 – Prairie View
http://2008.igem.org/Protocols_E.coliTransformation
Cocktail:

70 ul d.s.H2O
10 ul Ligation product
100 ul Competent cells
____
180 ul Total

Control:

70ul d.s.H2O
10ul ligation product
100ul Competent cells
____
180ul Total

Procedure:

Let product sit on ice for 10 minutes
Take out plates with corresponding antibiotics
Label plates
Heat shock in 42*C water bath for 1.5 minutes
Put on ice for 5 minutes
Turn on Bunsen Burner
Pipette all cells and put on plate
Spread cells on plate with streaks for cell/colony dillution
Put in 37*C incubator lid facing down for 8 hours or overnight

Ligation – 2008 – Prairie View
http://2008.igem.org/Protocols_Ligation
Cocktail:

5.5ul d.s.H2O
1ul Backbone (keep on ice)
2ul Insert (keep on ice)
1ul Buffer (T4 DNA buffer)
0.5ul Ligase (T4 DNA ligase)

== Negative Control 1 ==
Use 2mM Tris-Cl in place of insert

== Negative Control 2 ==
Use 2mM Tris-Cl in place of backbone

== Control for PNK ==
Use 2ul of d.s. H2O instead of insert

Let stand at room temperature for 20 minutes

Gel Purification – 2008 – Prairie View
http://2008.igem.org/Protocols_GelPurification
Extraction Procedure

1. Cut out DNA fragment
2. Add 600ul of Buffer QG
3. Incubate at ~50*C until gel has completely dissolved, vortexing occasionally
4. Check mixture is yellow in color
5. Add 100ul of isopropanol and mix by inverting the tube several times
6. Place a purple spin column in a provided 2mL collection tube in a suitable rack and pour mixture in it
800ul maximum per column
7. Centrifuge for 1 min and discard the flow-through and place the spin column in the same collection tube
8. Add 500ul of Buffer QG to the spin column and centrifuge for 1 minute
9. Discard the flow-through. Place the column in a clean 1.5mL microcentrifuge tube
10. Centrifuge for an additional 2 minutes
11. Place the column into another clean 1.5mL microcentrifuge tube
12. To elute DNA, add 50ul of 2mM Tris-Cl for backbone and 30ul 2mM Tris-Cl for insert into the center of the membrane, let stand for 1 minute, and centrifuge for 1 minute

To take away phosphates from backbone...

Add to purified Gel:
5 uL of 10X Antarctic Buffer (1/10 of total volume)
1 uL of Antarctic Phosphatase Enzyme
Incubate this for 15 min @ 37 C

Then heat inactivate for 5 min @ 65 C

Product ready for ligation

Electrophoresis – 2008 - Prairie View
http://2008.igem.org/Protocols_Electrophoresis
Making Gel

1% gel by dilution
=> 0.6g Low melt PCR agarose
=> 1.5mL 40x TAE
=> 58.5mL ddH2O ==> 1.5ul Ethidium Bromide

Running Gel

20ul digestion product
Add 5ul of 5x loading buffer
Load lane
10ul ladder marker

Run gel for 30-45 minutes

Protocols Test Digestion – 2008 – Prairie View
http://2008.igem.org/Protocols_Test_Digestion
Cocktail:

3 uL DNA
1 uL buffer
0.5 uL enzymes
5 uL H2O
____
10 uL total volume

Incubate at 37 C for 30min.
Run Gel.

Protocols Digestion – 2008 – Prairie View
http://2008.igem.org/Protocols_Digestion
Cocktail:

12ul d.s.H2O
3ul plasmid
2ul Buffer
1.5ul Enzyme I
1.5ul Enzyme II
____
20ul Total

1 hour incubation @ 37*C
(Up to 2 hours for more complete digestion)

Colony PCR – 2008 – Prairie View
http://2008.igem.org/Protocols_ColonyPCR
Procedure
1 Add 5 volumes (~250mL) of Buffer PBI to 1 volume (~50mL) of the PCR sample mix
2 Check that the color of the mixture is yellow (similiar to Buffer PBI w/o PCR sample)
If the color is orange or violet, add 10ul of 3M sodium acetate, pH 5.0 and mix

3 Place a QIAquick spin column in a 2mL collection tube (Purple Column)
4 To bind DNA, apply the sample to the QIAquick column and centrifuge for 30-60s
5 Discard flow-through. Place the QIAquick column back into the same tube

6 To wash, add 0.75mL Buffer PE to the QIAquick column and centrifuge for 30-60s
7 Discard flow-through and place the QIAquick column back in the same tube.
Centrifuge the column for an additional 1 minute to evaporate ethanol

8 Place QIAquick column in a clean 1.5mL microcentrifuge tube
9 To elute DNA, add 50ul Buffer EB (or 2mMTris) or water to the center of the QIAquick membrane and centrifuge the column for 1 minute.
Alternatively, for increased DNA concentration, add 30ul elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute and then centrifuge.
Maximum elution efficiency with pH 7.0-8.5

10 Store DNA at -20*C

PCR Purification – 2008 – Prairie View
http://2008.igem.org/Protocols_PCRPurification
Procedure
1 Add 5 volumes (~250mL) of Buffer PBI to 1 volume (~50mL) of the PCR sample mix
2 Check that the color of the mixture is yellow (similiar to Buffer PBI w/o PCR sample)
If the color is orange or violet, add 10ul of 3M sodium acetate, pH 5.0 and mix

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5 Discard flow-through. Place the QIAquick column back into the same tube
6 To wash, add 0.75mL Buffer PE to the QIAquick column and centrifuge for 30-60s
7 Discard flow-through and place the QIAquick column back in the same tube.
Centrifuge the column for an additional 1 minute to evaporate ethanol

8 Place QIAquick column in a clean 1.5mL microcentrifuge tube
9 To elute DNA, add 50ul Buffer EB (or 2mMTris) or water to the center of the QIAquick membrane and centrifuge the column for 1 minute.
Alternatively, for increased DNA concentration, add 30ul elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute and then centrifuge.
Maximum elution efficiency with pH 7.0-8.5

10 Store DNA at -20*C

PCR Mutation – 2008 – Prairie View
http://2008.igem.org/Protocols_PCR_Mutation
Set up two separate primer extension reactions
One for each top and bottom primer
Cocktail: 40ul H2O
5ul 10X Pfu Buffer
1ul dNTP (10mM)
1ul Pfu Polymerase
1ul of each Primer (10uM)
1ul Template
____
50ul

ThermoCycle Program
94*C.......30 sec

____
95*C.......30 sec
55*C.......60 sec
68*C.......2 min/kb

Run for a total of 4 cycles
Combine 25ul from each extension reaction above. Add 1ul Pfu Polymerase.
Incubate as above for a total of 18 more cycles.

Protocols PCR – 2008 – Prairie View
http://2008.igem.org/Protocols_PCR
Cocktail:
37ul H_2O
5ul 10X PCR Buffer
1ul dNTP (10mM)
0.5ul Polymerase
0.5ul of each Primer (100uM)
1ul Template
____
50ul

ThermoCycle Program
98*C.......30 sec
____
98*C.......10 sec
65*C.......30 sec
72*C.......(20 sec/kb {Min 20sec})
____
72*C.......5 min
10*C........Forever

Alternate ThermoCycle Program #1
90*C.......30 sec
____
92*C.......10 sec
62*C.......30 sec
72*C.......(20 sec/kb {Min 20sec})
____
72*C.......5 min
10*C........Forever

Alternate ThermoCycle Program #2
90*C.......30 sec
____
92*C.......10 sec
62*C.......60 sec
72*C.......(20 sec/kb {Min 20sec})

72*C.......5 min
10*C........Forever

Colony PCR – 2008- MIT
http://2008.igem.org/Team:MIT/Colony_PCR
Colony PCR
2 uL primer #1 (to 10uM) 2 uL primer #2 0.4 uL DNTPs 2 uL Buffer 5 uL colony culture 0.2 uL Taq 10.4 uL H20
92C 1:30 (92 0:30 50 :30 72 1:30) repeat x30 72 10:00

Transforming Lactococcus lactis – 2008 - MIT
http://2008.igem.org/Team:MIT/Transforming_Lactococcus_lactis

Pretreatment of L. lactis with lithium acetate and/or dithiothreitol was as follows: 109 cells were suspended at room temperature for 30 min in 8 ml of 100 mM LiAc, 10 mM DTT, 0.6 M sucrose, and 10 mM Tris-HCl, pH 7.5. Following treatment, the cells were pelleted, resuspended in 1.5 ml microcentrifuge tube, and washed as described above.
Electroporation: Overnight cultures of L. lactis of both strains grown at 37°C MRS broth were diluted 1:12.5 in 25 ml of MRS. Cells were harvested by centrifugation at 10,000 g for 10 min when the optical density at 660 nm was between 0.26 and 0.38. The cells were washed sequentially with the following ice-cold solutions by alternate centrifugation and resuspension: bidistilled water 2.0 ml, bidistilled water 1 ml, 50 mmol/l EDTA 1 ml, bidistilled water 1 ml, 0.3 mol/l sucrose 1 ml, 0.3 mol/l sucrose 0.3 ml. After the final suspension, the cells were immediately electroporated at a concentration of 1010 cells/ml, using an electroporator with pulse controller (Electro Cell Manipulator™ 600, BTX, USA). Electroporation was performed by a single pulse at 2.5 kV (E = 12.4 kV/cm), 200 Ω, and 25 µF (corresponding to pulse length of 4.6 ms), in 2 mm disposable electroporation cuvettes, using 1 µg of purified plasmid DNA. The cell suspension was diluted immediately by the addition of 5 ml MRS broth supplemented with 10 µg/ml CM and incubated for 2 h at 37°C before being placed on MRS agar supplemented with CM (10 µg/ml). Transformation was confirmed by selection in CM containing medium and plasmid analysis.

Tooth binding assay protocol – 2008 – MIT
http://2008.igem.org/Team:MIT/Tooth_binding_assay_protocol

Home	**The Team**	**The Project**	**Experiments**	**Parts Submitted to the Registry**	**Results**	**Notebook**

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2 Tooth binding assay (reference: PMID 9062560)
3 Why is it novel?
4 Reagents and Recipes

Culturing and maintaining S. mutans

Note: S. mutans needs to grow at 37C anaerobically. Estimated doubling time is 90 minutes.

To make a glycerol stock for long-term storage of live S. mutans, grow a 5mL culture until it is turbid (usually takes 16 hours). Add glycerol to a final concentration of 50%. The final 50% glycerol stock of S. mutans can be stored at -20C for 3 months or at -70C for years.

Night before binding assay--Inoculation--set up 3 tubes with 5 mL THB and add 10 (should be about OD 1.8 after 15 hours), 25 and 50 microL of Glycerol stock to the tubes.

Tooth binding assay (reference: PMID 9062560)

Wash 50 mg HA beads 3 times with 1mM phosphate buffer (PB) and equilibrate in PB for 2 hours (or overnight)
HA beads added to 500 microL 1:5 diluted saliva (with PBS) and stir suspension for 1h at 37 C
Aspirate saliva
Treat HA beads with 2 mg/mL BSA in PBS for 30 min. at 37 C
Take OD of overnight S. mutans culture, if initial OD is above 1 dilute with THB so that the OD is between .1 and .5
Dilute bacteria with 1mMolar PBS with 2 mg/mL BSA to get the final concentration of 1-2 *10^7 CFU (.075 to .16 OD--use .10)
Add 1mL S. mutans suspension to HA beads, incubate at 37 C at 20 rpm in microfuge tubes
Extract 100 µL supernatant after letting HA beads settle for 5 minutes. and transfer into fresh epindorf tube, leave at 37 C
Repeat above after 1h, 2h
Aspirate and wash beads twice with 1 mL PBS (with or without BSA), rotate tube vertically to mix instead of vortex
Add 1 mL PBS with 1 mM EDTA to beads, rotate vertically at 37 C for 10 min
Let beads settle and take 100 microL of supernatant
(proceed to serial dilution and spotting)
Count colonies (approximately CFUs) on the three samples and calculate the amount attached to HA beads through the relation "CFU supernatant time 0 – CFU supernatant time 1h, 2h = CFU on HA beads"
OD-CFU conversion factor: optical density at 600 nm of 0.75 to 0.8: 1 × 10^8 CFU/ml)

Why is it novel?

A couple of things are novel in the protocol. Firstly, we modified the protocol by including a dechaining step to prevent S. mutans from sticking to each other (We read an article which stated that S. mutans form long chains in solution). We dechanined them by passing them through a syringe 15 times and also vortexing them with glass beads. Secondly, we are coating the beads with BSA to prevent non specific binding. We increased the saliva coating time than what is reported in the literature after we got results indicating a correlation between time beads remain in saliva and binding. Thirdly, we washed the beads in EDTA and plated both the supernatant and the beads to confirm there is bacteria on the beads (the paper with the original protocol failed to prove that there was bacteria on the beads using the indirect assay). Finally, our most significant addition to the protocol has been the way we plate after incubation with HA beads. We are using the spotting method where we take some supernatant from each tube and serially dilute it in a 96 well plate. Then we spot, drawing about 5microliters from each microwell, onto a rectangular plate.

Reagents and Recipes

PBS — NaCL 8.0 g L-1, KCl 2.0 g L-1, Na2HPO4, 2H20 2.0 g L-1, KH2PO4 2.0 g L-1; pH 7.2 (Verify using a pH meter)
Todd Hewitt Broth (THB) liquid and agar - 15g THB powder, (10g agar), bring volume up to 500 mL using distilled water from white tap. Stir to dissolve THB powder. (agar remains undissolved until autoclaving) Autoclave using liquid cycle for 30 min. Let THB liquid cool to room temp then add 2 mL of 50 mg/mL Streptomycin sulfate (thaw and vortex well before pipetting; our S. mutans strain is resistant

to streptomycin so we use the antibiotic to reduce contamination) and 5 mL of 10% glucose. Seal bottle with cap to reduce evaporation. Keep the liquid THB at 37C so that it is conveniently pre-warmed.

Protein Purification – 2008 – MIT
http://2008.igem.org/Team:MIT/Protein_Purification
TEV Purification Protocol
These volumes are for 4L LB culture split into 4 cell pellets
Buffers:
Binding Buffer: 20mM tris +.5M NaCl
Elution Buffer: 20mM tris +.5M NaCl + 300mM Imidazol
Re suspend pellets in 30mL BB per cell pellet into centrifuge tube
Lyse cells with sonicator
Sonicate for 1min
Ice for 1min
Repeat 4-5 times
Centrifuge at 14,000 for 40miin
Save supernatant and pellet for gel
Filter supernatant
Prepare 2 NiNTA columns
Add 5mL NiNTA to each
Wash with 1 column volume ddwater
Equilibrate with 4 c.v. BB
Load filtered supernatant
Collect FT
Save some for gel
Wash with 8 c.v. BB
Collect W1-4
Save some of each for a gel
Elute with 5 c.v. EB
Collect E1-5
Save some of each for a gel
Add 1mM EDTA and 1mM DTT to all elution tubes
Run a gel.
Peptide Purification Protocol
First day:

Lysis buffer:
200mL 1x binding buffer
200 mg lysozyme
40 µL 5mM AEBSF
4 enzyme tablets
Thaw frozen cell pellets
Resuspend cells in 30mL lysis buffer.
Sonicate for one minute to homogenize.
Rock at 4C for 2 hrs to lyse
Spin lysate 40min and filter
Put 4mL Ni-NTA into flow tubes, wash w/ water and equilibrate w/ 8mL BB
Load filtered supernatant and collect flow through (FT)
Wash with 16mL BB
collect two washes (W1, W2)

Elute with 16mL EB and collect 4 elutions (E1-4)
Dialyze E1 and E2 into TEV cleavage buffer overnight
2L –
5mL EDTA
40mL 4M NaCl
100ml tris
Second day:
Add 300 µL tev protease to each
Cleave for two hours at rm temp
Spin down for 10min at 3500rpm
Add 1mL Ni-NTA resion to columns
Wash with water
Equilibriate with 8mL BB
Load supernatant from tev cleavage and collect flow through
Wash with 15mL BB and collect three washes
Elute with 20mL EB and collect two elutions

Preparing Antibiotic Stocks – 2008 - MIT
http://openwetware.org/wiki/Endy:Preparing_Antibiotic_Stocks

Ampicillin
Stocks & Usage
Stock Concentration - 50mg/ml in H_2O
Aliquots - 100µl and 500µl
Working Concentration - 50µg/ml
Preparation of 80ml stock solution
Ampicillin is kept in the 4C fridge in 68-564D. It is light sensitive.
Weigh 4g of ampicillin into a small weigh boat.
Add 80ml of milliQ to a 250ml bottle.
Add the ampicillin to the milliQ
Mix/vortex so all the ampicillin goes into solution.
Filter sterilize the solution into a falcon tube using a 20ml syringe and a 200nm filter.
Aliquot into pcr tubes and 1.7ml eppendorfs.
Store at -20C.
Store the small aliquots in the small box and the big aliquots in the larger box.
Kanamycin
Stocks & Usage
Stock Concentration - 10mg/ml in H_2O
Aliquots - 200µl and 1ml
Working Concentration - 20µg/ml
Preparation of 80ml stock solution
Kanamycin is kept in the 4C fridge in 68-564D. It is light sensitive.
Weigh 800mg of kanamycin into a small weigh boat.
Add 80ml of milliQ to a 250ml bottle.
Add the kanamycin to the milliQ
Mix/vortex so all the kanamycin goes into solution.
Filter sterilize the solution into a falcon tube using a 20ml syringe and a 200nm filter.
Aliquot into pcr tubes and 1.7ml eppendorfs.
Store at -20C.
Store the small aliquots in the small box and the big aliquots in the larger box.

Tetracycline
Stocks & Usage
Stock Concentration - 5mg/ml in 70% Ethanol
Aliquots - 200µl and 1ml
Working Concentration - 20µg/ml
Preparation of 80ml stock solution
Tetracycline is kept in the 4C fridge in 68-564D. It is light sensitive.
Weigh 400mg of tetracyline HCL into a small weigh boat.
Dilute 95% Ethanol to 70% using milliQ water.
Adding 20ml of milliQ to 60ml of 95% ethanol gives 80ml of 71% ethanol.
Add 80ml of 70% Ethanol to a 250ml bottle.
Add the tetracycline HCL to the ethanol.
Mix/vortex vigorously so all the tetracycline goes into solution.
Filter sterilize the solution into a falcon tube using a 20ml syringe and a 200nm filter.
Aliquot into pcr tubes and 1.7ml eppendorfs.
Store at -20C and protect any unused stock solution from light.
Store the small aliquots in the small box and the big aliquots in the larger box.
Chloramphenicol
Stock concentration - 34mg/ml in 100% Ethanol
Aliquots - 1ml
Working concentration = 25µ/ml (Stringent), 170µ/ml (relaxed)
Preparation of 80ml stock solution
Chloramphenicol is kept at room temperature. It is near our other chemicals
Weight 2.72g of chloramphenicol sulfate into a small weight boat.
Add 80ml 100% EtOH to the chloramphenicol
95% EtOH probably works just fine, but I haven't tried it.
Mix/vortex vigorously so all the chloramphenicol goes into solution.
Aliquot into 1.7ml eppendorfs.
N.B. There is no need to filter sterilize, as it is in EtOH.
Store at -20C.
Streptomycin
Stocks & Usage
Stock Concentration: 50mg/ml in H_2O
Aliquots - 0.5 ml
Working Concentration - 10-50µg/ml
Preparation of 40ml stock solution
Streptomycin is kept in the 4C fridge in 68-564D. I don't think it is light sensitive (not stored in opaque bottle).
Weigh 2g of streptomycin into a small weigh boat.
Add 40ml of milliQ to a 100 or 250ml bottle.
Add the streptomycin to the milliQ
Mix/vortex so all the streptomycin goes into solution.
Filter sterilize the solution into a falcon tube using a 60ml syringe and a 200nm filter.
Aliquot into 1.7ml eppendorfs.
Store at -20C.

MIT iGEM Restriction Digests – 2008 – MIT
http://openwetware.org/wiki/MIT_iGEM_Restriction_Digests
Restriction Digest protocol

For Analytical digests, aim for 500-1000ng of DNA; for a preparative digest (where you know you want to cut out a band) try to load as much DNA as possible (gel lanes w/ wide-toothed combs can hold up to 50µl).

Materials

DNA; the thing you want to cut. Usually plasmid or PCR product. Measure concentration in Nanodrop beforehand.

Appropriate NEB 10x Buffer (check the NEB enzyme chart or catalogue to find compatible buffers).

Appropriate enzymes.

ddH2O

BSA (100x from NEB)

Method

The following volumes apply to a 20µl analytical digest; for larger, preparative digests, simply scale up (eg. for a 30µl digest, use 3µl of 10x buffer, etc)

Add 20µlTotalVolume-(µlDNA + µlBuffer +µlBSA +µlEnzyme)µl ddH2O to PCR tube. (eg. 20µl-(1µlDNA+2µlBuffer+0.2µlBSA+0.5µlEnzymeA+0.5µlEnzymeB)=15.8µl ddH2O)

Add 0.2µl BSA to tube.

Add 2.0µl 10x Buffer to tube.

Add appropriate amount of DNA to tube.

Add 0.5µl of each enzyme to tube.

MIX THE REACTION BY PIPETTING HALF THE VOLUME UP AND DOWN.

Incubate the reaction at 37*C for 2hrs to ensure complete digestion.

Deactivate the enzymes by heating @ 80*C for 20min (in the thermocycler). This will inactivate most enzymes, though check to make sure that your enzyme can be heat deactivated by checking in the NEB catalogue.

Store digest at -20*C or run immediately on gel.

MIT iGEM Agarose Gels -2008 – MIT

http://openwetware.org/wiki/MIT_iGEM_Agarose_Gels

Running an Agarose Gel

Brief intro:

Agarose gels are analytical and functional tools for physical partitioning of DNA fragments. The DNA fragments are separated by size on the gel and are visualized by fluorescent dyes or ethidium bromide. The visualization of the DNA in the gel allows us to analyze restriction digests and the presence/absence of correct PCR product, among other things. We can also cut out desired fragments of DNA that have been separated from other DNA (such as a plasmid w/ an undesirable antibiotic marker) for further cloning.

Materials

DNA, the thing you want to analyze/cut out. Measure concentration in nanodrop beforehand.

1xTAE buffer. Make a 1/10 dilution from bought iGEM stock.

SybrSafe dye, 10,000x. Use iGEM aliquot.

Gel well and **comb**. Use appropriate comb for your need. For analytical gels, can use thin combs. For preparative gels, use wide-toothed combs.

Melted 1%Agarose in 1xTAE.

Prepare ahead of time by melting appropriate amount of Agarose mixed in TAE in the microwave. (eg. to prepare 500ml of melted 1% agarose, mix 5g of agarose in ~495ml 1xTAE in a foil-covered flask and microwave for 6+min; keep an eye on it every couple of minutes when you microwave so it doesn't boil over; be careful to wear gloves when handling hot liquid containers). Can keep the melted agarose in the 65*C oven for convenience or leave on bench and re-melt every time you need it.

Setting up and Running the Gel

Set up your *clean* gel tray by taping both sides w/ lab tape and putting the*clean* comb in place.
Thin-toothed combs will hold <20µl.
Wide-toothed combs will hold <50µl.
Measure out the needed amount of melted agarose in a cylinder and pour it in a beaker.
For small gels, use 35-40ml.
For large gels, use 95-100ml.
Add appropriate amount of 10,000xSybrSafe dye to the melted agarose. (eg. for 35**ml** of agarose, add 3.5**µl** of SybrSafe).
MIX the SybrSafe into the agarose by swirling the agarose.
POUR the agarose into the gel tray you prepared.
WAIT ~10min for the agarose to harden. It should turn a somewhat opaque light blue.
While waiting for the agarose to harden, MIX your DNA sample w/ 5x Ficoll Orange stain. (eg. for 10µl of PCR product, add 2-3µl of 5x Ficol Orange stain). The stain has dense glycerol in it and makes the DNA fall to the bottom of the well.
You don't need to stain the ladder DNA, since it's been pre-stained.
Once the agarose has hardened, REMOVE the tape and PLACE the gel in the gel box. Make sure the gel is covered in 1xTAE buffer.
Remove the comb GENTLY! This is easiest by pulling the comb up from one side first; don't try to pull the whole thing out at once or it'll break.
Load your DNA. 500ng of the ladder is usually plenty. If you can't see the wells, try placing a dark blue nitrile glove behind the gel box to increase contrast between the empty wells and the rest of the gel.
Hook up the leads to the correct plugs and turn on the power supply.
Typically, we run a small agarose gel at 100volts for 1hr, but you can vary the time and voltage according to your time/resolution needs.
Be careful not to let the current become >110mA or the gel might start to melt. You can watch the Ficol Orange stain travel ahead of the DNA, so once it reaches the end of the gel you know you can't run it any longer.

Viewing the Gel

Turn OFF the generator and power supply.
Using gloves, REMOVE the gel tray from the gel box. BE CAREFUL! Don't let it slip from the tray and break! Use both hands.
Bring the gel to the Endylab gel box in the supply room. Wipe down the viewing tray in the box to remove residual Ethidium Bromide that may have been left by the Sauer lab.
Place the gel on the viewing tray (can be left on the gel tray, but the image can be improved by removing the gel from the tray), slide it into the box, and close the sliding cover.
Open the GeneSnap software if it's not open already. Make sure the top left button is green. If it's red, click on it to freeze the image.
Set exposure time to 150ms, light source to Upper White, filter to No Filter. Click on the Green button in the upper left. You should now be able to view your gel in white light and adjust its position and the zoom and focus w/ which you view it.
Once you're happy w/ your gel's position/zoom/resolution, click on the red button in the upper left. This freezes the image. Now, change exposure time to 2s, light source to Transilluminator, filter to Short Band Path (this might take a couple of tries to click before the sofware accepts it; just keep clicking it).
Click on the green button. Your gel should appear as seen through UV light. If it looks OK, IMMEDIATELY PRESS THE RED BUTTON. If you leave the transilluminator on, the dye bound to your DNA will bleach and you won't be able to re-image the gel if you need to. Also, UV damages DNA, so if you need to cut out your DNA, you might be incurring damage.
If you are not happy w/ your gel image, re-adjust the settings and re-image until you are happy.
Sometimes simply playing w/ the exposure time can make a hard-to-see band easier to visualize.

Cutting out Gel Bands

Once you've imaged your gel in GeneSnap and have confirmed that the band you want to cut out is there, bring your gel to the UV lightbox next to the Gelbox setup.
Remove your gel from the tray and place it on the UV lightbox.
Be sure you have on hand a sterile razor blade and empty epp-tubes into which you can place your gel slices.
Don the appropriate protective gear (lab coat, eyewear) and close the door to protect yourself and other people from the UV.
Turn off the light in the room and turn on the UV lightbox using the knob on the front left of the box. One click clockwise goes to Analytical, which is very bright and damages you and the DNA; another click goes to Preparative, which is less bright; try to use the Preparative setting if you can.
Cut out the desired bands carefully using the razor blade. Work swiftly to minimize the amount of time the UV light is on and therefore the amount of damage to the DNA.
Once you are done w/ your gel, gather up the pieces and dispose of it in the hazardous waste bin labeled "SybrGold".
Use the Gel Extraction protocol from Qiagen to extract the DNA from your gel. Expect low yields. This kit at

Endy pcr – 2008 – MIT
http://openwetware.org/wiki/Endy_pcr
PCR in the Endy Lab
We generally use Invitrogen's Taq-based Platinum HiFi PCR supermix for most of our reactions. Following the manual is straightforward. The manual is availablehere (PDF). Read it before you use the mix for the first time and keep it for reference. If the link is broken, simply Google "Platinum PCR SuperMix."

Briefly, a typical reaction is set up as follows:

1. set up pre-labeled reaction tubes on ice

2. add the following components:
- 45µL Platinum PCR SuperMix (rock gently after thawing, quick spin before use)
- 200nM final concentration of each primer (insert calculation short cut here)
- template DNA
(note: the total volume of primers and template can be 0.5 to 20µL)

3. make sure reaction tubes are properly capped before placing in thermocycler

4. perform PCR with an initial heating step at 94C for 2 minutes followed by 25-35 cycles of 30sec at 94C, 30sec at 55C and 1Kb/min at 72C

DNA Sequencing – 2008 – MIT
http://web.mit.edu/biopolymers/www/DNA.html
General Information
We use the Applied Biosystems Model 3730 capillary DNA sequencer with Big Dye Terminator Cycle Sequencing Kit. Maximum read lengths are approximately 1000 bases. The sequence is readable about 30–50 bases downstream of the primer sequence. We anneal at 50°C and extend at 60°C so make your primer melting point (Tm) ca. 55°C. You may want to do a Google search for "Oligo Molecular Weight Calculator" to find web pages that will calculate Tm quickly.

Primer Considerations
High Purity
Appropriate concentration
No mismatches
No alternative hybridization sites in template (false priming)
No palindromic sequence present, particularly at the 3’ end of primer
Appropriate length to give Tm of ~55-60°C. (generally 20-24 bases)
GC "clamp" on the 3' end
Desirable [GC] = ~50-55%
Avoid strings of four or more of same the base if possible
Avoid low Tm (i.e. 40-45°C.) If Tm is low, make the primer longer.
Template Primer Quantity Table
Supply the required **template plus primer combined in a single tube** diluted to 12ul with H2O (submit only 6 ul if using 96 well plate and scale down amounts by 50%)

Template	**Quantity**
PCR products:	
100-200 bp	1.5-9 ng
200-500 bp	3-30 ng
500-1000 bp	6-60 ng
1000-2000 bp	15-120 ng
>2000 bp	30-150 ng
Single-stranded DNA	30-150 ng
Double-stranded DNA	400-900 ng
Primer	10 pmoles
For the following templates types submit sample and primer in **SEPARATE** tubes:	
Template	**Quantity**
BAC DNA	0.6-3.0 ug in total volume of 10ul
Bacterial genomic DNA	3-9 ug in total volume of 10ul
Primer	5ul at 10pmol/ul (10uM)

Template Preparation for Successful Automated DNA Sequencing
Pure DNA should give an OD260/280 of between 1.7-1.9 (1.5-1.7 is usually OK) and an OD200/260 of about 1.1. Low 260/280 indicates protein contamination, high OD260/280 indicates possible RNA or residual organics contamination. High OD200/260 indicates contamination by organics and/or salts.
Commercial Methods for Template Preparation
In addition to the Applied Biosystems Sequencing Chemistry Guide, also see Qiagen’s web page for DNA purification products. Qiagen has an excellent DNA Template Preparation Guide and Troubleshooting publication. Your final purification step should be a column spin using water. Substitute

water for “elution” buffer to obtain reproducible results. Elution buffer components (e.g. tris, NaCl, EDTA) cause run failures unless diluted out. Also see "ExoSAP-IT" from USB Corporation for PCR product cleanup.

Ensuring Template Quality

The quality of DNA in a reaction affects the performance of the DNA Analyzer. When preparing DNA templates, it is critical to avoid the following:

Residual salts

Proteins

Residual detergents

Residual RNA

The presence of residual salts, proteins, RNA, and detergents can interfere with capillary electrophoresis and electrokinetic injection. Your current template purification methods may have to be modified to remove residual salts, proteins, and detergents.

Effect of Residual Salts

The 3730 DNA Analyzer is especially susceptible to salt in samples from template preparation. The negative ions in salts can be preferentially injected into the capillary array during electrokinetic injection, leading to lower signal. In addition, the negative ions compete and interfere with the injection of larger DNA extension fragments, leading to shortened read lengths. If salts and unincorporated dyes are not removed from the
sequencing reaction, they will compete with extension fragments during electrokinetic injection and result in weak signals.

Effect of Proteins

Many DNA preparation methods for sequencing require the recovery of DNA from lysed bacterial cultures. Unless DNA is carefully purified, protein can remain in the DNA samples. Protein can be injected and adhere to the walls of the capillary array adversely affecting data resolution.

Effect of Residual Detergents

Some methods of template preparation, such as the Thermomax method for M13 preparation, use detergents such as Triton X-100 to lyse the protein coat of phage particles. Other detergents, such as sodium dodecyl sulfate (SDS), are used in plasmid purification protocols to lyse bacterial cells. Small, negatively charged detergents may be preferentially injected over DNA during electrokinetic injection. If present at high levels, detergents such as Triton X-100 and SDS will adversely affect the life of the capillary array and the quality of the sequencing data.

Effect of Residual RNA

Residual RNA that is present in DNA template preps competes with the DNA for injection into the capillary array. Residual RNA has the same effect as excess salt, that is, decreased signal and shortened read lengths.

Template Quantity

Effect of Too Little Template

Too little template or primer reduces the signal strength and peak height. In the worst case, the signal-to-noise level decreases so that bases cannot be called.

Effect of Excess Template

Excess template can affect data quality when present in sample loading onto the DNA Analyzer. Excess template inhibits the injection of extension fragments thus affecting signals generated from the instrument. Excess template can behave similarly to proteins and accumulate in the capillary array, which affects data resolution. (Applied Biosystems 3730/3720xl DNA Analyzers Sequencing Chemistry Guide, p.18-22).

Host Bacterial Strains

The host strain used for a specific template preparation can impact template quality.

Applied Biosystems reports the following on host strain variability vs. sequencing results

• DH5 alpha host strains consistently produce good results.

• HB101, MV1190, JM109 and XL1 Blue host strains show some variability in result quality. XL1 Blue

grows slower than most strains and can lead to decreased DNA yields and it does not respond to TB as do other strains (only showing a 2-3 fold increase in cell number per ml).
• JM101 (JM 100 series) is not recommended.
The following is anecdotal:
Avoid Terrific broth and other rich media
Avoid host strains TG1 and TG2 which contain high carbohydrate levels
Note: Template preparation or purification procedures which involve the use of phenol or chloroform should be avoided if possible. If use of phenol or chloroform can not be avoided an additional ethanol precipitation is recommended.

Some Primer Sequences Used in Fluorescent Sequencing (5'-3')

Bac Forward TTTTACTGTTTTCGTAACAGTTTT
Bac Reverse CGGATTTCCTTGAAGAGAGTA
Bac(+15) Reverse ACTTCAAGGAGAATTTCC
Bac1 ACCATCTCGCAAATAAATAAG
Bac2 ACAACGCACAGAATCTAGCG
BacMam 3' TCCCATATGTCCTTCCGAGTGA
BacMam 5' ACGTGCTGGTTGTTGTGCTGTCT
BGH Reverse TAGAAGGCACAGTCGAGG
BK Reverse ACAGGAAACAGCTATGACCTTG
Blue2S ACCGCTGCTGCTAAATTCGAA
Bluescript KS TCGAGGTCGACGGTATC
Bluescript SK CGCTCTAGAACTAGTGGATC
CBDcenA TCAACGGCACCACCTGCA
CBDcexLEAD TAGGTGCAACTGTTGTTCTG
CBDclos CAACACCAGTTGTAAATCCA
EBV Reverse GTGGTTTGTCCAAACTCATC
M13 Forward (-20) GTAAAACGACGGCCAGTG
M13 Forward (-41) GGTTTTCCCAGTCACGAC
M13 Reverse (-27) GGAAACAGCTATGACCATG
M13 Reverse (-48) AGCGGATAACAATTTCACAC
pCDM8 Reverse TAAGGTTCCTTCACAAAG
pCEP Forward AGAGCTCGTTTAGTGAACCG
PCMV Forward CGCAAATGGGCGGTAGGCGTG
pTRE 3' CCACACCTCCCCCTGAAC
pTRE 5' CGCCTGGAGACGCCATCC
pYESTrp Forward GATGTTAACGATACCAGCC
pYESTrp Reverse GCGTGAATGTAAGCGTGAC
SP6 TACGATTTAGGTGACACTATAG
T3 CAATTAACCCTCACTAAAGG
T3 Bluescript AATTAACCCTCACTAAAGGG
T7 GTAATACGACTCACTATAGGG
T7 Promoter pET AAATTAATACGACTCACTATAGGG
T7 Terminator GCTAGTTATTGCTCAGCGG
T7 Terminator pET GCTAGTTATTGCTCAGCGG

Vectors Incompatible with the Above Primers

Vector	Primer(won't work)	Alternate Primer(will work)
pT7T3	T3	M13 FWD
pT7T3	T7	T7 Promoter

pSP72	T7	T7 Promoter
pcDNA	T7	T7 Promoter

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Cost
See Pricing Chart.
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How to Order
We use a laboratory information management system (dnaLIMS) to handle orders and distribute data. Templates are mixed with primers and submitted in 12 ul as described in the **Template Primer Quantity Table** section. Also see the **Sample Submission in Detail** section for additional information. Requests are submitted to:
http://dnalims.mit.edu/
First time users must create an account before logging into the LIMS. Once logged in, follow the instructions and call if you have any problems. Your account number must be exactly seven digits with no spaces, dashes etc.
The LIMS asks if the template is "difficult to sequence". We will use extra polymerase, blend polymerases and / or alter the thermocycling conditions if you select "difficult template" and also indicate the nature of the difficulty (e.g. hairpin or GC rich) next to the samples in question when you fill out the web request form.
Print out 2 copies of your request—one for yourself and one to submit to the lab. Drop off your samples and request forms in the refrigerator at the front of the lab. The file name of your data will consist of your sample name and primer. For example, if your sample name is AB123 and the primer is M13F the electropherogram file name will be AB123-M13F.ab1. Submit samples in 8-strip PCR tubes or 96-well plates. Use only ABI (PN: N8010560) plates which you may obtain from us. Place strips and tubes into 50cc tubes and write the assigned "Order Number" on the tube or plate.
Additional LIMS helpful notes are located below in the **dnaLIMS User Notes** section.
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Sample Submission in Detail
We ask for 12 ul but use only 6 in case we have to repeat an analysis.
If you have any questions about sample preparation for DNA sequencing that are not addressed on this page we suggest that you download the Applied Biosystems DNA Sequencing Chemistry Guide and/or the Qiagen publication: "The QIAGEN Guide to Template Purification and DNA Sequencing, 2nd edition" from their web sites. We will also email these documents to you upon request.
How to Prepare samples for DNA sequencing
Individual tubes
Template and primer should be diluted to 12 ul with water. Submit samples in 8 strip PCR tubes with caps (ie.VWR/MIT Stockroom PN 20170-004) Label tubes 1,2,3 etc. DO NOT try and squeeze your sample name on the tubes. It makes it very difficult to read and can result in a delay if we need to contact you because we cannot read the tubes. Submitting samples in eppendorf vials takes longer to set up so PLEASE USE STRIPS. Group individual tubes/strips into 50 ml Falcon tubes. (we have recycled 50 ml tubes in the lab near the refrigerator) Label with NAME and ORDER NUMBER. Place in refrigerator at front of lab and leave forms in the plastic pocket.
96 well plate(>75 samples)
ONLY use ABI plates (PN: N8010560) which can be obtained from us. Scale down the template and primer requirements by 50% and dilute to 6 ul with water.
To receive the discounted price you MUST follow these instructions for submitting directly in 96 well plates:

Contact the lab and we will supply you with the correct plate to use. If samples are submitted in plates other than those specified, we will have to transfer the samples into the correct plates, therefore, the price will not be discounted.
Load the plate vertically NOT horizontally. The instrument picks up vertical columns at one time. When the data is posted it is arranged by vertical wells, A1-H1, A2-H2, A3-H3...A12-H12. If you load horizontally your data will not post in the same order as you see on the request sheet. We cannot change the order in which data posts.
You MUST use only 6ul if directly loading a well plate. If you use 12ul we will have to transfer 6ul of all the samples to another plate defeating the whole purpose of submitting it in the plate, and therefore, the price will not be discounted.
Leave A1 and/or H12 empty for our standard. It is preferable to have a standard in one odd well(A1) and one even well(H12). A single standard is OK if you have 95 samples. We can run a plate of 96 samples but please be aware there will be no control sample to verify instrument performance.
Please Note: An order of fewer than 76 samples will not be eligible for a discounted price. If you have fewer than 76 samples it is preferable to use the 8 strip tubes
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Troubleshooting Guide

Frequent Reasons for Failure to Get Good Data

Failure results when there is an insufficient level of fluorescent termination products for the computer software to assign a sequence. Some possible reasons:
1. Our new capillary sequencer (and corresponding new sequencing polymerase kits) provides longer reads but is also more sensitive to residual salt and ethanol in sample.
If you are experiencing variable results(e.g. in silica based purification schemes where 10 mM Tris is the recommended elution buffer), try a post spin-out ethanol PPT or elute in water instead of E.B.
2. Too little template results in reactions with little or no signal and poor or no base calling.
3. Too much DNA producing reactions which terminate prematurely, often with fewer than 250 bases of reliable sequence data.
4. Poor quality template DNA. Template DNA must be free of residual ethanol and salt.
5. Insufficient primer concentration or poor quality.
6. The Tm of the primer is << 50°C.
7. The template does not contain a sequence complementary to the primer.
8. Primer and/or template was not added to the reaction.
9. Using the same primers for sequencing as were used for PCR. One of the primers used to generate the PCR product does not work under fluorescent cycle sequencing reaction conditions.
10. Template from two different colonies was submitted inadvertently. You may have picked the biggest colony or plated too densely in which case many of your colonies are actually two different colonies rather than the one of interest. This results in two overlapping sequences on the electropherogram. The fix is to re-plate at a lower density and re-sequence.

Some Common Mistakes with Qiagen Plasmid Kits which Affect the Sequence Quality

The directions for cell growth are not followed resulting in overloading the Qiagen resin. Usually, a poor yield of plasmid DNA results, presumably due to competition with RNA fragments for binding to the Qiagen resin. Use the recommended quantity of LB broth (don't use Terrificbroth) for cell growth.
The isopropanol-precipitated DNA is not washed with 70% ethanol to remove excess salt. Wash the DNA pellet at least once as directed with 70% ethanol. Residual salt in the final template will interfere with the activity of Taq polymerase resulting in sequence data which extends fewer than 200 bases from the primer and exhibits a low signal to noise ratio.
The template DNA is not dried completely before final resuspension in H2O. To remove residual ethanol, dry the DNA for 5 min. in a properly operating speedvac. If air-drying, make sure that the DNA is dry (no fluid in the tube, the DNA pellet does not look wet). When air drying, a brief 15 min incubation of the open tube at 65 oC is often sufficient to completely dry the DNA. Residual ethanol is detrimental to Taq cycle sequencing resulting in data with a drastically reduced signal.

DNA Sequencing Troubleshooting Table

Observation	Possible Cause	Solution
Poor quality template		
Weak , noisy signal. Signal strength in raw channel is usually less than 100	Contaminated Template: Template DNA must be free of residual ethanol and salts. And also free of cellular components such as RNA, proteins, polysaccharides, and chromosomal DNA	Re-precipitate template
Large stop peaks	Degraded DNA	Re-isolate DNA
Multiple, overlapping peaks	More than one template present Double pick of two colonies	Clean up PCR products Re-isolate DNA
Large dye blob at ca. 60 and 100 bases	Insufficient template	Increase amount of template
Primer related problems		
No sequence generated	Insufficient template	Increase amount of template
	Contaminant	Clean up template
	Insufficient primer	Increase amount of primer
	Primer has no annealing site Poor primer design Incorrect sequence	Re-design primer
Noisy data with weak signal	Not enough DNA	Use more DNA in reactions
	Primer anneals poorly	Re-design annealing temp
Noisy data with good signal strength	Contaminated template	Clean up template
	Multiple templates	Examine template on agarose gel
	Multiple priming sites	Redesign primer
	Multiple primers with PCR products	Purify PCR template
	Too much DNA	Use less DNA
Noise up to or after a certain point	Mixed plasmid prep Multiple PCR products	Ensure only one template present
	Long homopolymer A or T (slippage)	1. Use an anchored primer(ie. a sequencing

		primer that is PolyT containing an A, C or G base at the 3’ end of a polyA region) The 3’ base will anchor the primer into place at the end of the homopolymer region. 2. Sequence from both directions

(includes data from ABI 3730 Sequencing Chemistry Guide “Appendix B: Troubleshooting”)
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Viewing the Sequencing Results
You will be notified by email when the results are ready. Inspect each electropherogram for errors in base calling. Occasionally, there are miscalls that are obvious to the eye but difficult for a computer to recognize. As a rule, when you reach the point where there is one N per 20 bases, the sequence is not usable.
You can view and print the electropherogram (sequencing traces) and text output along with a quality score. Files can be downloaded to your computer. For rapid turnaround, on weekends we may send out raw data without editing. If subsequent editing was needed you will receive a new mailing. Turnaround time is typically 48 hours.
The following web sites offer free programs for viewing electropherograms (.ab1 files):

Software	**Operating System**	**Comments**
4 PEAKS	Mac OS X	http://www.mekentosj.com/4peaks/
Sequencer Scanner	Windows	http://www.appliedbiosystems.com/)
Sequencher	Mac OS 9 and below	Sequencher (free demo but will not save; from Gene Codes www.genecodes.com)
Chromas	Windows	(http://www.technelysium.com.au/chromas.html)
Codon Code Aligner	Mac, Windows and Unix	Codon Code Aligner nee Trace Viewer(http://www.codoncode.com/TraceViewer/)

Pay software to view electropherogram outputs
Your department may have a site license for DNA alignment, searching and manipulation programs such as DNASTAR, Sequencher etc.
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dnaLIMS User Notes
Reprinting order requests
If you forgot to print out the order request you cannot get back to that page. You can go to “Display Order Summary” to get a list of all current orders and obtain a printout. Select the order you wish to print. You will get a summary page similar to the original printout.
Deleting an order
To delete an order go to “View your requests” enter the order number you wish to delete and choose the “delete order” radio button. You can only delete orders that have not yet been put into a sample sheet. You would generally use this to delete an order that you have maybe decided not to submit, or had problems with and just want to start fresh. If you have problems contacts us and we can also delete the order for you.
Downloading Sequencing Results

If you are looking in the “Download sequence Results” page and see your order # but it has no data and says ‘There are 0 samples” this means the plate is processing and data will post when done.
If you are using a MAC and download individual files you may see a .txt extension after the .ab1 in the file name for the chromatogram files. The file will not open. You can simply delete the .txt and the file will revert to the .ab1 file which you can now open. To avoid this, choose to download all at once (Button at the top of the page). This will not attach the .txt to the files.
Samples that are ready to download sometimes will not appear in the "Order Number Search" box when you are trying to download your samples. If you think your data should be ready but do not see the order number, use the "Plate Number Search" box and select the plate at the top of the list. You should then be able to see the order and download it. If you think it should be ready but don't see it in either box then contact the lab.
Trouble submitting an order
If you get an error message after submitting an order, such as “database does not exist” or “user ID not found” contact the lab immediately. This indicates a problem with the server.
Results may be listed out of order
Occasionally you may notice the list order of your data is not in the same order as on the request form. This is due to the fact that the software arranges data according to the well # starting with A1-H1, A2-H2, etc. We try to load the plate so you get the data back in the same order as submitted but it is sometimes unavoidable.

Knight:Electroporation – 2008 – MIT
http://openwetware.org/wiki/Knight:Electroporation
This protocol is for transforming plasmid DNA into *Escherichia coli* cells.
Materials
Electrocompetent cells
Plasmid DNA (from a ligation reaction)
Ice
Ice bucket
For the following, you need one per DNA sample
Electroporation cuvette (either 1mm or 2mm gap width)
Electroporator
1.5 mL eppendorf tube
LB-agar plate with appropriate antibiotic
1mL SOC at room-temperature
Procedure
Chill electroporation cuvettes, DNA samples and tubes on ice.
Place LB-agar plates in 37°C incubator to warm.
Once cuvettes are cold, remove electrocompetent cells from -80°C freezer and thaw on ice. Alternatively, freshly prepared electrocompetent cells may be used immediately.
If electrocompetent cells are not already in individual aliquots, then aliquot out into pre-chilled 0.6mL tubes.
Turn on electroporator and set voltage to either 1.25 kV (1mm cuvettes) or 2.5 kV (2mm cuvettes).
Dial a P2 pipetman to either 1 or 2µL depending on the salt content of your DNA sample and . Use 2µL for samples that have been purified in some way.
Dial a P200 pipetman to 50µL or whatever volume of electrocompetent cells you want to use. Usually 20-50µL.
Dial a P1000 pipetman to 950µL and pipet in SOC. Place pipetman on counter such that tip doesn't touch anything.
Pipet 1-2µL of DNA sample and add to electrocompetent cells. Swirl tip around gently in cells to mix DNA and cells. Do not pipet up and down.

Place cells back on ice to ensure they remain cold.
Transfer cell-DNA mixture to cuvettes using P200 pipetman. Try not to handle cuvette base too much so that it stays cold.
Tap the cuvette on the counter gently so that cells are at the bottom and to remove any air bubbles.
Wipe off excess moisture from outside of cuvette.
Place in chamber of electroporator.
Slide the chamber in so that the cuvette sits snugly between electrodes.
Pulse the cells with a shock by pressing button on electroporator.
Remove cuvette from the chamber and immediately add SOC. This step should be done as quickly as possible to prevent cells from dying off.
Transfer SOC-cell mixture to chilled eppendorf tube.
Chill sample on ice for 2 mins to permit the cells to recover.
Transfer eppendorf tube to 37°C incubator and shake to promote aeration. Incubate for 1 hr to permit expression of antibiotic resistance gene.
Plate transformation onto prewarmed LB-agar plate supplemented with appropriate antibiotic. I generally plate 200µL but appropriate plating volume depends on efficiency of the transformation.
Incubate plate overnight at 37°C.
Leave remaining SOC-cell mixture on the benchtop overnight.
If you don't have any transformants, plate the rest of the transformation in the morning.

Notes

If you are in a hurry and your selection marker is ampicillin, you can go ahead and plate immediately because ampicillin takes a while to be pumped into cells at a high enough concentration to have an effect.

Knight:Electroporation – 2008 - MIT

http://openwetware.org/wiki/Knight:Electroporation
This protocol is for transforming plasmid DNA into *Escherichia coli* cells.
Materials
Electrocompetent cells
Plasmid DNA (from a ligation reaction)
Ice
Ice bucket
For the following, you need one per DNA sample
Electroporation cuvette (either 1mm or 2mm gap width)
Electroporator
1.5 mL eppendorf tube
LB-agar plate with appropriate antibiotic
1mL SOC at room-temperature
Procedure
Chill electroporation cuvettes, DNA samples and tubes on ice.
Place LB-agar plates in 37°C incubator to warm.
Once cuvettes are cold, remove electrocompetent cells from -80°C freezer and thaw on ice. Alternatively, freshly prepared electrocompetent cells may be used immediately.
If electrocompetent cells are not already in individual aliquots, then aliquot out into pre-chilled 0.6mL tubes.
Turn on electroporator and set voltage to either 1.25 kV (1mm cuvettes) or 2.5 kV (2mm cuvettes).
Dial a P2 pipetman to either 1 or 2µL depending on the salt content of your DNA sample and . Use 2µL for samples that have been purified in some way.
Dial a P200 pipetman to 50µL or whatever volume of electrocompetent cells you want to use. Usually 20-50µL.

Dial a P1000 pipetman to 950µL and pipet in SOC. Place pipetman on counter such that tip doesn't touch anything.
Pipet 1-2µL of DNA sample and add to electrocompetent cells. Swirl tip around gently in cells to mix DNA and cells. Do not pipet up and down.
Place cells back on ice to ensure they remain cold.
Transfer cell-DNA mixture to cuvettes using P200 pipetman. Try not to handle cuvette base too much so that it stays cold.
Tap the cuvette on the counter gently so that cells are at the bottom and to remove any air bubbles.
Wipe off excess moisture from outside of cuvette.
Place in chamber of electroporator.
Slide the chamber in so that the cuvette sits snugly between electrodes.
Pulse the cells with a shock by pressing button on electroporator.
Remove cuvette from the chamber and immediately add SOC. This step should be done as quickly as possible to prevent cells from dying off.
Transfer SOC-cell mixture to chilled eppendorf tube.
Chill sample on ice for 2 mins to permit the cells to recover.
Transfer eppendorf tube to 37°C incubator and shake to promote aeration. Incubate for 1 hr to permit expression of antibiotic resistance gene.
Plate transformation onto prewarmed LB-agar plate supplemented with appropriate antibiotic. I generally plate 200µL but appropriate plating volume depends on efficiency of the transformation.
Incubate plate overnight at 37°C.
Leave remaining SOC-cell mixture on the benchtop overnight.
If you don't have any transformants, plate the rest of the transformation in the morning.
Notes
If you are in a hurry and your selection marker is ampicillin, you can go ahead and plate immediately because ampicillin takes a while to be pumped into cells at a high enough concentration to have an effect.

MIT iGEM T4 and Quick Ligation – 2008 - MIT

Calculating Insert Amount
For all ligation reactions, optimal insert:vector molar ratio is 6:1. The appropriate mass ratio for the ligation can be calculated using the following
formulas:

$$Insert\ Mass\ in\ ng = 6 \times \left[\frac{Insert\ Length\ in\ bp}{Vector\ Length\ in\ bp}\right] \times Vector\ Mass\ in\ ng$$

I've also used 6:1 crude mass ratios (eg. 60ng of insert, 10ng vector), and this has worked OK, though it's probably not optimal ~~Felix moser

One can use either Quick ligase or T4 ligase for these reactions. Quick is faster but more expensive; T4 is time-tested and proven but takes longer. Here's the protocols for both:
***Quick* Ligase ligation**
Materials
2x Quick ligase buffer (in 40µl aliquots; these are 1-time use since freeze-thaw cycles degrade the ATP in the buffer).
Quick Ligase from NEB
ddH2O
Purified, linearized vector (likely in H_2O or EB)
Purified, linearized insert (likely in H_2O or EB)*Italic text*
Procedure

For 10µl reaction
Larger volumes can be scaled up if needed
5 µL 2X Quick ligase buffer
0.5 µl *Quick* ligase
6:1 Molar ratio of insert to vector (~10ng vector). Try to keep total DNA concentration <100ng/rxn for optimal efficiency.
Add (4.5 - vector and insert volume)µl ddH_2O
Method
Add appropriate amount of deionized H_2O to sterile PCR tube
Add in appropriate amounts of vector and insert. Heat the mixture to 42*C for 2min to free up sticky ends (can set up a thermocycler for this).
Add 5 µL of 2X ligation buffer to the tube.
Pipette buffer up and down before pipetting to ensure that it is well-mixed.
Add 0.5 µL of *Quick* ligase. PIPETTE half the volume of the mixture UP AND DOWN to ENSURE MIXING OF THE ENZYME.
Also, the ligase, like most enzymes, is in some percentage of glycerol which tends to stick to the sides of your tip. Just touch your tip to the surface of the liquid when pipetting to ensure accurate volume transfer.
Let the 10 µL solution incubate at room temp for 5-10min.
Denature the ligase at 65°C for 10min.
Store at -20°C

T4 ligase ligation
Materials
T4 DNA Ligase
10x T4 DNA Ligase Buffer --> make sure it smells bad (like "wet dog"); if it doesn't smell, it might be bad.
Deionized, sterile H_2O
Purified, linearized vector (likely in H_2O or EB)
Purified, linearized insert (likely in H_2O or EB)*Italic text*
Procedure
10µl Ligation Mix
Larger ligation mixes are also commonly used
1.0 µL 10X T4 ligase buffer (use 10µl aliquots in -20 freezer; repeated freeze-thaw cycles can degrade the ATP in the buffer that's critical for the ligation rxn)
6:1 Molar ratio of insert to vector (~10ng vector)
Add (8.5 - vector and insert volume)µl ddH_2O
0.5 µL T4 Ligase
Method
Add appropriate amount of deionized H_2O to sterile PCR tube
Add in appropriate amounts of vector and insert. Heat the mixture to 42*C for 2min to free up sticky ends (can set up a thermocycler for this).
Add 1 µL ligation buffer to the tube.
Pipette buffer up and down before pipetting to ensure that it is well-mixed.
Add 0.5 µL T4 ligase. PIPETTE half the volume of the mixture UP AND DOWN to ENSURE MIXING OF THE ENZYME.
Also, the ligase, like most enzymes, is in some percentage of glycerol which tends to stick to the sides of your tip. Just touch your tip to the surface of the liquid when pipetting to ensure accurate volume transfer.
Let the 10 µL solution incubate at 16*C for 1hr.

Denature the ligase at 65°C for 10min.
Store at -20°C
Notes
Make sure the buffer is completely melted and dissolved. Precipitate is DTT (or BSA?). Probably best to aliquot this buffer into smaller portions, to reduce the freeze/thaw cycles. In general, make sure the buffer still smells strongly like "wet dog" (Checking if the DTT is still good.)

MIT iGEM Top10 ChemComp Ecoli transformation protocol – 2008 – MIT
http://openwetware.org/wiki/MIT_iGEM_Top10_ChemComp_Ecoli_transformation_protocol - Regular_Transformation_protocol_for_Top10_cells

Regular Transformation protocol for Top10 cells
Take out an appropriate aliquot of Top10 ChemComp cells from -80 freezer (Very top right rack on top shelf, bottom slide-out shelf).
Let cells thaw ON ICE! for ~5-10min. Cells MUST be kept cold until heat shock! This is critical!
Aliquots are either 50µl (small PCR tubes) or 200µl (bigger tubes). If you're transforming multiple (>=4) DNA samples, aliquot 50µl into PCR tubes from the 200µl aliquot.
Transform 50 µl of cells with 3-4µl of ligated DNA
Keep ON ICE 30min. This step is to let the salt from the ligation equilibrate over the cells.
Heat shock 60 sec at 42C. You can set a thermocycler to 42*C instead of making a water bath.
Put cells back on ice for 2min.
Add your 50µl of cells to 200 µl SOC in a 2ml epp tube.
Incubate at 37 C for 1 hour. You can just place the 2ml epp tubes in a little plastic cup and put them on the shaker in the 37*C room.
Using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
For plasmids pSB1AC3 and pSB1AT3, which are chloramphenicol and tetracycline resistant, we find growing for 2 hours yields many more colonies. This might be because the cells double in that time or it might be because more Ab-resistance protein is made.
Ampicillin and kanamycin appear to do fine with 1 hour growth
Plate 200 µl (or less if you're expecting a ton of transformants) on the appropriate antibiotic plates. Use either hockey stick in ethanol or sterilized glass beads to spread.
To Test Competence:
Transform 50 µl of cells with 1 µl of standard pUC19 plasmid (Invitrogen)
This is at 10 pg/µl or 10^{-5} µg/µl
This can be made by diluting 1 µl of NEB pUC19 plasmid (1 µg/µl, NEB part number N3401S) into 100 ml of TE
Hold on ice 0.5 hours
Heat shock 60 sec at 42C
Add 250 µl SOC
Incubate at 37 C for 1 hour in 2 ml centrifuge tubes rotated
using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
For our plasmids (pSB1AC3, pSB1AT3) which are chloramphenicol and tetracycline resistant, we find growing for 2 hours yields many more colonies
Ampicillin and kanamycin appear to do fine with 1 hour growth
Plate 20 µl on AMP plates using sterile 3.5 mm glass beads
Transformation efficiency is 15 x colony count x 10^5
We expect that the transformation efficiency should be between $5x10^8$ and $5x10^9$

Analytical Gel Standard Protocol – 2008 – Michigan
http://2008.igem.org/Team:Michigan/Notebook/GelProtocol

1. Mix 5 µL of DNA with 1 µL of loading dye
2. Pour heated liquid gel (with stoppers and combs in place) according to the number of samples you have to run, and the type of gel electrophoresis apparatus you have
3. Allow gel to cool and solidify
4. Remove all stoppers and combs
5. Fill gel box with TAE until it completely covers the gel
6. Load 3-4 µL of dye/DNA mixture
7. Load ladders for comparison
8. Turn on current to ~45 V
9. Once DNA enters gel, turn up current to ~100 V
10. Allow gel to run until dye is ~3/4 of the way across the gel, depending on the size of your DNA

Gel Extraction Standard Protocol

All of our gel extractions are performed using QIAGEN kits and protocols.

QIAGEN's website can be found HERE.

Plasmid Prep Standard Protocol – 2008 – Michigan
http://2008.igem.org/Team:Michigan/Notebook/PlasmidPrepProtocol
Plasmid preps are used to clean and isolate a plasmid from a liquid culture of cells. The type of prep you wish to use depends on the amount of culture you have, which determines how much DNA you will obtain.

Protocol
All of our minipreps, midipreps, and maxipreps are performed using QIAGEN kits and protocols.

QIAGEN's website can be found HERE.

PCR Standard Protocol – 2008 – Michigan
http://2008.igem.org/Team:Michigan/Notebook/PCRProtocol
PFU turbo enzyme or Taq polymerase were used as enzymes. Taq is cheaper, but error prone, so only used for side screening.

1. Determine the total volume you wish to use - usually 50 or 100 µL
2. Add 1 µL of your DNA vector for every 50 µL total volume to PCR tube
3. Add 1 µL of each primer for every 50 µL total volume
4. Add 5 µL of dNTP for every 50 µL total volume
5. Add 5 µL of the correct buffer solution (depending on enzyme used) for every 50 µL total volume
6a. If using PFU, use 1 µL for every 50 µL total volume, and the balance deionized water (add enzyme last)
6b. If using Taw, use 0.5 µL for every 50 µL total volume, 1.5 µL of MgCl, and the balance deionized water (add enzyme last)
7. Follow protocol for running PCR machine - This will depend on the type of PCR machine used. Be aware that some PCR machines may also require different mixtures to run properly. This is the protocol specified for our machine, the PCR Express.

Following this protocol, your PCR mixtures should look similar to the following:

EXAMPLE PCR 1: 50 µL total volume using Taq polymerase
35 µL dd water
5 µL dNTP
5 µL Taq buffer
1 µL Primer 1
1 µL Primer 2
1 µL DNA vector
1.5 µL MgCl
0.5 µL Taq polymerase

EXAMPLE PCR 2: 100 µL total volume using PFU turbo
72 µL dd water
10 µL dNTP
10 µL PFU buffer
2 µL Primer 1
2 µL Primer 2
2 µL DNA vector
2 µL PFU turbo

PCR Clean Up Standard Protocol
All of our PCR clean ups are performed using QIAGEN kits and protocols.

QIAGEN's website can be found HERE.

Transformation Standard Protocol - 2008 – Michigan
http://2008.igem.org/Team:Michigan/Notebook/TransformationProtocol

Transformation of Plasmids (using heat shock)
1. Precool 1.5 mL tubes, 1 for each transformation and 1 for a positive control
2. Defrost competent cells (usually XL1 blue) on ice
3. Mix 100 µL (or 50 µL if doing many transformations) of cells with 1-2 µL of prepped plasmid (or 10 µL of plasmid if coming from a ligation)
4. Incubate mixture on ice for 30 minutes
5. Heat shock at 42 degrees Celsius for 45 seconds
6. Incubate on ice for 5 min
7. Add 900 µL pre-warmed LB media & allow to recover on spinning wheel in incubator (37 degrees Celsius) for 1 hour
8. Spin down and resuspend in 50 µL LB media
9. Selectively plate and grow in incubator overnight

Transformation of Linearized DNA via Electroporation
This technique was used for crossing our linearized landing pad plasmids onto the chromosome.
1. Add 1-2 µL of DNA to 50 µL of electrocompetent cells into electroporation tube
2. Electroporate (using BIORAD Micropulser) and immediately add 0.9 mL of LB media
3. Incubate cells for 1 hour to recover
4. Spin down and resuspend in 100 µL of LB
5. Selectively plate and grow in incubator overnight

Ligation Standard Protocol – 2008 – Michigan
http://2008.igem.org/Team:Michigan/Notebook/LigationProtocol
Ligations are used to combine DNA fragments.

Protocol
1. Generally, use 20 µL for total ligation volume
2. Use 10% ligase
ALWAYS add the ligase last to the ligation mixture!!!
3. Add the ligase buffer
Buffers are 20X, so use 2 µL for each 10 µL total volume
4. Usually have between 2 and 6 µL of DNA in ligation mixture
This can be altered depending on the results you desire. For example, if you have an insert you wish to add to a vector, sometimes you may want to have up to 3x as much insert as vector in your ligation mixture.
5. Balance of ligation mixture should be deionized water
6. Allow ligation to set in 4 oC fridge or on desktop overnight
Fridge is preferred

Following these guidelines, your ligation mixtures should look similar to these:

EXAMPLE LIGATION 1:
2 µL DNA Vector
6 µL DNA Insert
4 µL Ligation Buffer
2 µL Ligase
6 µL dd water

EXAMPLE LIGATION 2:
4 µL DNA Vector
4 µL DNA Insert
4 µL Ligation Buffer
2 µL Ligase
6 µL dd water

Digest Standard Protocol – 2008 – Michigan
http://2008.igem.org/Team:Michigan/Notebook/DigestProtocol
Digests are used to cut your DNA with restriction enzymes.
We always used buffers and enzymes from New England Biolabs, whose website can be found HERE. Their site has great tools that can be used to find compatible enzymes, determine which buffers to use and whether to use BSA or not.

Digest Protocol
1. Generally, use 20 µL for total digest volume
If linearizing a plasmid, use more than 20 µL
2. NEVER use more than 10% enzyme
Even with a 40 µL digest, you can use 1 µL of enzyme for an overnight digest
3. Add the correct buffer for your enzyme
Buffers are 10X, so use 1 µL for each 10 µL total volume
4. Check to see if your digest requires BSA

BSA is 10X, so use 1 µL for each 10 µL total volume
5. Balance of digest mixture should be your vector (DNA you wish to cut)
6. ALWAYS digest in incubator
Usually leave overnight, but at least 4 hours

Following these guidelines, your digest mixtures should look similar to these:

EXAMPLE DIGEST 1: one enzyme w/ BSA
15 µL Vector
2 µL NEBuffer 3
2 µL BSA
1 µL EcoRV

EXAMPLE DIGEST 2: one enzyme w/ no BSA
17 µL Vector
2 µL NEBuffer 1
1 µL NAE1

EXAMPLE DIGEST 3: two enzymes w/ BSA
14 µL Vector
2 µL NEBuffer BamHI
2 µL BSA
1 µL BamHI
1 µL NotI

Digest Purification Protocol
All of our digest purifications are performed using QIAGEN kits and protocols.

QIAGEN's website can be found HERE.

Antibiotics – 2008 – Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Antibiotics
Ampicillin
1000X ampicillin stock is a 100 mg/ml solution.
Combine 1 g ampicillin with 10 ml nanopure H_2O.
Sterile filter using a 0.2 µm Corning filter.
Aliquot into eppendorf tubes and store at -20C.
Kanamycin
1000X kanamycin stock is a 50 mg/ml solution.
Combine 0.5 g kanamycin with 10 ml nanopure H_2O.
Sterile filter using a 0.2 µm Corning filter.
Aliquot into eppendorf tubes and store at -20C.
Spectinomycin
1000X spectinomycin stock is a 100 mg/ml solution.
Combine 1 g spectinomycin with 10 ml nanopure H_2O.
Sterile filter using a 0.2 µm Corning filter.
Aliquot into eppendorf tubes and store at -20C.
Streptomycin
1000X streptomycin stock is a 50 mg/ml solution.
Combine 0.5 g streptomycin with 10 ml nanopure H_2O.

Sterile filter using a 0.2 µm Corning filter.
Aliquot into eppendorf tubes and store at -20C.

Solid growth media – 2008 – Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Solid_growth_media

LB agar
Materials
25 g LB broth mixture (Difco)
15 g agar
1 L ddH_2O
Procedure
Mix dry ingredients together with 1 L ddH_2O using a magnetic stir bar.
Autoclave to sterilize. Let cool to 55C.
Pour 28 ml LB solution for thin plates; 50 ml for thick plates.
LB + amp_{100}
Materials
1 L LB agar
1 ml 1000X ampicillin stock solution
Procedure
Make LB agar as directed.
When mixture is cooled to 55C, add 1 ml 1000X ampicilllin stock solution.
Plate as usual.
LB+kan_{20}+amp_{50}
Materials
1 L LB agar
0.5 mL 1000X ampicillin stock solution
0.4 mL 1000X kanamycin stock solution
Procedure
Make LB as directed.
After cooling to 55C, add ampicillin and kanamycin.
Plate as usual.
LB+sp_{100}
Materials
1 L LB agar
1.0 mL 1000X spectinomycin stock solution
Procedure
Make LB as directed.
Cool to 55C, then add spectinomycin.
Plate as usual.
BG-11 agar
Materials
940 ml nanopure H_2O
20 ml 50X thiosulfate (final conc. of 1mM)
20 ml 50X TES buffer, pH 8.0 (final conc. of 10mM)
20 ml 50X BG-11
15 g agar
Procedure
Combine agar and nanopure H_2O. Stir mixture with a magnetic stir bar.
Autoclave to sterilize. Cool to 55C.

Add TES, thiosulfate, and BG-11
Test pH (should be ~8.0).
Plate 28 ml for thin plates; 50 mL for thick plates.
BG-11 + 5% LB
Materials
1.25 g LB broth mixture (Difco)
15 g agar
940 ml nanopure H_2O
20 ml 50X BG-11
20 ml 50X thiosulfate
20 ml 50X TES buffer
Procedure
Combine LB and agar with the nanopure H_2O.
Autoclave to sterilize.
Add TES, thiosulfate, and BG-11.
Test pH (~8.0).
Plate as usual.
BG-11+$sp_{2.5}$+$sm_{2.5}$
Materials
BG-11 agar
0.15 ml 1000X spectinomycin stock solution
0.3 ml 1000X streptomycin stock solution
Procedure
Make BG-11 agar as directed.
After adding TES, thiosulfate, and BG-11, add spectinomycin and streptomycin.
Plate as usual.

Transformation PCC6803 – 2008 – Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Transformation_PCC6803

Theory behind tri-parental matings
"Mobilization of a plasmid into a recipient cell is often used for cloning, transposon mutagenesis, or other procedures. As mentioned, mobilizable plasmids have an advantage over self-transmissible plasmids in being smaller. Nevertheless, difficulties can be encountered before these plasmids can be mobilized. For example, the self-transmissible plasmid and the plasmid to be mobilized may be members of the same Inc group and so do not stbaly coexist in the same cell. Also, the self-transmissible plasmid may express its *tra* genes only for a short time after entering a recipient cell so that transfer is inefficient.
"Triparental matings help overcome some of the barriers to efficient plasmid mobilization. As the name implies, three bacterial strains participate in the mating mixture. The first strain contains a self-transmissible plasmid, the second contains the plasmid to be mobilized, and the third is the evenaul recipient. After the cells are mixed, some of the self-transmissible plasmids in the first strain are transferred into the strain carrying the plasmid to be mobilized. Becauase it is fertile when it first enters the cell, the self-transmissible plasmid quickly spreads through the population of cells containing the mobilizable plasmid. It is then able to mobilize the mobilizable plasmid into the third strain with a high efficiency because new transconjugants retain their ability to transfer for at least six generations. Contrast this to a mating involving only two strains, one of which contains both the self-transmissible plasmid and the mobilizable plasmid. Only a small fraction of the cells are fertile and can mobilize the mobilizable plasmid into the recipient strain. Also in a triparental amting, even if the two plasmids are members of the same Inc group, they coexist long enough for the mobilization to occur."

Reference:
Snyder, Larry and Wendy Champness. *Molecular Genetics of Bacteria*. 3rd ed. Washington: ASM Press, 2007. 259.

Protocol

As dictated by Dr. Sean Callahan
Based on *Anabena* experiments
Grow cyanobacteria into exponential phase (OD750 = ~0.5) in liquid BG-11.
Streak out fresh plates of transformed *E. coli* one day before. Use to innoculate 40 ml SOB and allow *E. coli* to grow until OD600=0.6.
Pellet *E. coli*. Wash with 40 mL BG-11. Pellet and wash again.
Resuspend pellet in 1.4 ml BG-11.
Spot *E. coli* and cyanobacteria together on BG-11 + 5% LB plate. Use 5 µl of each *E. coli* strain and 10 µl of cyanobacteria.
Wait for spot to dry on plate.
Incubate for 2 days in light and CO_2 at 30C.
Use a loop to scrape the growth on the BG-11 + 5% LB plate.
Streak innoculate on BG-11+sp+sm.
Incubate in light and CO_2 at 30C.
Note: Because E. coli may curtail the growth of cyanobacteria (sometimes even killing them), it is suggested that 1/1, 1/10, and 1/100 dilutions of E. coli *cultures be made before spoting with cyanobacteria.*

Discussion

From beginning to end, triparental conjugation between *E. coli* and PCC6803 takes 13 days. Exploring other methods of transformation that would minimize this lag time would be extremely advantageous.

Colony PCR – 2008 - Hawaii

http://2008.igem.org/Team:Hawaii/Protocols/Colony_PCR

Protocol

Pick a colony and suspend in 200 µl LB (or other appropriate media).
Vortex ~8 sec.
Heat at 97C for 10 min.
Vortex again briefly.
Use 1 µl in PCR reaction.

PCR reaction

Combine:
1 µl colony sample (above)
0.5 µl 10mM forward primer
0.5 µl 10mM reverse primer
3 µl nanopure water
5 µl *Taq* (we used EconoTaq Green Taq)
Run for 30 cycles of denaturing, annealing, extension
Initial denature @ 94C for 2 min.
Denature @ 94C for 30 sec.
Anneal @ 62C for 30 sec.
Extend @ 72C for 90 sec.
Final extension @ 72C for 10 min.
Hold @ 4C inifinitly.
Run PCR products on a gel to verify desired DNAs.

Reference
Protocol dictated by Dr. Sean Callahan, Department of Microbiology, University of Hawaii at Manoa

Plasmid prep – 2008 - Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Plasmid_prep
Miniprep Protocol
1. Grow single colony of *E. coli* at 37C overnight in 5 ml LB w/ antibiotic selection.
2. Microcentrifuge 1.5 ml cells for 20 sec at 16,000g. Discard supernatant.
3. Resuspend pellet in 100 µl GTE solution.
50 mM glucose
10 mM EDTA
25 mM Tris-HCl (pH 8.0)
4. Let sit for 5 min. at room temperature.
5. Add 200 µl NaOH/SDS solution.
0.2 M NaOH
1% SDS
6. Mix by inverting the tube a few times.
7. Incubate on ice for 5 min.
Incubate no more than 5 min. to allow for maximum release of plasmid DNA while minimizing genomic DNA release and overexposure to denaturing conditions.
8. Add 150 µl potassium acetate solution.
Precipitation of cellular debris may be enhanced by using chilled KOAc.
9. Invert a few times to mix.
10. Incubate on ice for 5 min.
11. Microcentrifuge for 3 min. at 16,000g.
12. Transfer supernatant to a new tube.
13. Add 0.8 ml 95% ethanol.
14. Incubate for 2 min. at room temperature.
15. Microcentrifuge for 1 min. at 16,000g at room temperature. Remove supernatant.
16. Wash pellet w/ 1 ml 70% ethanol. Aspirate to dry (dry in hood).
17. Resuspend pellet in 30 µl TE buffer.
Reference: Short Protocols in Molecular Biology
Add 1 µl RNase after step 12. Incubate at 55C for 30-90 min. (Re: SC)
Add 1 µl RNase after step 7. Incubate at 55C for 60 min. *-GK*
Large Scale Prep
Innoculate 5mL LB containing selective agent with a colony of plasmid bearing *E. coli*. Grow at 37C with vigorous shaking (~235 rpm) overnight.
Inoculate 500mL LB containing selective agent with ~1mL of over night culture. Grow at 37C with vigorous shaking until OD_{600}~4.0 is reached (saturation).
use baffled 2L flask
for this prep, we used a 300mL culture.
Centrifuge 10 minutes at maximum 894 rcf (maximum rcf for our centrifuge), 4°C. Use 50mL aliquots.
The protocol recommends using 6,000 x g.
The 300mL prep is divided into 6 50mL tubes.
Combine 3 tubes by resuspending in 4mL of GTE solution. Incubate 10 minutes at room temperature.
Add 10mL NaOH/SDS solution, mix (gently) by inverting 4 times. Incubate on ice for 10 minutes.
Solution should become cloudy because cells have lysed.
Add RNase at a concentration of 50 µg/ml after this step if desired.
Add 7.5mL potassium acetate, mix (very gently) by inverting (slowly) 4 times. Incubate on ice for 10 minutes.

A white precipitate forms.
Incubating on ice longer (i.e. "freezing" proteins) will help pellet the proteins better
Centrifuge for 15 minutes at 894 rcf, 4°C.
Recommended to spin at 20,000 x g for 10 minutes.
Centrifuge until a good pellet forms (may take up to 90 min). Some material will be floating, remove as much as possible with pipette tip.
Decant supernatant to a new tube.
Do this step with a pipette and avoid the white precipitate.
Add 0.6 volume of isopropanol. Mix by inversion, let stand 5-10 minutes at room temperature.
For a 21.5mL prep (total volume up to this point) add 12.9mL isopropanol.
Centrifuge 15 minutes at max rcf (894 rcf for us) at room temperature. Discard supernatant.
Recommended to spin at 15,000 x g.
Centrifuge until really good pellet forms. Avoid the pellet!
Wash pellet with 2mL 70% ethanol.
Centrifuge for 5 minutes at 894 rcf at room temperature. Aspirate ethanol.
Recommended to spin at 15,000 x g briefly.
Centrifuge until really good pellet forms.
Dry pellet in the hood.
In a really good plasmid prep, the pellet should be clear (free of protein).
Recommended to dry the pellet under vacuum.
Resuspend in 100uL TE, lightly vortex.
Recommended to store indefinitely at 4°C (but does not specify if buffer is needed).
Resuspend in a smaller volume of TE for higher concentration of plasmid.
If solution is viscous (like egg whites), there is a lot of protein contamination.
Heat at 65°C for 30 minutes.
The product was cloudy so taking extra purification step.
Centrifuge 10 minutes at 894 rcf, room temperature.
Aspirate clear liquid, avoiding pellet.
Wash pellet with 100uL TE, centrifuge, aspirate and combine with product.
Check the concentration of the plasmid using a spectrophotometer (need protcol).
Verify presence of plasmid DNA by first using a restriction digest, followed by gel electrophoresis.
UV Spectroscopy for the Quantification of Plasmid DNA
used to asses purity and concentration of nucleic acids
A_{260} measurements are quantitative for relatively pure nucleic acid preps in microgram quantities
Cannot be used to discriminate between RNA and DNA
Ratio of A_{260}/A_{280} indicates purity, as protein absorbs at 280nm.
A_{325} indicates particulates in solution or dirty cuvette
A_{230} for contaminants containing peptide bonds or aromatic moieties such as protein and phenol
NanoDrop Protocol
Turn on computer, select spec icon, choose nucleic acids setting
Pull up lever of spec (DON'T PULL with WIRE!), wash top and bottom with small amount of water.
Blank with 2uL TE, click blank
Load 2uL sample, click measure
If results significant, can print. If not, repeat steps with a dilution of sample.

Transformation E coli – 2008 – Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Transformation_E_coli

Protocol
Thawed cells on ice.

Transformed 50 µl cells w/ 1 µl DNA
Incubated on ice 30 min.
Heat shocked 60 sec. in 42C water bath
Added 250 µl SOC
Incubated @ 37C for 1 hour with shaking (150 rpm)
Plated 20-250 µl onto LB+antibiotic plates
Plate more if needed.
Notes
Adjust DNA amount as necessary. Transformation is saturated at >10ng DNA. DNA should not exceed 10% of cell volume (Re: SC).
Incubating 10 min. on ice after adding DNA and returning cells to ice for 2 min. after heat shock seems to improve transformation efficiency. -*GK*

Competent Cells – 2008 – Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Competent_Cells

Overview
This protocol is a variant of the Hanahan protocol *Hanahan91* using CCMB80 buffer for DH10B, TOP10 and MachI strains. It builds on Example 2 of the Bloom05 patent as well. This protocol has been tested on TOP10, MachI and BL21(DE3) cells. See Bacterial Transformation for a more general discussion of other techniques. The Jesse '464 patent describes using this buffer for DH5α cells. The Bloom04 patent describes the use of essentially the same protocol for the Invitrogen Mach 1 cells.
This is the chemical transformation protocol used by Tom Knight and the Registry of Standard Biological Parts.
Materials
Detergent-free, sterile glassware and plasticware (see procedure)
Table-top OD600nm spectrophotometer
SOB
CCMB80 buffer
10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
80 mM $CaCl_2.2H_2O$ (11.8 g/L)
20 mM $MnCl_2.4H_2O$ (4.0 g/L)
10 mM $MgCl_2.6H_2O$ (2.0 g/L)
10% glycerol (100 ml/L)
adjust pH DOWN to 6.4 with 0.1N HCl if necessary
adjusting pH up will precipitate manganese dioxide from Mn containing solutions.
sterile filter and store at 4°C
slight dark precipitate appears not to affect its function
Procedure
Preparing glassware and media
Eliminating detergent
Detergent is a major inhibitor of competent cell growth and transformation. Glass and plastic must be detergent free for these protocols. The easiest way to do this is to avoid washing glassware, and simply rinse it out. Autoclaving glassware filled 3/4 with DI water is an effective way to remove most detergent residue. Media and buffers should be prepared in detergent free glassware and cultures grown up in detergent free glassware.
Prechill plasticware and glassware
Prechill 250mL centrifuge tubes and screw cap tubes before use.
Preparing seed stocks
Streak TOP10 cells on an SOB plate and grow for single colonies at 23°C

room temperature works well
Pick single colonies into 2 ml of SOB medium and shake overnight at 23°C
room temperature works well
Add glycerol to 15%
Aliquot 1 ml samples to Nunc cryotubes
Place tubes into a zip lock bag, immerse bag into a dry ice/ethanol bath for 5 minutes
This step may not be necessary
Place in -80°C freezer indefinitely.
Preparing competent cells
Inoculate 250 ml of SOB medium with 1 ml vial of seed stock and grow at 20°C to an OD600nm of 0.3
This takes approximately 16 hours.
Controlling the temperature makes this a more reproducible process, but is not essential.
Room temperature will work. You can adjust this temperature somewhat to fit your schedule
Aim for lower, not higher OD if you can't hit this mark
Centrifuge at 3000g at 4°C for 10 minutes in a flat bottom centrifuge bottle.
Flat bottom centrifuge tubes make the fragile cells much easier to resuspend
It is often easier to resuspend pellets by mixing *before* adding large amounts of buffer
Gently resuspend in 80 ml of ice cold CCMB80 buffer
sometimes this is less than completely gentle. It still works.
Incubate on ice 20 minutes
Centrifuge again at 4°C and resuspend in 10 ml of ice cold CCMB80 buffer.
Test OD of a mixture of 200 µl SOC and 50 µl of the resuspended cells.
Add chilled CCMB80 to yield a final OD of 1.0-1.5 in this test.
Incubate on ice for 20 minutes
Aliquot to chilled screw top 2 ml vials or 50 µl into chilled microtiter plates
Store at -80°C indefinitely.
Flash freezing does not appear to be necessary
Test competence (see below)
Good cells should yield around 100 - 400 colonies
Thawing and refreezing partially used cell aliquots dramatically reduces transformation efficiency by about 3x the first time, and about 6x total after several freeze/thaw cycles.
Measurement of competence
Transform 50 µl of cells with 1 µl of standard pUC19 plasmid (Invitrogen)
This is at 10 pg/µl or 10^{-5} µg
Hold on ice 0.5 hours
Heat shock 60 sec at 42C
Add 250 µl SOC
Incubate at 37 C for 1 hour in 2 ml centrifuge tubes rotated
using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
For our plasmids (pSB1AC3, pSB1AT3) which are chloramphenicol and tetracycline resistant, we find growing for 2 hours yields many more colonies
Ampicillin and kanamycin appear to do fine with 1 hour growth
Plate 20 µl on AMP plates using 3.5 mm glass beads
Transformation efficiency is 15 x colony count x 10^5

Experimental Results

Using this protocol and the pUC18 plasmid, we were able to produce cells with a transformation efficiency of 4000-6000 transformants per mL of cells.

References

<biblio>
Hanahan91 pmid=1943786

Reusch86 pmid=3536850
Addison04 pmid=15470891
Bloom04 US Patent 6,709,852 Media:pat6709852.pdf
Bloom05 US Patent 6,855,494 Media:pat6855494.pdf
Jesse05 US Patent 6,960,464 Media:pat6960464.pdf
</biblio>

Cryopreserve cells – 2008 – Hawaii
http://2008.igem.org/Team:Hawaii/Protocols/Cryopreserve_cells

Purpose
Cryopreserve bacteria for long term storage in -80C deep freezer.
Materials
Cryovials
Cryoboxes
Mr. Frosty (optional)
Reagents
General Bacteria
80% Glycerol Stock
Growth Medium
Cyanobacteria
DMSO solution
1x BG-11
H_20
Equipment
-80C Freezer
Procedure
Freezing
Label (strain, date, medium) on cryovials, do at least 3 vials /strain.
E. coli (26% glycerol is higher than normal E. coli cryo-preservation protocols, this concentration allows easy scraping of top without thawing out entire tube according to Dr. Callahan)
dispense 0.4mL 80% sterile glycerol stock into cryovial
add 0.8mL E. coli culture grown overnight in TB medium, so final glycerol concentration in vial is ~26%
perform quick freeze (prevent water-molecules to organize and form large crystals and rupture membrane) by immediately putting cryotube into eppendorf rack holes prefilled and -80C prechilled w/ 70% ethanol. Place rack in -80C for freezing.
after freezing is complete in -80C, transfer vials to final cryoboxes at -80C
Record cryopreserved bacteria addition in -80C Deep Freezer Inventory.
Thawing
Record cryopreserved bacteria removal in -80C Deep Freezer Inventory.
Use one of the following thawing methods:
General Bacteria: Waterbath Method
Thaw quickly in 37C water bath.
Cells are immediately pelleted by centrifugation of the cryovial, and the supernatant is discarded. One ml of fresh growth medium is placed into the vial to resuspend the pellet.
Dispense contents on media agar plate or in liquid media.

Notes
According to a writeup (local mirror) by Jerry Brand at UTEX

[3] Although glycerol is an effective cryoprotective agent for many bacteria, it is not effective for most cyanobacteria. Methanol at approximately 5% (v/v) is suitable for most strains. However, we have been successful with concentrations of methanol ranging from 2% to 12.5%, and DMSO ranging from 4 to 15 %, depending on the culture. A small fraction of some cultures survive with no added cryoprotective agent.
[5] Cells are killed by exposure to bright light when in cryoprotective solution. Keep the culture in subdued room light while handling, and in complete darkness at other times.

Acknowledgments

References

See Harvard iGEM 2006 Cyanobacteria Protocol

Team:Hawaii/Protocols/ExoSAP – 2008 – Hawaii

http://2008.igem.org/Team:Hawaii/Protocols/ExoSAP

Overview

ExoSAP removes ssDNA and dNTPs

Protocol

Add 2 µl ExoSAP to 5 µl of the PCR reaction.
Incubate in a 37C water bath for 15 min.
Incubate in a 80C water bath for 15 min. to inactivate ExoSAP.

Gel Extraction – 2008 – Hawaii

http://2008.igem.org/Team:Hawaii/Protocols/gel_extraction
Using Qiagen MiniElute Gel Extraction Kit

Protocol
Cut band(s) from gel.
Determine weight of gel.
If weight of gel >400mg, run as 2+ extractions in different tubes.
Add Buffer QG.
For gels <2% agarose, add 3 gel volumes of QG.
For gels >2% agarose, add 6 gel volumes of QG.
Incubate at 50C, shaking every 2-3 minutes, until gel is dissolved.
Check the color of the solution is yellow. If the solution is orange, add 3M sodium acetate until solution is yellow (indicates neutral pH).
Add 1 gel volume of isopropanol and mix by inverting.
Transfer solution to elution spin column (purple).
Centrifuge 1 min. at room temperature at 13,000 rpm.
Discard flow through.
Add 500 µl Buffer QG.
Centrifuge 1 min. at room temperature at 13,000 rpm.
Discard flow through.
Add 750 µl Buffer PE and incubate 2 min.
Centrifuge 1 min. at room temperature at 13,000 rpm.
Discard flow through.
Transfer spin column to fresh eppendorf tube.
Add 10 µl Buffer EB and incubate 1 min.
Add buffer directly onto filter in the spin column.
Centrifuge 1 min. at room temperature at 13,000 rpm.

Collect flow through and store at -20C.
Notes
DNA recovery is ~80%.

DNA restriction digest – 2008 - Hawaii
http://2008.igem.org/DNA_restriction_digest

Overview
Restriction digest of DNA for downstream electrophoresis (to verify fragment existence) or BioBrick ligation assembly
Materials
List reagents, supplies and equipment necessary to perform the protocol here. For those materials which have their own OWW pages, link to that page. Alternatively, links to the suppliers' page on that material are also appropriate.
Restriction Enzyme (from NEB)
EcoRI
XbaI
SpeI
PstI
Matching NEB Buffer for the Restriction Enzyme

	NEBuffer 1	NEBuffer 2	NEBuffer 3	NEBuffer 4
EcoRI	100%	100%	100%	100%
XbaI	0%	100%	75%	75%
SpeI	75%	100%	25%	75%
PstI	75%	75%	100%	50%

X µL DNA (usually ~500 ng depending on downstream uses).
1 µL BioBricks enzyme 1 (regardless of the volume of the reaction, 1 µL enzyme is used because generally this represents a 10-25 fold excess of enzyme and is therefore sufficient for most digests. Also, it can be difficult to accurately pipet less than 1 µL of enzyme since it is sticky due to the glycerol content.)
1 µL BioBricks enzyme 2
15.6 µL deionized, sterile H_2O
0.5 µL 100X BSA (added to all digests because BSA never hurts a restriction digest)
Procedure
Postfix Vector (SpeI/PstI)
Combined 0.4 µl DNA (from plasmid prep), 15.6 µl nanopure water, 2 µl 10X NEBuffer 2, 1 µl SpeI, and 1 µl PstI.
Incubated 2.5 hours at 37C (recommended incubation of at least 1 hour)
Stopped reaction by incubation 10 min. at 65C.
Postfix Insert (XbaI/PstI)
Combined 0.4 µl DNA (from plasmid prep), 15.6 µl nanopure water, 2 µl 10X NEBuffer 2, 1 µl XbaI, and 1 µl PstI.
Incubated 2.5 hours at 37C (recommended incubation of at least 1 hour)
Stopped reaction by incubation 10 min. at 65C.
Prefix Vector (EcoRI/XbaI)
<under construction>
Prefix Insert (EcoRI/SpeI)

<under construction>

Notes

Remember to check that REs are compatible.
Reaction can also be stopped by adding 5µl 10X loading buffer.
<10% of digest should be RE
REs are stored in glycerol. <5% glycerol should be present in digest. Therefore, a minimum 10-fold dilution is necessary so the enzymes don't act funky.
It's always a good idea to gently shake and spin down RE and RE buffer prior to use.
References: Short Protocols in Molecular Biology. Vol. 1. 5th edition. 3-3 to 3-4.
NEB

References

http://openwetware.org/wiki/Restriction_digest
http://openwetware.org/wiki/Endy:Restriction_Digest
http://openwetware.org/wiki/Knight:Restriction_Digest
http://2008.igem.org/Team:Hawaii/Initial_Synth._Oligo_Assembly
Short Protocols in Molecular Biology. Vol. 1. 5th edition. 3-3 to 3-4.
New England BioLabs Restriction Enzyme Product Insert

Contact

Grace Kwan
or instead, discuss this protocol.

DNA ligation – 2008 – Hawaii

http://2008.igem.org/Team:Hawaii/Procols/DNA_ligation
Protocol
Combine 4 ul of construct one with 4 ul of construct 2.
Add 1 ul of nanopure H_20.
Add 10 ul of 2x quick ligation reaction Buffer. In order to avoid shearing the DNA, mix by resuspending very slowly.
Add 1 ul of Quick T4 DNA ligase and mix thoroughly by resuspending very slowly.
Incubate at room temperature for 5 minutes.
Cool on ice then transform, or store at -20°C
Notes:
Adjust amount of DNA as necessary. For ligation of 2 pieces of DNA, use 1:1 ratio of parts. For ligation of insert to vector, use 3:1 ratio of insert to vector. ~180ng of DNA can be ligated using the above protocol.
Reference: Quick Ligation Kit from NEB.
The Endy lab recommends using ~10 ng vector in a reaction of with 6:1 ratio of insert to vector. Total DNA concentration should not exceed 100ng for maximum ligation efficiency.
Reference: OpenWetWare

Oligo Hybridization of Part – 2008 - Hawaii

http://2008.igem.org/Team:Hawaii/Protocols/oligo_hybridization
Protocol
Mix:
3 µl 100 µM sense oligo
3 µl 100 µM anti-sense oligo
3 µl 10 x PNK (polynucleotide kinase) buffer
2 µl 10mM ATP
2 µl T4 polynucleotide kinase (PNK)

17 µl distilled water
Total volume = 30 µl
Incubate at 37C for 1.5 hours.
Add 4 µl 0.5 M NaCl.
Heated mixture in PCR machine to 97C for 2 min. Cooled mixture at each degree between 80 and 96 for 2 min (Tm range of oligos). Cooled mixture at each degree between 25 and 79 for 1 min.
Reference: Pam Silver Lab. Oligonucleotide Inserts.

Transforming *Shewanella oneidensis* – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Grow bacteria to log phase (OD 0.4-0.6)
Aliquot 1mL of culture to a 1.5mL tube. Make as many aliquots as you need for the number of plasmids you need to transform
Centrifuge aliquots at 12,000g for 1 minute
Remove media and wash with 0.33 volumes (330µL) of 1M sorbitol
Centrifuge at 12,000g for 1 minute
Remove wash and resuspend in 0.05 volumes (40µL) 1M sorbitol.
Place tubes in ice and use within 15 minutes.
Add 100-500ng of plasmid DNA to the tube
Electroporate at 0.55kV
Flush cuvette gently with 800ul SOC
Allow to recover for 1-2 hrs at 30 degrees C, shake at 200rpm
Spin down cells briefly, pour off most of supernatant, gently resuspend cells and plate
Colonies will appear overnight at 30 degrees C, although they will be significantly smaller than E. coli grown for a similar period of time. After 2 nights of growth, colonies become visibly pinkish orange.

Freezing these cells for later use is not recommended. Even with storage at -80C, subsequent transformations often fail.
Efficiency is variable; 200ng DNA general yields 20-100 colonies.

Ligation protocol, using Roche Rapid Ligation Kit – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Brief Protocol
Mix:
5x DNA dilution buffer – 2uL
Vector (cut and CIP treated) – 0.25-1uL
Insert DNA – 0.5-6.5 uL
ddH2O – to final volume of 10uL

Vortex and quick centrifuge above mixture, then add:
10uL of 2X rapid ligation buffer
1 uL ligase
Vortex and quick centrifuge again. Hold at room temp ~20 minutes, then use 5uL of the ligation reaction to transform 50uL of chemically competent E. coli (eg, TOP10 or DH5-alpha).
Protocol Notes
Reagents
Make sure buffers are completely thawed before use. The 2x rapid ligation buffer contains a reducing agent that looks like white flakes when thawed. These white flakes must be completely dissolved back into the buffer before use.

Troubleshooting

What to try if your ligation isn't working (in this order).

Make sure your gel box uses long wave UV. Short wave UV will cause thymine dimer formation that can destroy your sticky ends (e.g. the TTAA overhang of EcoRI)

Repeat the ligation. Try a range of insert to vector ratios from 2:1 to 10:1.

Review all of your digestion steps – are you sure you cut the vector and the insert with the correct enzymes? Did you let it digest for long enough?

Try a different tube of enzymes kit.

Still not working? Send the parts you are trying to clone for sequencing. You may not be cloning what you think you are cloning.

Bacterial Transformation Protocol – 2008 - Harvard

http://2008.igem.org/Team:Harvard/GenProtocols

Chemically competent E. coli

To make chemically competent cells

Day 1:

Grow 5mL culture of bacteria in LB media overnight, 37° C , shaking

Day 2:

Inoculate fresh 100mL LB culture with 1mL bacteria from 5mL culture

Grow culture at 37°, shaking, to an OD600 of 0.2-0.4 (takes 1-3h.)

Perform the remainder of the protocol in the cold room OR take care to make sure cells remain cold!!!

Decant culture into two 50mL falcon tubes and chill on ice 15 minutes.

Spin for 15 minutes at 2500 RPM, 4°C

Remove supernatant and wash pellets in 50 mL ice cold 100mM $MgCl_2$ (add 25 mL per tube and combine tubes)

Spin for 15 minutes at 2500 RPM, 4°C

Remove supernatant and resuspend pellet in 50mL ice cold 100mM $CaCl_2$

Incubate on ice 30 minutes

Spin for 15 minutes at 2500 RPM, 4°C

Remove supernatant and gently resuspend pellet in 10 mL ice cold 100mM $CaCl_2$ + 15% glycerol (v/v).

Incubate on ice 30 minutes.

Aliquot 100uL to 0.6mL tubes and store at -80°C. (makes ~100 aliquots)

To transform chemically competent cells

Soak the spots in 5 µL of the warmed TE for 20 minutes. This allows the maximum concentration of DNA in solution. Start thawing the competent cells on wet crushed ice.

Chill labeled 2 ml conical bottom tubes on wet ice. Add 2 µL of DNA in TE and 50 µL of thawed TOP10 competent cells to the tubes. In our experience, these volumes have the best transformation efficiency. The 2 ml tubes allow better liquid movement during incubation. Extra eluted DNA may be held at least several weeks frozen or at refrigerator temperature.

Hold the DNA and competent cells on ice for 30 minutes. This improves transformation efficiency by a significant amount.

Heat shock the cells by immersion in a pre-heated water bath at 42ºC for 60 seconds. A water bath is important to improve heat transfer to the cells.

Incubate the cells on ice for 2 minutes.

Add 200 µl of SOC broth (check that this broth is not turbid, which would indicate previous contamination and bacterial growth). This broth should contain no antibiotics.

Incubate the cells at 37ºC for 2 hours while the tubes are rotating or shaking. We have found that growth for 2 hours helps in transformation efficiency, especially for plasmids with antibiotic resistance other than ampicillin.

Label an LB agar plate containing the appropriate antibiotic(s) with the part number, plasmid, and antibiotic resistance. Plate 250 µl of the incubated cell culture on the plate.
Incubate the plate at 37ºC for 12-14 hours, making sure the agar side of the plate is up. If incubated for too long the antibiotics, especially ampicillin, start to break down and un-transformed cells will begin to grow.

Restriction Digest, using New England Biolabs (NEB) Enzymes – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Pick a volume where it will be easy to calculate the quantities of 10x and 100x buffers to add and write out a quick recipe (See samples).
(You can add a little water or EB buffer to make this easy, without hurting the reaction)
Pipette the DNA to cut into a 1.5ml eppendorf tube.
(Keep a few micoliters to run on a gel against the product, as a negative control)
Add water if you need (See example below)
Add 10X reaction buffer
(See the enzyme(s) page in the NEB manual or website for the correct one)
Add 100X BSA if needed
(BSA is recommended for some reactions (see manual), and will not hurt any)
Add restriction enzyme(s) last
(Keep these on ice or in a freezer box, **Always**) (Also, keep the percentage of enzyme in the reaction below 5% by volume to avoid nonspecific cutting)
Let the reaction run at the temperature recommended in the NEB manual for at least 2 hours.
(Overnight is ok)
Run a bit of the digest sample against the undigested control to confirm that the digstion worked.
Sample single digest (50 ul total volume)
30ul DNA
13.5ul Water
5ul 10X Buffer
0.5ul 100X BSA
1ul Restriction Enzyme
Sample double digest (50 ul total volume)
First, check the NEB manual double digest page for optimal conditions or whether it is recommended against for your enzymes
30ul DNA
12.5ul Water
5ul 10X Buffer (Check NEB double digest table - many not be what is used for the single digests
0.5ul 100X BSA (Add if it is required for *either* enzyme)
1ul Restriction Enzyme 1
1ul Restriction Enzyme 2

PCR protocol – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Colony PCR with Platinum Taq
Touch the tip of a p2 pipet tip gently to the center of one colony
Swish the tip around in 100uL of sterile water
Vortex for a few seconds then hold on ice
Thaw PCR SuperMIx and keep it on ice
Reconstitute all primers to 100µM
Make a 1:5 working dilution of your primer from the 100µM stock, using sterile water
PCR reactions set up

Reaction	PCR SuperMix (uL)	Template from colony (uL)	5' Primer (uL)	3' Primer (uL)	Water to 50 uL
A	45	1	1	1	2
B	45	3	1	1	--

Set up an A and a B reaction for each colony and for each primer set (ps)
ex: colony #1 1-A-PS1, 1-B-PS1, 1-A-PS2, 1-B-PS2
Cycle:
94 C for 30 sec
55 C for 30 sec
72 C for 1 min/ kb of sequence
Cycle 35 times
hold at 4 C when cycles are complete

PCR with Phusion polymerase **– 2008 - Harvard**
http://2008.igem.org/Team:Harvard/GenProtocols
Rx mix:
10µL 5x HF buffer
1µL 10mM dNTPs
1.25µL each primer
0.5µL template
0.5µL Phusion polymerase
35.5µL H_2O

Initial denaturation: 30s @ 98°C (2:30 if doing colony PCR)
Denaturations: 10s @ 98°C
Annealing: 30s
Extension: 15-30s/kb @ 72°C (20s is usually enough for plasmids)

Colorimetric LBB Assay Protocol (from Colleen Hansel) – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
LBB Reagent: 0.04% LBB in 45 mM acetic acid
(0.04 g LBB in 100 mL 45 mM acetic acid)
45 mM acetic acid (2.81 mL of concentrated (16M) acetic acid in 100 mL)

STANDARDS
Mn(III/IV) standard made with potassium permanganate. Add 2mM stock concentrations in 25 mL.

Standards (uM)	2 mM Stock (uL)
10	125
15	187.5
20	250
40	500
50	625

For Reaction:

Add standard (or sample) to LBB at 1:3 (0.25 mL standard:0.75 mL LBB)
Use DI water as blank before running standard curve
Incubate in dark for 15 minutes
Record Absorbance on a spec at 620 nm

Note: The linear range for the LBB colorimetric method is 10-50 uM. If your samples are above this limit, dilute accordingly to bring into linear range. Do not force standard curve through zero – there is a background for the LBB solution.
Setting up Samples for Sequencing

Testing IPTG Inducible GFP Systems – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Grow overnight culture of cells (including positive control w/o repressor and negative control w/o fluorescence)
In morning do a 1:20 dilution in LB w/ resitance markers
Incubate for ~2hrs
OD and measure fluorescence (for GFP ex 489 and em 509) - OD levels should be between .3 and .5
Add 1mM IPTG to half of each sample
Incubate for 2hrs
OD and measure fluorescence

Pouring LB plates w/ antibiotics – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Heat LB-agar in microwave until it melts - take it out when it starts to boil (about 4 min). Be careful to make sure it doesn't boil over
Gently swirl media and let cool to below 45-50°C (able to hold w/o pain for 10 s). Try to avoid creating too many bubbles. Adding amp at a higher temperature will ruin it. To speed the cooling process, you can swirl it in ice water; however, if you let the temperature drop too low the agar will begin to harden and you will have to re-melt it.
All following steps must be done by a flame
Once cool enough add antibiotic in a 1:1000 dilution (Amp, Sm, Kan, Cm are all stored as 1000x stock)
Quickly pour into petri dishes- add enough to each plate so that the agar just covers the entire plate
Flame bubbles or move them to the side of plate by gently tilting plate
Leave plates on table until they solidify with lids on
Label sleeve or individual plates. Kan= kanamycin, Amp= ampicillin, Cm= chloramphenicol, Sm= spectomycin

Ethanol precipitation of Takara ligations – 2008 - Harvard
http://2008.igem.org/Team:Harvard/GenProtocols
Place a 15mL falcon of 200 proof ethanol in ice (there's an aliquot in -20°C most likely)
Put a microcentrifuge into a 4°C fridge (you might have to take the rocker out of the one in the small room)
Add 1/10 volume's (of original ligation) worth of pH 5.2 3M sodium acetate; mix well
Add 2 volume's (of original ligation) worth of ice cold 200 proof ethanol; mix well
Stick in -80°C for 15-20 minutes
Spin at max speed (at least 13,000 rpm) for 15-20 minutes at 4°C
Remove supernatant- be careful not to remove the DNA, which may or may not be a visible pellet at the bottom of the tube. It's better to leave some ethanol than to lose your DNA.

Add 1mL 70% EtOH; mix by vortexing briefly
Spin at max speed in microfuge for 1min
Remove supernatant- be careful not to remove the DNA, which may or may not be a visible pellet at the bottom of the tube. It's better to leave some ethanol than to lose your DNA.
Use the vacuum centrifuge (on widget group's bench) to evaporate the remaining ethanol
Resuspend in 10-15µL sterile nuclease-free water. Be sure to pipet the water repeatedly onto the sides of the bottom of the tube and vortex gently a few times. Spin down everything.

FimE Inversion Assay – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/FimE

Grow 5mL LB cultures with appropriate antibiotics overnight.
Backdilute culture 1:100 into fresh 5mL LB with appropriate antibiotics.
When the cultures reach 0.4 OD at 600nm, add 0.1% arabinose and grow at 37C for 1 hour.
Wash and resuspend cells in phosphate buffer solution (100mM, 7.0pH).
Measure GFP fluorescence of 200 uL of culture at 498nm on plate reader.

Flow Cytometry – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/Flow_cytometry
Set up 10mL LB cultures with appropriate antibiotic and aTc concentrations. Grow overnight at 37C while shaking.
Take out cultures and measure OD at 600nm. If it's below 0.4, place back at 37C for another 3 hours until it reaches OD ~0.5.
Spin down cultures in 15mL falcons at 10min, 3000xg and 22C.
Resuspend in 2mL BioAssay Buffer (BAB).
Spin 2 min to pellet.
Resuspend in BAB + antibiotic + aTc (at same antibiotic and aTc concentrations as the original overnight cultures).
Measure OD and make sure it's between 0.4 and 0.6. If it's below 0.4, spin and pellet and try again.
Put in 37C shaker for 4 hours.
Measure fluorescence of 1.5mL samples.

Bioassay Buffer – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/BioAssay_Buffer

To 1L ddH20 you will add:
0.5 g Tryptone
0.3 mL 100% Glycerol (or, I use 0.6 mL sterile 50% glycerol)
5.8 g NaCl
5.0 mL 1M MgSO4
Autoclave to sterilize. Small aliquots are preferred, since contamination is difficult to detect.

para-Aminobenzoic Acid (pABA) HPLC Assay Protocol – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/PABA_HPLC_assay

Purpose

To test for para-aminobenzoic acid levels in *E. coli* overexpressing the pABA synthesis genes *pabA* and *pabB*.

Materials

Buffer A: 0.1% formic acid in H_2O

Buffer B: 0.1% formic acid in MeOH

A high performance liquid chromatography machine.

HPLC Column: Eclipse XDB-C18 column, 5um particles, 4.6x150mm column, with attached guard column (all from Agilent)

Procedure

Sample Prep

Centrifuge the cell culture max speed, 10 minutes.

Separate pellet from supernatant.

Resuspend the pellet in 1mL 0.1M Tris-HCl buffer

Sonicate the resuspended pellet for 1 minute, alternating between 0 and 12Hz.

Filter sterilize the cell lysate and the supernatant.

Load 500uL of each into the HPLC.

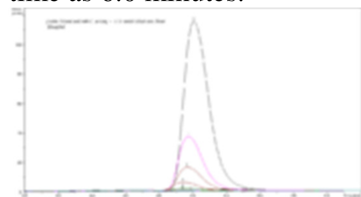
HPLC Method

Injection volume was 20uL, column temperature was kept at 40°C.

Flush the lines individually with Buffer A and B for two minutes each, flush the column with 92% A, 8% B for 10 minutes.

From the source: "The starting eluent was 92% A mixed with 8% B; the proportion of B was increased linearly to 50% in 7 min, then to 100% in 3 min. The mobile phase was then immediately adjusted to its initial composition and held for 4 min in order to re-equilibrate the column."

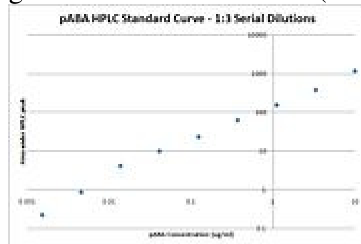
We found the retention time of pABA to be 4.9-5.0 minutes, however the literature states the retention time as 6.0 minutes.



pABA eluting at 4.9-5.0 minutes for the standard curve.

Standard Curve

1. Run 1:3 dilutions starting from 10ug/ml pABA in wild type bacterial lysate using the same protocol to generate a linear standard (see below).



pABA HPLC standard curve with 1:3 dilutions starting from 10ug/ml pABA. The integral of the peak is plotted against the known pABA amount.

Sources

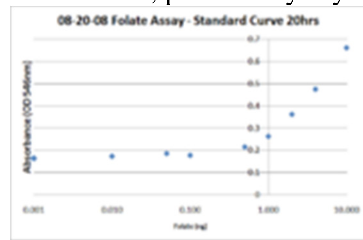
Guo-Fang Zhang, Kjell A. Mortier, Sergei Storozhenko, Jet Van De Steene, Dominique Van Der Straeten, Willy E. Lambert. *Free and total para-aminobenzoic acid analysis in plants with high-performance liquid chromatography/tandem mass spectrometry.* **Rapid Communications in Mass Spectrometry**: Volume 19, Issue 8 , Pages 963 - 969, 2005.

Contacts
Victoria Hsiao

Folate Microbiological Assay – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/Folate_assay

Purpose
To quantify and measure levels of folate production in the cell lysate and supernatant of 'E.coli' transformed with high copy folate biosynthesis genes *folB, folKE, folBKE*.
The assay uses growth of the folate-dependent strain *L. rhamnosus* as an indicator of folate concentration in the sample. Growth is measured by taking the OD at 546nm.
Important Notes
Almost every ingredient in this assay is light sensitive -- take extreme caution to limit exposure of buffers, folic acid mixtures, and sample tubes to light. I usually wrapped the buffers, assay media, and standard curve folic acid dilutions in aluminum foil. I also started covering the entire rack of samples with foil during incubation, but decided it would be ridiculous to covered each sample tube with foil.
Previously, we had been using this protocol with the other folate-dependent strain *Entercoccus hirae* ATCC 8043, and were able to generate a linear standard curve from 0.1-1 ng. However, this strain did not grow when combined with our samples, so we switched to *L. rhamnosus*. Lactobacilli Broth AOAC is recommended for *L. rhamnosus*, so we used this media in place of the recommended Lactobacilli MRS broth.
Materials
Folic Acid Assay Medium (BD Biosciences)
Lactobacilli Broth AOAC (BD Biosciences)
Folic Acid (for standard curve)
0.1M sodium acetate buffer (pH4.8) -1% ascorbic acid
Ascorbic acid (Sigma, A4544-25G)
0.1M 2-mercaptoethanol-0.5% sodium ascorbate (We used the above 0.1M sodium acetate - 1% ascorbic acid buffer in place of the sodium ascorbate)(for deconjugation mix)
Sodium ascorbate
Human plasma (Sigma, P9523-1ML)(for deconjugation mix)
Lactobacillus rhamnosus (ATCC 7469)
Saline (0.9% NaCl in H20)
Tween 80
Folic Acid Assay Media
Add 100g of the dehydrated assay media to water. Light sensitive, cover with foil.
Autoclave the media using normal liquid autoclaving procedures.
Add 0.4 mL/L of filter sterilized Tween 80 to the media. This prevents any oleic acid present in the sample from causing a lag phase in the bacterial growth.
Deconjugation Mixture
Dilute 1 g of human plasma in 5ml of 0.1M 2-mercaptoethanol-0.5% sodium ascorbate
Clear from precipitates by centrifugation (10,000xg,2 min)
Add 2.5% (vol/vol) concentration of the clarified human plasma solution to the folate samples.
L. rhamnosus inoculum preparation
Inoculate 5 mL of Lactobacilli Broth AOAC with *L.rhamnosus.*
Incubate for 12-24 hours.
Centrifuge the culture, (13,000x g, 10 min, 20C), remove the supernatant.
Wash twice with saline (0.9% NaCl in H20)
Resuspend in folate assay media, adjust OD (546nm) to approximately 1.0.
Sample Preparation

Centrifuge the full-grown cell culture (5ml) after centrifugation (13,000x g, 10 min, 20 C).
Recover both cells and supernatant.
Dilute the supernatant 1:1 with 0.1M sodium acetate buffer (pH4.8) -1% ascorbic acid.
Wash with the 0.1M sodium acetate-1% ascorbic acid and resuspend in 5 mL of the same buffer.
Release folate from the cells by incubating the samples at 100C for 5 min (determined to be optimal for folate release + the heat inactivates the bacteria)
Add the deconjugation reaction mixture (2.5% vol/vol)
Incubate for 4h at 37C, you should see obvious cell debris at the bottom of the cell lysate tubes.
Filter sterilize 2.5 mL of each sample into a clean glass tube.
Add 2.5 mL of folate assay media to each tube, bringing the volume up to 5mL.
Add 50uL of prepared *L.rhamnosus* inoculum to each assay tube.
Incubate for 16-20 hours at 37C.
Read the absorbances of the samples at 546 nm on a plate reader (200uL per well). The protocol recommends refrigerating the samples at 4C for 15-30 minutes prior to reading, but I don't think it makes a difference, particularly if you intend on returning the samples to the incubator to take later timepoints.
Standard Curve
Prepare folic acid dilutions from 0- 10 ng/mL. Usually we used 0.001, 0.01, 0.05, 0.1, 0.5, 1, 2, 4, 8, 10ng samples. With *L. rhamnosus* the linear range of the assay appears to be from 0.1 to 10 ng.
Add 2.5 mL of folic acid assay media to each tube, along with 2.5 mL folic acid + water to make up a total volume of 5mL.
Add 50uL of the prepared *L. rhamnosus* inoculum.
Incubate for 16-20 hours at 37C.
Read the absorbances of the samples at 546 nm on a plate reader (200uL per well). The protocol recommends refrigerating the samples at 4C for 15-30 minutes prior to reading, but I don't think it makes a difference, particularly if you intend on returning the samples to the incubator to take later timepoints.



A fantastically linear standard curve
Sources
Sybesma et al. *Increased Production of Folate by Metabolic Engineering of Lactococcus lactis.* **Applied and Environmental Microbiology**, June 2003, p. 3069-3076, Vol.69, No. 6 [1]
Horne et al. *Lactobacillus casei Microbiological Assay of Folic Acid Derivatives in 96-Well Microtiter Plates* **CLIN. CHEM.** 34/11, 2357-2359 (1988) [2]
Folic Acid Assay Medium [3]
Contacts
Victoria Hsiao

LacZ Assay – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/LacZ_Assay

Grow cells overnight in 5 mL culture of LB Media with proper antibiotic at 37˚C and 225 RPM.
In the morning, back-dilute overnight cultures 1/100 (50 µL into 5mL) and grow for 3 hours at 37˚ and 225 RPM. The OD should be between 0.09 and 0.20 for 200 µl in a 96-well plate measured in a plate reader (Spectramax M2).

Combine 20 µL of cell culture and 380 µL permeabilization solution, mix, and place at 37˚C for 10 minutes.
Permeabilization Solution: 100mM Na_2HPO_4, 20mM KCl, 2 mM $MgSO_4$, 0.6 mg/mL CTAB, 0.4 mg/mL sodium deoxycholate, and 5.4 µl/mL ß-mercaptoethanol.
Combine 100 µL of lysed cells with 600 µL substrate solution.
Substrate Solution: 60 mM Na2PO4, 40 mM NaH_2HPO_4, 1 mg/mL ONPG, and 5.4 µL/mL ß-mercaptoethanol.
When a faint yellow color is observed, add 300 µl 1M sodium carbonate to stop the reaction.
Miller units are calculated using the following equation: $MU = 1000 (ABS_{420} / (0.02 * t * ABS_{600}))$.

Titering Bacteriophage – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/Titering

Grow fresh overnight cultures of the bacteria of interest (i.e. LE392, D1210, etc.)

An example plate. Dark spots are cleared zones in a lawn of bacteria.
Incubate the cultures until they reach an OD600 of roughly 0.1
More specifically, when the culture is swirled, cloudiness is observed
Do NOT titer with bacterial cultures that have just been diluted from a culture past OD600 of 0.8 (complications will occur)
Place agar/agarose plates in the 42 C oven and ready the 48 C water bath
Prepare fresh 10 mM MgSO4 solution from the 1 M MgSO4 stock solution (100 ul MgSO4 in 10 mL sterile water works conveniently)
In 1.5 mL eppendorf tubes, add 1 mL of the 10 mM MgSO4 solution.
Prepare phage dilutions by adding appropriate amount of phage into MgSO4 solution (i.e. 1000-fold dilution is 1 ul phage in 1 ml MgSO4)
After addition of phage to solution, invert tube at least 12 times for sufficient mixing
For further dilutions, can simply dilute made phage dilutions (i.e. 1,000,000-fold can be obtained from taking 1 ul 1000-fold diluted phage and adding to another 1 mL of 10 mM MgSO4
Aliquot 0.1 mL cell solution (i.e. LE392, D1210, etc.) into new 1.5 mL eppendorf tubes
To begin infection, add appropriate amount of diluted phage to cell solution (10 ul usually works well for starters) and start the timer
During this 30 minute infection period, label the plates and place back in the 42 C oven
~3 minutes before the end of infection, can take 3 mL aliquots of the top medium in 13 mL falcon tubes or 5 mL round-bottom tubes and microwave till complete liquification has occurred.

Note that top agar is the same composition as is used to pour the plates, only with half the agar (so ~7g/L agar rather than 15 g/L).
Right before the end of infection, take out the plates and place on the bench with the cover on top. Have a flame close-by to prevent contamination. Also, set the pippette to the right volume of infected cell solution and have sterile pipette non-filter tips ready
Very quickly with pipette in hand, pippette out infected cell solution, add to top medium, invert the tube at least 2 times, and pour the mixed contents onto the corresponding plate.
Rock the plate gently to allow the top medium to uniformly cover the plate and use the sterile tips to poke any bubbles.
Let the plate cool on the bench for at least 5 minutes (10 minutes is usually enough) with the cover completely on the plate.
Place the plate in the 37 C incubator.

rcsA Lysogen Induction – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/rcsA_Lysogen_Induction

Grow fresh overnight cultures of lysogens of bacteria carrying the rcsA construct.
Grow fresh overnight cultures wildtype E. coli.

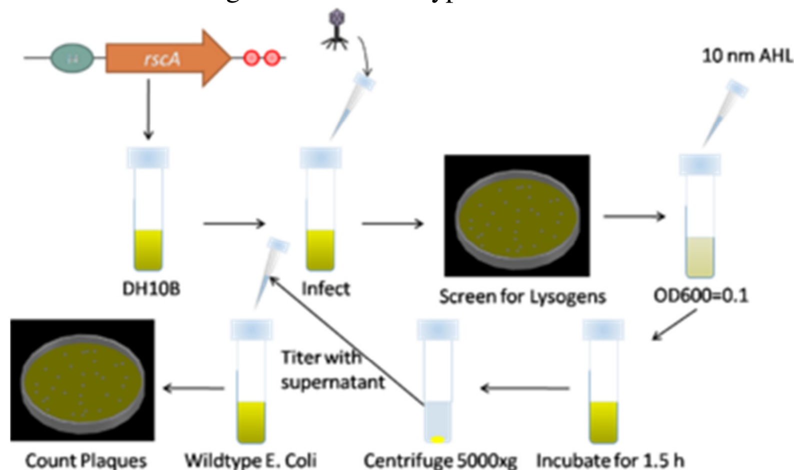
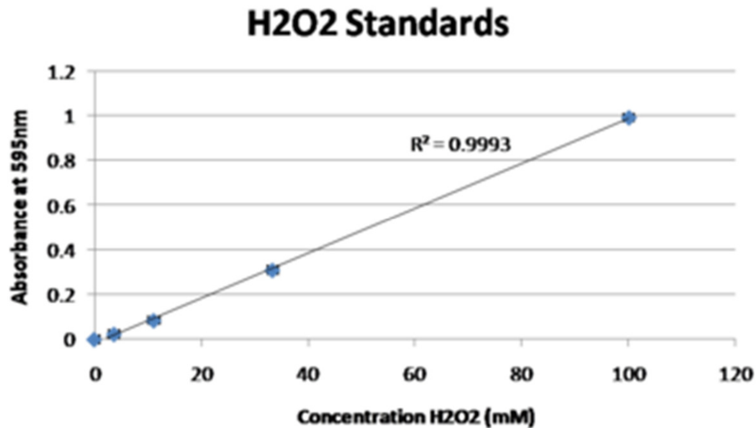


Diagram of the induction process.
Make a 1:1000 dilution of lysogen culture and incubate the cultures until they reach an OD600 of 0.1
More specifically, when the culture is swirled, cloudiness is observed. Check OD on plate reader as it is important to keep OD constant between trials and experiments.
At this time also make a 1:1000 dilution of the wildtype E. coli culture.
Add AHL to bring the concentration within the Lysogen culture to 10 nM.
Resume incubation at 37 degrees C for 1.5 hours.
At 1.5 hours, add 5% v/v formaldehyde to the lysogen culture and vortex vigorously.
Spin down cells at 5000xg for 5 minutes.
Remove an aliquot of the supernatant.
Follow the phage titering protocols using the supernatant as the phage solution, and titer against the culture of wildtype E. coli.
Count plaques the following day to estimate the concentration of phage in supernatant.

Measuring H_2O_2 – 2008 – Caltech
http://2008.igem.org/Team:Caltech/Protocols/Measuring_H2O2

Note: Based on manufacturer's instructions.
Spin down approximately 100 uL of cell culture
Measure peroxide concentration using a commercial colorimetric assay following the manufacturer's instructions.
After incubating the reaction at room temperature for at least 20 minutes, measure the absorbance at 595 nm on a plate reader (Spectramax M2).
Calculate the concentration of hydrogen peroxide by reference to a standard curve (100uM, 33.3uM, 11.1 uM, 3.7 uM and 0.0 uM H2O2) made in the corresponding media.
NB: Dilute culture supernatants as appropriate to bring measurements into the assay's linear range.



An example of a typical standard curve generated by the assay. Error bars (1SD, n=3) are too small to be seen.

Coculture Inhibition Assay – 2008 - Caltech
http://2008.igem.org/Team:Caltech/Protocols/Coculture_Inhibition_Assay

Strains
The engineered strain was JI377 transformed with K137076 (spxB) in pSB1A2 (AmpR).
The target strain was JI377 transformed with B0015 (a transcriptional terminator) in pSB1AK3 (AmpR KanR).
The negative control was JI377 transformed with a modified pUC18 vector (AmpR) containing galactose oxidase. (Kindly provided by Professor Arnold at Caltech.)
Protocol
Grow overnight cultures of each strain in LB + Amp.
In the morning, back dilute cultures 1:100 into SOC + IPTG + Amp and grow to an OD600 of ~0.8.
To begin the assay, inoculate the target strain into 2.5 ml cultures of the engineered or control strains in amounts of (A) 1:1,000 (B) 1:10,000 and (C) 1:100,000.
Immediately serially dilute aliquots of the cocultures and plate to single colonies on LB+Kan plates for CFU counting.
Co-culture "A" should be plated at dilutions of 1:100, 1:1,000, and 1:10,000.
Co-culture "B" should be plated at dilutions of 1:1,000 1:10,000 and 1:100,000.
Co-culture "C" should be plated at dilutions of 1:10,000 1:100,000 and 1:1,000,000.
Induce the co-cultures to produce hydrogen peroxide by bringing them to 10 nM AHL.
Incubate co-cultures for 6hrs and then plate to single colonies as before.
After incubation at 37C, count the CFU of each plate.

Measuring Minimum Inhibitory Concentration – 2008 - Caltech
http://2008.igem.org/Team:Caltech/Protocols/MIC_Assay

Grow one 5 ml culture to saturation at 37C with shaking in the indicated media and antibiotics as necessary.
Back dilute parent culture 1:50 into 5 ml of the same media and antibiotics plus differing amounts of hydrogen peroxide and let shake at 37C overnight.
Record MIC as the concentration of hydrogen peroxide necessary to prevent overnight turbid growth.

H_2O_2 Production Assay – 2008 - Caltech
http://2008.igem.org/Team:Caltech/Protocols/H2O2_Production_Assay

Grow an overnight culture of JI377 transformed with K137076 or luxR (negative control) on pSB1A2 in SOC + Amp.
In the morning, dilute culture 1:100 into 5 ml SOC +Amp +IPTG in triplicate.
Grow cultures to an OD600 of ~0.8, then assay supernatant for hydrogen peroxide approximately every 30 minutes.
1hr after reaching an OD600 of ~0.8, induce cultures with 10 nM AHL.

Purification of His_Z_alpha and His_Z_omega – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Culture *E. coli* producer strain in 10 ml of liquid LB medium for 8 hours. Then use it to inoculate 1000 ml of liquid LB medium with 0.5 mM IPTG and grow it overnight. In the morning spin down the culture (5000 RPM, 10 min, 4°C). Resuspend the pellet in PBS buffer and disrupt cells by sonication. Spin down sonication mixture (13200 RPM, 10 min, 4°C) and discard supernatant – protein is present in sonication debris. Resuspend it in sterile ice cold ddH2O and Spin down (13200 RPM, 10 min, 4°C). Discard supernatant and resuspend it in sterile ice cold ddH2O and store at 4°C.

Purification of His_A_alpha – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Culture, induce and disrupt *E. coli* in the same way as to purify His_Z_alpha. The protein is present in supernatant (about 10% of total protein) and can be added to selection medium without further purification. Nevertheless we purified it to determine how much exactly should be added:
Swing sonication products with Ni-nta-agarose bed for 2 hours at 4°C
Load them onto column
Wash the bed with 20 mM imidasole buffer
Elute purified protein with 100 mM imidasole

Testing various hunter/prey combinations – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Setup of culture E. coli carrying "hunter" with kanamycin and 0.2 mM IPTG
Inoculate liquid LB medium with kanamycin, 50 μg/ml ampicillin, 0.2 mM IPTG and "prey" (the control is medium without "prey")

Grow it 4-16h
Observe growth, or its lack
Isolate the plasmid DNA
Perform control digestion

Plasmid DNA isolation – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
We use "Plasmid Mini" plasmid DNA isolation kit from A&A Biotechnology and follow the protocol of producer.

DNA isolation from agarose gel – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
We use "Gel-Out" DNA isolation kit from A&A Biotechnology and follow the protocol of producer.

DNA purification after enzymatic reaction – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
We use "Clean-Up" DNA purification kit from A&A Biotechnology and follow the protocol of producer.

Genomic DNA isolation – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
We use "Genomic-Mini" universal genomic DNA isolation kit from A&A Biotechnology and follow the protocol of producer.

DNA digest – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
We use restriction enzymes and buffers provided by Fermentas. Overall volume of digest mix is either 20 μl, either 50 μl in case of digesting for ligation. We usually use 1 μl of restriction enzyme and the buffer in 10x dilution (as they initially are 10x concentrated). The rest of mix is plasmid DNA.

Enzyme & buffer combinations
(as recommended by Fermentas)

Buffer	**Enzyme**	**Enzyme**
BamHI buffer	BamHI	SacI
BamHI buffer	NdeI	SacI
BamHI buffer	PstI	KpnI
BamHI buffer	PstI	BamHI
Tango 1x	XbaI	PstI
BamHI buffer	EcoRI	BcuI (SpeI)
BamHI buffer	SacI	NotI

Tango 2x NdeI BamHI

Control digests are set up for 1 hour.
Digests for cloning take 3 hours or are left overnight.
Enzymes are deactivated by high temperature or by putting on gel, according to producer's recommendations.

Preparation of chemocompetent bacteria – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Keep the bacteria on ice during the procedure. Pour ca. 25 ml of bacteria into a falcon tube and spin at 4°C at 4 krpm, 8 min with prolonged acceleration and deceleration. Remove supernatant. The pellet mustn't run dry. You can pour another portion of bacteria onto it and spin again. After desired amount of bacteria in pellet is collected, add $CaCl_2$ in an amount of 10% of initial culture used for spinning. Suspend the pellet until no debris is visible on the bottom. Incubate 45 min on ice. Then spin 8 min at 4 kg and remove supernatant. Suspend the pellet in 3 ml $CaCl_2$ and divide into aliquots of 100 µl.

Preparation of electrocompetent bacteria – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Set up bacterial culture in 10 ml.
Use the culture for inoculation of 1 L of medium and let it grow at 18°C until it reaches OD 0.6 - 0.8.
Spin for 10 min at 6 krpm.
Remove supernatant and suspend the pellet in **1 L** of H_2O.
Spin for 10 min at 6 krpm.
Remove supernatant and suspend the pellet in **1 L** of H_2O.
Spin for 10 min at 6 krpm.
Remove supernatant and suspend the pellet in **0.5 L** of H_2O.
Spin for 10 min at 6 krpm.
Suspend the pellet in 20 ml 10% glycerol.
Spin for 10 min at 6 krpm.
Suspend the pellet in 3 ml 10% glycerol.
Divide into aliquots of 40 µl and freeze in liquid nitrogen.

Electrotransformation – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Pour 100 ml H_2O plus desired amount of DNA into electroporation cuvette.
Add 40 ul of bacteria.
Electroporate.
Add 0.5 ml of LB.
Incubate with shaking at 37°C.
Plate.

Chemotransformation – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o

Add desired volume of DNA to the 100-µl-culture in eppendorf tube. Incubate 30 min on ice. Heat shock for 90 s at 42°C. Incubate 10 min on ice. Add 0.9 ml of culture medium and let the bacteria grow at 37°C.

Ligation – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
We use the following mixture:
appropriate volumes of vector and insert DNA (usually concentration of insert 3X higher than that of vector)
2 µl of ligation buffer
1 µl of T4 DNA ligase (purchased from Fermentas)
nuclease-free water
Overall mix volume is 20 µl.
If ligated DNA has sticky ends - incubate 2h at room temperature; if ligated DNA has blunt ends - perform overnight incubation at 18°C.

DNA ends blunting – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Prepare digestion mix in overall volume of 50 µl.
Add to reaction mix:
1.5 µl of 2 mM dNTPs
0.5 µl Klenow fragment (for 5' sticky ends)
0.5 µl T4 DNA polymerase (for 3' sticky ends)
Incubate overnight at 37 degrees.

Standard concentrations of antibiotics and other supplements – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Ampicillin
100 µg/ml for high copy number plasmids (pET15b)
30 µg/ml for one-copy plasmid (pZC320)
50 µg/ml for testing various hunter/prey combinations

Tetracycline
12-15 µg/ml

Kanamycin
30 µg/ml

Rifampicin
300 µg/ml

Chloramphenicol
35 µg/ml

X-Gal

40 µg/ml

IPTG
For liquid LB broth: variable concentration, depends on induced protein
For plates (blue-white screening): 0.1 mM

Rifampicin test – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Transform competent *E. coli* GM2163 or Top10 cells with:
pMPM-T5+AID
pMPM-T5+AID+T7 (transcriptional fusion)
pMPM-T5+AID-T7 (translational fusion)
pMPM-T5+AID+AID-T7
pMPM-T5+T7
and plate on LB + Amp 30 µg/ml + Tet.

Inoculate two tubes with 3 ml LB + Amp30 µg/ml + Tet + 100 µl of 20% L-arabinose with colonies of transformants (negative control without arabinose)
Incubate overnight at 37°C
Plate 200 µl of bacterial culture on LB + 300 µg/ml Rifampicin + Tet
Incubate overnight at 37°C.

Removing of 5' phosphate groups from DNA ends – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Make digestion mix in overall volume of 50 µl.
Add 1 µl of Calf Intestinal Alkaline Phosphatase to the reaction mix.
Incubate 2h or overnight at 37°C.

TAXI protocol (Tet+Ap 30+X-Gal+IPTG) – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o

Single transformations of competent *E. coli* GM2163 (or TOP10 - in this case steps with GM2163 used were omitted, induced transformants were plated immediately on TAXI) carrying plasmid pZC320 with:
pMPM-T5+AID
pMPM-T5+AID+T7 (transcriptional fusion)
pMPM-T5+AID-T7 (translational fusion)
pMPM-T5+T7
Induction using of L-arabinose (100 µl 20% inductor/3 ml LB broth Ap 30 µg/ml + standard Tet) and negative control of each probe
Isolation of plasmids (pMPM-T5+AID, pMPM-T5+AID+T7 (transcriptional fusion) , pMPM-T5+AID-T7 (translational fusion) andpMPMT5+T7)
Transformations of competent *E. coli* TOP10 with the isolated plasmids.
Plating on TAXI (Tet+Ap 30+X-Gal+IPTG)

Protein concentration measurement (BCA method) – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Place 10 µl of sample in a cuvette. Use 10 µl of solution, in which the sample is suspended, as control.
Mix BCA (bicinchoninic acid) with $CuSO_4$ (concentration?) at ratio of 50:1 .
Add 1.99 ml of BCA with $CuSO_4$ to the cuvetes.
Incubate 30 min at 37°C.
Measure absorbance at 562 nm.
Read protein concentration from reference curve.

PCR – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
Most PCR was carried out in following conditions:
3 min 94°C - preincubation
30 s 94°C - melting
30 s annealing (temperature depends on primer sequence)
72°C (elongation time depends on length of product)
The above steps were repeated 15 - 35 times (depending on PCR efficiency)
5 min 72°C additional elongation
hold 4°C
PCR standard mix
For colony PCR (10 µl of reaction)
1 µl of each primer
1.2 µl $MgCl_2$ (25 mM)
0.8 µl dNTPs mix (2 mM)
1 µl Pfu buffer
0.2 µl Pfu turbo polymerase water up to 10 µl
template - bacterial cells suspended in PCR mix
To obtain PCR product for cloning (50 µl)
5 µl of each primer
6 µl $MgCl_2$ (25 mM)
4 µl dNTPs mix (2 mM)
5 µl Pfu buffer
1 µl Pfu turbo polymerase
template depends on DNA concentration
water up to 50 µl

Polymerase Chain Ligation – 2008 – Warsaw
http://2008.igem.org/wiki/index.php?title=Wiki/Team:Warsaw/protocols#Z_a_Z_o
It's PCR reaction on two partially complementing templates. In our project all fusions containing linkers were put together using this technique. Typical mix:
5 µl of each primer
6 µl $MgCl_2$ (25 mM)
1 µl dNTPs mix (10 mM)
5 µl Pfu buffer

1 µl Pfu turbo polymerase
achieving equal amounts of both templates is crucial
water up to 50 µl
Note about cycling conditions: It's very important to check that melting temperature of complementing region is lower than elongation temperature, thus in all of our PCL reactions elongation was carried out at 68°C

Glycerol Stock – 2008 – Valencia
http://2008.igem.org/Team:Valencia/Notebook/Protocols
2 ml Eppendorf containing:
Sterile Glycerol (stock 50 %) 1ml
Half volume of a culture plate

SD Medium – 2008 – Valencia
http://2008.igem.org/Team:Valencia/Notebook/Protocols
Yeast Nitrogen Base 0.67%
Glucose 2%
Agar 2%
Casamino acids 0.1%
Tryptophan 20 mg/l
Adenine 40 mg/l

SP liquid medium – 2008 – Valencia
http://2008.igem.org/Team:Valencia/Notebook/Protocols
Yeast Nitrogen Base 0.67%
Potassium phosohate 0.1%
Ammonium sulfate 0.12%
Glucose 0.05%
Lactic acid 2%
Casamino acids 0.1%
Tryptophan 20 mg/l
Adenine 40 mg/l

YPKAc Medium – 2008 – Valencia
http://2008.igem.org/Team:Valencia/Notebook/Protocols
Yeast extract 1%
Peptone 2%
Potassium acetate 2%
Tryptophan 20 mg/l
Adenine 40 mg/l

Making Agarose Gel – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Standard 1%
Time:
~1 hour
Required:
Gel tank, with combs, casting tray and relevant power supply
Agarose powder

TAE buffer, 1x
Ethidium bromide

Method
1.) The gel tank we normally use holds 150ml, therefore, take 150ml of 1x TAE Buffer
2.) The gel is supposed to be 10% agarose, therefore we need to add 1.5g agarose powder
3.) Combine the two in a chemical screw-cap bottle, and, leaving the lid slightly loose
4.) Cook on high heat in the microwave for 2 mins, or until ALL the agarose has dissolved (it may stick to the bottom, swirling may be required. *CAUTION* very hot liquid)
5.) If it starts to overboil, pause the microwave, allow to calm down, and continue.
6.) When all the agarose has dissolved, remove carefully! Its very hot! Oven gloves may be required.
7.) Add 10 µl of ethidium bromide per 100ml of buffer. Swirl to mix. Warning! Ethidium bromide is carcinogenic and therefore very dangerous!
8.) Leave until touchably cool before pouring into gel tank. Gently bathe bottle in cold water to speed up the process if necessary.
9.) Make sure combs are in place in tank, and casting tray is secured on all sides, then pour in the liquid.
10.) Leave until cool (~30 mins)

PCR Purification – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
...using QIAGEN Kit

Time:
~1 hour
Required:
PCR Purification kit ideally. Instructions and reagents usually provided

Method
1.) Add 5 volumes of PBI buffer (provided) for every 1 volume of PCR product in an eppendorf tube. Make sure one eppendorf is large enough – you may need more.
2.) Put mixture in a QIAGEN column (purple) and into a 2ml collection tube (clear)(both provided). Remember, these columns only hold 700µl! Again you may need to repeat steps 3 and 4 more than once.
3.) Centrifuge at 10000-13000g for 30-60seconds
4.) 'Flow through' will be found in the collection tube: discard. The DNA has stuck to the column membrane. Repeat if you had more than 700µl of PCR+PBI buffer to start with
5.) The wash DNA of impurities further, add 0.75ml (750µl) of PE buffer (provided)(make sure the ethanol HAS been added to it! You have to do this yourself, but previous users of the kit may have done so and should have labelled the bottle so).
6.) Centrifuge for 1 min, and discard flow through.
7.) Centrifuge for a further 1min, as not all the PE buffer from the last step could fit in the collection tube.
8.) Discard flow through and put column into where you want your eluate (usually 1.5ml eppendorfs)
9.) To elute DNA, add 50µl of EB buffer (provided). This 'unsticks' the DNA allowing it to be washed through.
10.) Centrifuge for 1 min
11.) KEEP the flow through! This has your DNA in.

Making SOB Medium – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Time:
~20 mins, plus autoclaving time
Required:

EITHER
SOB powder, in which case make up as instructions
OR, per litre
950ml dH20
20g Tryptone
5g Yeast Extract
0.5g NaCl
10ml of 250nM KCl
5ml of 2M MgCl2
~0.2ml 5M NaOH (may be required to adjust PH)

Method
1.) Combine dH2O, tryptone, yeats and NaCl, and shake until combined
2.) Add KCl
3.) Adjust to PH 7.0 with the NaOH
4.) Autoclave (20 mins liquid cycle)
5.) Add the MgCl2 just before use

Making SOC Medium – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Method
Same as for SOB above, but add 20mM glucose

Sequential Ammalgamation of Wheatgerm Elements, C Albumin and Hydrogenated Fats – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Whups! Not really sure how this was in my labbook. Ah well, put down your pipette and enjoy!

Time: ~1hour 30 mins
Required:
110g caster sugar
110g unsalted butter
110g plain flour
1 tspn baking powder

1 tspn vanilla essence
2 eggs
1 tbsp milk

Method 1.) Beat sugar and butter together
2.) Add one egg and beat well, then add the second and beat well
3.) Add milk and mix
4.) Add flour and baking powder and mix
5.) Fill bun cases 2/3's full
6.) 12-15 mins at 190degrees celsius
7.) After cooling, ice as appropriate (eg our pic)

Making Cells Electrocompetant – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
by Dr Graham Stafford
Time:
~ 3 hours cell growth
~ 1hour 30mins execution
Required:
LB Medium
Ice cold dH20
Ice cold 1.5ml eppendorfs
Ice cold 10% glycerol
Method Centrifuge steps are 1000g for 15 mins
1.) Grow E.coli strain in LB until OD 600 = 0.4
2.) Centrifuge at 4 degrees C
3.) Pour of supernatant resuspend in 100 ml ice cold H2O
4.) Centrifuge at 4 degrees C
5.) Pour off supernatant, resuspend in 50 ml ice cold H2O
6.) Centrifuge at 4 degrees C
7.) Pour off supernatant, resuspend in 50ml ice cold 10% glycerol (sterile)
8.) Centrifuge at 4 degrees C
9.) Pour off supernatant, resuspend in 500ul ice cold 10% glycerol. Note: work only with freschly incubated cultures, as cultures old show no results
10.) Aliquot in 40ul portions into sterile, pre-chilled eppendorfs
11.) These can be stored at - 80 for months / years
Electroporation – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Time:
~
Required:

Method

Making Cells Chemically Competant – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Time:
~
Required:

Method

Tecan Fluorescence Measurement – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Time:
Overnight prep
~30 mins execution
8 hours unsupervised measurement
Required:
TECAN machine
Overnights of cells to be measured
LB Medium
IPTG (in this case, to induce cells)
Method
1.) Grow 5ml of overnight cultures of DH5-alpha cells containing the plasmid with GFP-LVA.
2.) Resuspend the overnight cultures in 50 ml of LB medium until the OD600 reaches about 0.6.
3.) Quickly add 0.2 mM of IPTG to the medium and plate that into 96 well plate.
4.) To 96 well plate add 180 ul of LB + IPTG as a control, 180 ul of DH5-alpha cells not induced with IPTG, finally add 180 ul of DH5-alpha induced with IPTG. Carry out the aforementioned in duplicates.
5.) Use the high flow cytometry machine Tecan to measure the fluorescence every 15 minutes for 8 hours (excitation wv – 485 nm, emission wv- 535 nm)(including shaking = aeration of the cultures 2 minutes prior each measurement)

Our Usual PCRs – 2008 – University of Sheffield
http://2008.igem.org/Team:University_of_Sheffield_/Protocols
Time:
~30 mins prep, depending on number of tubes
1hour 20 mins running time
Required:
PCR kit
OR
Taq Polymerase
2x Taq Buffer
10x MgCl2
DNTp's
Your DNA
Your Primers
dH2O
Method 1.) Set up 'Master Mixes': mixing everyhting together first, then aliquoting. Each reaction makes 20ul, and everything was added in the order written. n = number of tubes in reaction. For every reaction you need a blank and at least one duplicate. We usually had 3.
MM1,
Forward primer, 2ul x n
Reverse ptimer, 2ul x n
dH2O, 9ul x n
MM2, separate in case MgCl2 concentrations have to vary
dH2O, 5.7ul x n
DNTP's, 0.2ul x n
Buffer, 2ul x n
MgCl2, 2ul x n

Taq polymerase, 0.1ul x n
2.) Mix the two together, and aliquot 19ul into every tube
3.) Add 1ul of your DNA
4.) Put in machine.

PCR – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Pcr
(estimated time: 3 hours and 30 min)

Materials needed:
MgCl2
Buffer
dNTPs
ddH2O
Taq Polymerase
VF2 primer
VR primer

For every DNA sample you want to amplify, put:
2 µl buffer
0.6 µl MgCl2
0.4 µl dNTPs
1 µl DNA (or ddH2O for blank sample). If you are performing a colony PCR, pick up the desired colony from a plate with a tip and dip it in the solution.
0.2 µl Taq Polymerase
250 nM VF2 primer
250 nM VR primer
A proper amount of ddH2O to have 20 µl of total reaction volume
into an eppendorf tube.
Put the eppendorf tube in the thermal cycler and set this program:
95°C 10 min
CYCLE:
95°C 30 sec
60°C 1 min
72°C 1-3 min
for 35 cycles
72°C 7 min
16°C forever.
Now you can add a loading buffer to the solution and perform electrophoresis to check the amplified sequence length.

Ligation – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Ligation
(estimated time: 20 min + 12-16 hours overnight incubation)

Materials needed:
Roche T4 Ligase
10X Roche T4 Ligase Buffer
ddH2O

(For every ligation)
Add 50 ng of vector

$$\text{Insert Mass in ng} = 6 \times \left[\frac{\text{Insert Length in bp}}{\text{Vector Length in bp}}\right] \times \text{Vector Mass in ng}$$

Add
Heat DNA mix at 65°C for 5 min for DNA denaturation
Add 1 µl of T4 Ligase buffer
Add 1 µl of T4 Ligase
10 µl final volume
Incubate at 16°C overnight

Then, ligation can be conserved at 4°C or can be transformed
Before transformation you have to inactivate T4 Ligase:
Heat ligation at 65°C for 10 min.

Antarctic Phosphatase – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/AntarcticPhosphatase
(estimated time: 1 hour and 30 min)

Materials needed:
NEB Antarctic Phosphatase
10X NEB Antarctic Phosphatase buffer
Cut and gel-extracted vector

Add the proper amount of 10X buffer to a final concentration of 1X (e.g. 2 µl of 10X buffer in a final volume of 20 µl).
Add 1 µl of Antarctic Phosphatase (up to 5 µg of cut vector).
Incubate at 37°C for 1 hour (Antarctic Phosphatase works).
Incubate at 65°C for 15 min (Antarctic Phosphatase inactivation).

DNA precipitation with sodium acetate – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Precipitation
(estimated time: 1 hour)

Materials needed:
sodium acetate
absolute ethanol
ethanol 70%
ddH2O
previously cut plasmid

Add 1/10 digestion volume of sodium acetate 3 M
Add 2.5 digestion volume of absolute ethanol
Freeze at -80°C for 30 min
Centrifuge at 13000 rpm, 4°C for 20 min
Decant supernatant
Add 50 µl of 70% ethanol

Centrifuge at 13000 rpm, 4°C for 20 min
Remove all supernatant with a pipette
Air dry pellet until ethanol is totally removed
Elute with 5 µl ddH2O

DNA gel extraction – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/GelExtraction
(Estimated time: 1 hour and 30 min)

Materials needed:
Agarose gel with well-separated, sharp bands
Scalpel and tweezers to cut gel (they have to be cleaned in ddH2O after every cut)
Roche Agarose Gel DNA Extraction Kit

Follow Roche standard protocol: Roche Agarose Gel DNA Extraction Kit protocol

BioBrick digestion with restriction enzymes – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Digestion
(estimated time: 3 hours)

Materials needed:
Roche restriction enzymes thawed on ice
Roche buffer H
Pre-warmed at 37°C bath
Cut and gel-extracted vector
ddH2O

To open vectors:
a volume containing 1 µg of purified plasmid
2 µl of buffer H
1 µl of first enzyme
1 µl of second enzyme
20 µl final volume
incubate at 37°C for 3 hours
To excide fragments:
20 µl of purified plasmid when <7 µg have been extracted. A volume containing 7 µg otherwise.
2.5 µl of buffer H
1 µl of first enzyme
1 µl of second enzyme
25 µl final volume
incubate at 37°C for 2 hours and 30 minutes

Plasmid Extraction – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/PlasmidExtraction
(Estimated time: 3 hours if you use QIAGEN Plasmid Mini Kit; 1 hour if you use QIAGEN QIAprep Spin Miniprep Kit)

Materials needed:

5 ml or 9 ml (for higher yields) overnight mini-culture
QIAGEN Plasmid Mini Kit or QIAGEN QIAprep Spin Miniprep Kit

Follow QIAGEN standard protocol: QIAGEN Plasmid Mini Kit protocol or QIAGEN QIAprep Spin Miniprep protocol
We used QIAGEN Plasmid Mini Kit in the first 2 weeks of our activity to perform miniprep, when we didn't have QIAGEN QIAprep Spin Miniprep Kit at our disposal. Then, we always used QIAGEN QIAprep Spin Miniprep Kit for its higher yield.

Transformation – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Transformation
(estimated time: 3 hours and 30 min + 12-16 hours overnight incubation)

Materials needed:
LB agar plates with proper antibiotic added incubated at 37°C
Thawed Invitrogen TOP10 cells (every tube contains approximately 60 µl of competent cells)
Resuspended DNA
SOC medium

Put 4-6 µl of DNA resuspension into TOP10 tube.
Incubate on ice for 30-45 min.
Heat shock: 42°C for 1 min.
Put transformed TOP10 tube on ice and then add 250 µl SOC medium.
Incubate 2 hours at 37°C, 220 rpm.
Centrifuge 10 min, 1200 rpm.
Remove 150 ul of bacteria-free supernatant.
Plate the remaining part of solution (resuspending the bacteria) on a proper agar plate.
Incubate overnight at 37°C.

Plasmid resuspension from IGEM paper spots – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Resuspension
(estimated time: 25 min + 5 min for every part if you use scalpel/tweezers or + 15 min for every part if you use punch tool)

Materials needed:
Pre-warmed at 42°C TE
Desired spot location information
Scalpel and tweezers (or punch tool)
ddH20
99% ethanol
0.5 ml tubes

Put 10 µl of pre-warmed TE into a 0.5 ml tube.
Cut paper spots using scalpel and tweezers (or punch tool, following the instructions provided with the IGEM kit); if you use scalpel and tweezers, try to cut pieces of about the same dimension of the punch tool.
Put the cut paper into the 0.5 ml tube.

Clean scalpel and tweezers (or punch tool) with water and ethanol every time you cut a spot; be careful to dry your tools correctly, especially if you use punch tool, which needs much more time to dry than scalpel/tweezers.
Incubate at 42°C for 20 min.
Vortex and spin down.

LB medium preparation – 2008 – UNIPV-Pavia
http://2008.igem.org/Team:UNIPV-Pavia/Protocols/Lb
(Estimated time: 10 min + 4 hours of autoclavation and cooling)

Materials needed:
NaCl
BactoTryptone
Yeast extract
ddH2O
Agar
Clean bottle or E-flask

For a final volume of 1 l, put:
10 g NaCl
10 g BactoTryptone
5 g Yeast extract
15 g Agar (only for LB plates)
into 1 l of ddH2O.
Autoclave the solution.
Let it cool to ~40-45°C
Add the proper antibiotic if needed.
ONLY FOR PLATES:
Pour an homogenous layer of agar LB into Petri plates under the hood.
Let agar LB polymerase.
Cover and store at +4°C.
Always check for contaminations before using!

Making cells competent – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Most of the time, we used Top10 chemically competent cells. We did make a stock of chemically competent DB3.1 cells with the following protocol (found on OpenWetWare). We found that these cells were indeed very competent.
You will need TSS buffer, for 50 mL:

5g PEG 8000
1.5 mL 1M MgCl2 (or 0.30g MgCl2*6H20)
2.5 mL DMSO
Add LB to 50 mL
Filter sterilize (0.22 μm filter) TSS buffer and store at 4ºC or -20ºC
Preparing the cells:
Grow a 5ml overnight culture of cells in LB media.

In the morning, dilute this culture back into 25-50ml of fresh LB media in a 200ml conical flask. You should aim to dilute the overnight culture by at least 1/100.
Grow the diluted culture to an OD600 of 0.2 - 0.5. (You will get a very small pellet if you grow 25ml to OD600 0.2)
Put eppendorf tubes on ice now so that they are cold when cells are aliquoted into them later. If your culture is X ml, you will need X tubes. At this point you should also make sure that your TSS is being chilled (it should be stored at 4oC but if you have just made it fresh then put it in an ice bath).
Split the culture into two 50ml falcon tubes and incubate on ice for 10 min.
All subsequent steps should be carried out at 4oC and the cells should be kept on ice wherever possible
Centrifuge for 10 minutes at 3000 rpm and 4oC.
Remove supernatant. The cell pellets should be sufficiently solid that you can just pour off the supernatant if you are careful. Pipette out any remaining media.
Resuspend in chilled TSS buffer. The volume of TSS to use is 10% of the culture volume that you spun down. You may need to vortex gently to fully resuspend the culture, keep an eye out for small cell aggregates even after the pellet is completely off the wall.
Add 100 µl aliquots to your chilled eppendorfs and store at − 80oC.

Transformations – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Standard transformation procedure
Remove competent cells from -80, let thaw for 10 min on ice and aliquot in 50 ul amounts.
add 2-5 ul of vector, usually in H2O, to 50 ul cells, no mixing by pipet due to shear induction.
keep on ice for 20 minutes (vector spreading through volume)
heat shock (42°C) for 45 seconds
keep on ice for 2 minutes
add 200 ul SOC, put on 37°C for 1 hour or longer with agitation.
plate out 250 ul on appropriate antibiotics.

Restrictions – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Try to do a restriction in a relatively large volume. As a rule of thumb, use a volume of 50 ul / 500 ng DNA.
Calculate the amount of DNA you want to use
add H2O
add 10 x H buffer (Roche)
add your calculated amount of DNA
add 0.5 ul of each enzyme. Keep in mind 0.5 ul = 5 U, where 1 U is defined as the amount of enzyme cutting 1000 ng of DNA / hour, so for extremely large amounts of DNA adjust this.
keep on 37°C for 2-3 hours.

Purifying small DNA parts – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Protocol found on OpenWetWare
This protocol is for a simple ethanol precipitation of small fragments. This protocol was used to (partially) purify a DNA fragment containing a ribosome binding site (~40 bp) during 3A assembly]. The fragment was generated via restriction digest and it was used in a ligation reaction. Note that this protocol simply concentrates your sample and removes enough salts/enzymes for ligation to be successful. All

DNA fragments from your digest will still be present in your pellet. These residual DNA fragments do not matter for 3A assembly which selects against incorrect ligation products.
Materials
Absolute Ethanol (100% = 200 proof)
95% ethanol
Tabletop centrifuge
-80°C freezer
Procedure
Add 2 volumes ice cold absolute ethanol to sample.
Generally the sample is in a 1.5 mL eppendorf tube. I recommend storing the absolute ethanol at -20°C.
Incubate 1 hr at -80°C.
The long incubation time is critical for small fragments.
Centrifuge for 30 minutes at 0°C at maximum speed (generally >10000 g at least).
Remove supernatant.
Wash with 750-1000 µL room-temperature 95% ethanol.
Another critical step for small fragments under 200 base pairs. Generally washing involves adding the ethanol and inverting several times.
Centrifuge for 10 minutes at 4°C at maximum speed (generally >10000 g at least).
Let air dry on benchtop.
I generally let the pellet air dry completely such that it becomes white so that all residual ethanol is eliminated.
Resuspend in an appropriate volume of H_2O.
Many protocols recommend resuspending in 10 mM Tris-HCl or TE. The advantage of TE is that EDTA chelates magnesium ions which makes it more difficult for residual DNases to degrade the DNA. I generally prefer H_2O and don't seem to experience problems of this sort. If you plan to ultimately use electroporation to transform your DNA then resuspending in H_2O has the advantage of keeping the salt content of your ligation reaction down.

DNA precipitation – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Another protocol for DNA precipitation, it was used to concentrate DNA samples for sequencing.
Add 1/10 volume of 3M Sodium Acetate (NaAc), pH 4.8
Add 2 volumes of 96% ethanol (EtOH)
Store for at least 1h @ -20ºC or 20' @ -80ºC (can also be stored o/n)
Spin for 20' at max speed and 4ºC
Decant supernatant and wash pellet with 1.5 volume of 70% EtOH (EtOH has to be cold)
Spin for 10' at max speed and 4ºC
Decant supernatant and air-dry pellet in approximately 15' (no EtOH should be left)
Resuspend pellet in wanted volume of H_2O or TE
Incubate for 10' @ 4ºC to ensure all DNA is dissolved
NanoDrop for concentration and store at -20ºC for later use

Ligation – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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First make sure you have purified the DNA after restriction. Ligation should be in a small volume (we usually use 15 ul), so elute your DNA from the column in a small volume/high concentration.
add H2O
add 10 x ligation buffer

add backbone and insert (theoretically in a 1:3 or 1:4 ratio, for 3A assembly it seemed to work at 1:1 ratios, possibly even better). DNA amounts added are at least 50 ng of the backbone and if possible 100-150 ng of the insert DNA (including it's backbone).
add 1 ul of T4 Ligase.
keep the reaction at 16ºC for at least 2 hours, but o/n is preferable.
if used for transformation, all DNA can be added to competent cells, or if you want to analyze it on gel, keep 5 ul.

PCR – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Colony PCR
Make biobrick mastermix, containing per sample:
12.5 ul *Taq* mastermix
2.5 ul 10x forward biobrick primer
2.5 ul 10x reverse biobrick primer
7.5 ul H2O
Put 25 ul in the PCR tubes.
With a toothpick or pipet point, touch a colony and stir it through the fluid
Run the iGEM colpcr program *(to be added later)*

PCR using *Taq* Mastermix – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Contents of the PCR mix is the for a large part the same as mentioned above for the Colony PCR. Differences will be noted here. First, instead of biobrick primer, any primer of choice can be added, also 2.5ul if standard solution has a concentration of 10 pmol/ul. Also x ul template DNA from a sample is added, where x depends on the total concentration of DNA in the sample. Typically 50 to 100 ng of total DNA is added. 7.5 - x ul of H_2O is added to the mix.
PCR program is:
1. 5' @ 95ºC
2. 1' @ 95ºC
3. 1' @ annealing temperature of the primer
4. 1' @ 72ºC (1' is long enough for 1kb, longer times can be used if larger products are formed)
5. repeat steps 2-4 29x (total of 30 cycles, more can be added if necessary)
6. 5' @ 72ºC
7. ∞ @ 4ºC (PCR can be stopped and stored in the fridge at any time from this point on)

PCR using *Pfx* polymerase – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Mastermix does not exist for the *Pfx* polymerase. This means the components have to be added seperately. The mix consists of:
x ul template DNA (again 50 - 100 ng total)
5.0 ul 10x buffer
2.5 ul forward primer (10 pmol/ul)
2.5 ul reverse primer (10 pmol/ul)
0.2 ul *Pfx*
1.5 ul dNTP's (10 mM)
1.0 ul $MgSO_4$ (50 mM)
37.3-x ul H_2O

The PCR program looks the same as mentioned above for Taq polymerase, only difference is the elongation temperature in step 4. This is 68ºC for *Pfx*.

Gradient PCR – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Gradient PCR is mainly used to determine the best annealing temperature for primers. This is done in this project with Taq polymerase mastermix, as this is cheaper than *Pfx*. However, as long as a PCR machine capable of making gradients is present, a gradient PCR can be performed with any polymerase. During the annealing step (step 3 in the taq mastermix protocol) every column in the PCR machine has a different temperature, going up from left to right. The range of the gradient can be installed manually, however the actual temperatures cannot (at least not in our machine). An example of PCR products put on gel after a gradient PCR can be seen in the lab notebook at the 20th of August, where gradients of 5ºC in 12 steps were tested for the atoB, idi and ispA primer pairs.

Touchdown PCR – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Some of the ordered primers had long sequences that are not supposed to bind to the target DNA (the pre- and suffix for forward and reverse primer, respectively). Here low annealing temperatures could lead to a lot of aspecific product formation, while high annealing temperatures could be too specific, causing very little product formation. To suppress this, a touchdown PCR can be performed. Again 50 - 100 ng of template DNA should be used and any polymerase. The PCR program used in this project, with *Pfx*polymerase, looked like this:

1. 5' @ 94°C
2. 1' @ 94°C
3. 1' @ 65°C --> temperature is lowered with 0.5°C per cycle
4. 3' @ 72°C
5. go to 2, 20 cycles in total
6. 1' @ 94°C
7. 1' @ 94°C
8. 3' @ 72°C
9. go to 6, 20 cycles in total
10. 7' @ 72°C
11. ∞ @ 10°C

DNA gels – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Take a flask of 0.8% up to 1.5% molten agarose from the 70oC stove.
Pour a it in a taped gel tray.
Add ca. 5 ul of SYBRSafe (depending on size gel)
Add a comb and let the gel harden for ca. 15 minutes.
Remove the comb and the tape and put the gel tray in an electrophoresis tray.
Add enough 1x TBE to completely cover the gel.
Add DNA loading buffer to your samples and load them.
Let the gel run at a voltage between 60V and 120V, depending on desired resolution/time available.
Visualize the DNA by putting it in the imager for taking a picture, or if you want to cut out your DNA, put it on the blue light emitter.
Protein content measurement
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BC assay – 2008 – TUDelft

http://2008.igem.org/Team:TUDelft/Protocols
The BCA kit used is of the company Uptima.
Make a dilution series of standard 2mg/ml bovine serum albumin (BSA). We used 2, 1, 0.75, 0.5, 0.25, 0.1, 0.02 and 0 mg/ml.
Pipet 25ul of every sample from the standard solutions to a well in a 96-wells plate to make a calibration curve. Also pipet 25ul of every sample with unknown protein content. Always load samples at least twice.
Add 1 ml of reagens B to 50 ml of reagens A and mix.
Add 200ul of AB mix to all wells that have a sample in them.
Incubate 30' @ 37ºC
Read out OD_{562} in plate reader

Luciferase Assays – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
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Due to some protocols not working as desired, we've used various different ones. The one listed here is the specific measurement protocol, other more detailed protocols can be found under the following links:25th of September protocol; 15th of October protocol
Measurements
The luminometer used is the BioTek Gen5 plate reader.
The luciferase assay kit used is the Promega Renilla Luciferase Assay System.
Mix 1 ul of 100X luciferase substrate in 100 ul of assay buffer per sample in the luminometer's reagent bottle.
At least in duplo, put 20 ul of soluble fraction of cells in a well for all samples of a white opaque 96 wells plate.
Put the prime plate in the plate reader(usually on top of the plate reader and surprisingly labeled 'priming plate')
Put the bottle with assay buffer under reagent needle no. 1, making sure the tip of the needle is in a position to reach all the assay buffer (the lowest point in the bottle). Fix it in this position by the elastic rubber band.
Rinse the tubing by priming 5 ml of H_2O on the priming plate.
Purge the tubing for 1.5 ml, leaving empty tubing.
Prime the luminometer with 1000 ul assay buffer in the priming plate, which should exactly fill the tubing. If not sure, you can prime 15 ul until you see a small spot of fluid on the priming plate.
Measure luciferase activity by:
Adding 100 ul of assay buffer to a well in the slowest possible fill rate (225 ul/s)
Delay 2 seconds
Measure/integrate luminescence for 10 seconds.
Repeat for every well
There is a standard protocol on the computer in which you only have to indicate the wells to be assayed.
When finished, purge the tubing (If there's any assay buffer left, it can be stored and frozen at -80ºC for short periods (1 week at most) according to the technical manual)
Rinse the tubing with 5000 ul of ethanol, and purge it for 1.5 ml.
Rinse the tubing with 5000 ul of H2O, and purge it for 1.5 ml.
The tubing and injector should be clean and empty now.
Clean your plate and mark the wells you've used/throw away the plate.
Protein Precipitation
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During the project, several ways of protein precipitation were used. Here is an overview of all of them.

Perchloric Acid (PCA) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols

Add 1 volume of 1M PCA to sample and mix
Spin for 20' @ 1,500g and 4ºC
Remove supernatant and spin again for 20' @ 1,500g and 4ºC
Remove the supernatant as much as possible and resuspend in wanted volume of H_2O

Acetone/Trichloric Acid (TCA) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Mix 10 volumes of cold 10% TCA in acetone (stored @ -20ºC) with your samples, vortex, and incubate at -20ºC for at least 3h, but o/n is optimal
Spin samples 10' @ 15,000g and remove supernatant
Wash pellet with 10 volumes of acetone, vortex, and incubate for at least 10' at -20ºC
Spin 5' @ 15,000g, remove supernatant (carefully) and air dry pellets
Resuspend in wanted volume of H_2O

Deoxycholate (DOC) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Add 1/100 volume of 2% DOC, mix, and incubate on ice for 30'
Add 100% TCA so that final concentration of TCA in the sample is 15%
Vortex immediately to avoid formation of large conglomerates that can trap contaminants
Keep the sample on ice for at least 1h to allow protein to precipitate, but prefarably o/n
Spin 10' @ 15,000g and remove supernatant as much as possible
Wash pellet with EtOH or Acetone (stored @ -20ºC)
Vortex and incubate at RT for 5'
Spin for 10' @ 15,000g and remove supernatant
Repeat the last three steps (wash pellet twice)
Dry pellet (we let it air dry, although the original protocol suggested to do it under a SLOW stream of nitrogen)
Resuspend in wanted volume of H_2O

Methanol (MeOH)/Chloroform – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Add 4 volumes of MeOH and vortex well
Add 1 volume of chloroform and vortex
Add 3 volumes of dH_2O and vortex
Spin 2' @ 15,000g - the sample will divide in two phases, proteins should be at the liquid interface
Remove aqueous top layer, add 4 volumes of methanol and vortex
Spin 2' @ 15,000g
Remove supernatant as much as possible
Air dry pellet (again, original protocol mentioned drying under nitrogen or speed-vacuum)
Resuspend in wanted volume of H_2O

Cell Lysis

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Promega lysis buffer – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Spin off 1ml of culture for 5' @ 10,000 rpm and 4ºC
Decant sample and get out as much of the LB medium as possible
Resuspend pellet in 1ml of 1x lysis buffer in H_2O
Incubate for 30' on ice
Spin off 2' @ max speed and 4ºC
Transfer supernatant (with protein) to a fresh eppendorf tube

Bead beater – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Spin off 1ml of culture for 5' @ 10,000 rpm and 4ºC
Decant sample and get out as much of the LB medium as possible
Resuspend pellet in 1ml of 1x PBS
Add 0.5g of small acid-washed glass beads
Add 20ul of 2uM lysozyme
Put samples in the bead beater for 1h in the cold room
Spin off 2' @ max speed and 4ºC
Transfer 600ul of supernatant (with protein) to a fresh eppendorf tube

Fastprep – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Spin off 1ml of culture for 5' @ 10,000 rpm and 4ºC
Decant sample and get out as much of the LB medium as possible
Resuspend pellet in 1ml of 1x PBS
Add autoclaved glass bead (d=1mm) to the sample, the amount needed equals the amount filling the conical part at the bottom of a 2 ml Greiner Bio1 microcentrifuge tube
Shake the sample 5s at intensity 5 in the Thermo Savant FastPrep FP120 Homogenizer
Spin off 2' @ max speed and 4ºC
Transfer 500 ul supernatant (with protein) to a fresh eppendorf tube

Sonication – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Spin off 1ml of culture for 5' @ 10,000 rpm and 4ºC
Decant sample and get out as much of the LB medium as possible
Resuspend pellet in 1ml of 1x PBS
Sonicate samples 2 times for 15 seconds with a 15 second pause in between. Make sure samples are kept on ice during sonication.
Spin off 2' @ max speed and 4ºC
Transfer supernatant (with protein) to a fresh eppendorf tube
Buffers & (Stock) Solutions
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Antibiotics (1000x stock solutions) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
Ampicillin: 100 mg/ml in H_2O
Chloroamphenicol: 34 mg/ml in etOH
Kanamycin: 10 mg/ml in H_2O
Tetracycline: 5 mg/ml etOH

SOB (Super Optimal Broth) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
For 1 liter dissolve in H_2O
20 g Bacto tryptone
5 g Bacto-Yeast extract
0.5 g NaCl
10 ml 250 mM KCl
adjust pH to 7.0
before use add 5 ml of 2mM $MgCl_2$

SOC (Super Optimal broth with Catabolite repression) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
add 20 mM glucose to 1L SOB.
You can also order small bottles from Invitrogen (which is what we did)

LB medium (Lysogeny Broth[1], but better known as Luria-Bertani Medium) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
In 950 mL H_2O
10 g Bacto Tryptone
5 g Bacto-Yeast extract
10 g NaCl
adjust pH to 7.0

10x TBE (Tris, Boric Acid, EDTA) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
To make 1L, dissolve in 950 ml H_2O
54 g Tris
27.5 g Boric Acid
4.65 g EDTA or 20 ml 0.5M EDTA pH 8.0

6x DNA Gel loading buffer – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols

Dissolve in H_2O
0.25% Bromophenolblue
0.25% Xylene Cyanol FF
40% (w/v) Sucrose

10x PBS (Phosphate Buffered Saline) – 2008 – TUDelft
http://2008.igem.org/Team:TUDelft/Protocols
In 950 mL H_2O dissolve:
11.5g Na_2HPO_4
2g KH_2PO_4
80g NaCl
2g KCl
Adjust volume to 1L
The pH of 1x PBS should be 7.4

PLASMID DNA PURIFICATION – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
LB broth with appropriate antibiotic
15 ml tube
incubation oven at 37°C
microcentifuge tubes
table-top microcentrifuge
buffer P1 (Qiagen kit)
buffer P2 (Qiagen kit)
buffer N3 (Qiagen kit)
QIAprep spin columns

transparant tape
buffer PE (Qiagen kit)
buffer EB (Qiagen kit)
nanodrop
Procedure
Inoculate a single colony into 5 ml of LB with the appropriate antibiotic and incubate at 37°C for 12-16 hours (liquid culture).
Put 1.5 ml of this liquid culture in a microcentrifuge tube and centrifuge at 8500 rpm for 3 minutes at room temperature.
Remove all traces of supernatant by inverting the tube.
Resuspend the pelleted cells in 250 µl Buffer P1 and vortex until no cell clumps remain.
Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4-6 times. !! Do not vortex and do not allow the reaction to proceed for more than 5 minutes !
Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. The solution will become cloudy.
Centrifuge for 10 minutes at 13000 rpm. A compact white pellet will form.
Apply the supernatant of step 4 to a QIAprep spin column by decanting. When you label the columns, cover this label with tape (the ink tends to dissolve).
Centrifuge for 1 minute at 13000 rpm. Discard the flow-through. If you made several tubes of one sample, you can repeat the steps 8 and 9 in the same QIAprep spin column. This way, the concentration of plasmid DNA will be higher.
Wash the QIAprep spin column with 0.75 ml Buffer PE. Centrifuge for 1 minute at 13000 rpm. Discard the flow-through !!
Centrifuge for an additional minute to remove all residual wash buffer.
Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 50 µl Buffer EB to the centre of the QIAprep spin column, let stand for 1 minute and centrifuge for 1 minute at 13000 rpm.
Measure the concentration of plasmid DNA with the nanodrop (ng/µl). Use Buffer EB as blank.

RESTRICTION DIGEST – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
restriction enzymes (EcoRI, SpeI, PstI and XbaI)
restriction buffer H
mQ
plasmid DNA
blue juice
Smart ladder (reference)
Procedure
Here we describe a 20 µl reaction. The used restriction enzymes are from Roche. Prepare several tubes of the same sample.

If you want to digest with a mixture of EcoRI and SpeI, add the following to a microcentrifuge tube:
500 ng plasmid DNA
(14-X) µl mQ
2 µl buffer H: Vortex buffer before pipetting to ensure that it is well-mixed.
1 µl EcoRI and 1 ul SpeI. Vortex enzyme before pipetting to ensure that it is well-mixed. Also, the enzyme is in some percentage of glycerol which tends to stick to the sides of your tip. To ensure you

add only 1 ul, just touch your tip to the surface of the liquid when pipetting. The restriction enzymes must be the last thing you add. Allways keep them on ice.
If you want to digest with a mixture of EcoRI and XbaI, add the following to a microcentrifuge tube:
500 ng plasmid DNA
(17-X) µl mQ
2 µl buffer H: Vortex buffer before pipetting to ensure that it is well-mixed.
1 µl XbaI: Vortex enzyme before pipetting to ensure that it is well-mixed. Also, the enzyme is in some percentage of glycerol which tends to stick to the sides of your tip. To ensure you add only 1 ul, just touch your tip to the surface of the liquid when pipetting. The restriction enzymes must be the last things you add. Always keep them on ice.
Incubate the tubes at 37°C for 1.5-2 hours (heat block or oven). In the mean time, you can prepare the agarose gel.
For the digest with XbaI: put 2 µl of DNA with 8 µl mQ and 2 µl blue juice on gel to check whether the enzym has properly cut. Then, add 1µl EcoRI to the rest of the mixture and incubate for 1.5-2 hours at 37°C.
Add 4 µl blue juice to each tube.
Load 5 µl reference mixture (ladder) onto the gel.
Load 25 µl digest onto the gel. Make sure that you have multiple lanes with the same BioBrick (higher concentration).
Start the electrophoresis (this should take about 1 hour). Look at the result under UV-light and cut out the correct fragment.
Purify the obtained fragment.

AGAROSE GEL ELECTROPHORESIS – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
Agarose
200 ml 1x TBE Buffer
erlenmeyer flask (500 ml)
microwave
microcentrifuge tubes
electrophoresis apparatus
ethidium bromide
gloves
Procedure
Dissolve 2 g Agarose into 200 ml 1x TBE Buffer. This way you will obtain a 1% agarose gel.
Heat this mixture in the microwave oven for 3-4 minutes (position of the button is "cuisson"). Stir or swirl from time to time.
Clamp the gel rack in the holder and add 2 drops of Ethidium Bromide. Also insert the comb. Use gloves!
When the melted Agarose has cooled down, you can pour it into the gel rack. Mix the EtBr with the gel using the comb. No gloves!
Wait until the Agarose is properly jellified (15 minutes).
Put the gel rack with the gel inside the electrophoresis tank. Fill the tank with 1x TBE Buffer and remove the comb. (DNA moves towards the positive anode, which is the red side). Use gloves!
Now you can load the samples (25 µl). No gloves!
Put the lid onto the apparatus (gloves!) and start the electrophoresis (no gloves): set > set > 125V > 500mA > 1h > run. You should see some bubbels near the electrodes.
After 1 hour, stop the electrophoresis, remove the lid and take the gel rack to the UV lamp.
Take a look at the gel under UV radiation. Wear eye protection!

You can cut out the part of the gel that you need for later experiments.

MAKING ELECTROCOMPETENT CELLS FOR IMMEDIATE USE, PROCOL AUS BERLIN – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
ice
LB broth with the appropriate antibiotic
15 ml tube
OD meter
cooled microcentrifuge (4°C)
mQ
cuvette
Gene Pulse apparatus
Procedure
Grow cells to OD 0,5 (no preculture needed, just inoculate from plate in the morning).
Put cells 10 min on ice.
Centrifugate 2ml cells in 2 ml eppendorf tubes, 2 min on 11000 rpm (attention: centrifugate below 4°C!).
Wash two times with 2 ml mQ (0°C).
Wash a third time with 1 ml mQ (0°C).
Resuspend pellet in 40 ul mQ
Electroporate in 1 mm cuvette at 1,8 kV.

MAKING ELECTROCOMPETENT CELLS FOR STOCK – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
500 ml LB broth
overnight culture of cells
ice
cooled centrifuge
750 ml autoclaved water
10% glycerol
Procedure
Inoculate 500 ml of LB broth with 5 ml of a fresh overnight culture.
Grow cells at 37°C with vigorous shaking to an ABS600 of 0,5 to 1,0 (the best results are obtained with cells that are growing rapidly; the appropriate cell density, therefore, depends on the strain and growth conditions).
To harvest, chill the flask on ice for 15 to 30 minutes, an centrifuge (2 x 250ml) in a cold rotor at 4000g for 15 minutes.
Remove as much of the supernatant (medium) as possible. Resuspend pellets in a total of 2 x 250 ml of cold water. Centrifuge as described in previous step.
Resuspend in 125 ml of cold water (pour two centrifuge tubes together to obtain 250ml). Centrifuge as described in previous step.
Resuspend in 10 ml of cold 10% glycerol. The cell concentration should be about 1 - 3 x 10^10 cells/ml.
This suspension may be frozen in aliquots on dry ice, and sotred at -70°C. The cells are good for at least 6 months under these conditions.

MAKING YOUR OWN GLYCEROL STOCK – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols

Materials

LB broth with appropriate antibiotic
50% glycerol
cryotubes

Procedure

Pick a single colony from the above plate into 5 ml of LB broth with appropriate antibiotic and grow 12-14 hours to create an overnight liquid culture.
Combine 900 µl of overnight culture and 900 µl of a sterile 50% glycerol solution in a screw-top cryotube.
Vortex briefly.
Incubate at room temperature for 1/2 hour, and place directly into a –80°C freezer.
Prior to freezing, label the tube with the Biobrick part number, plasmid, and antibiotic resistance.

ELECTRO-TRANSFORMATION – 2008 – KULeuven

http://2008.igem.org/Team:KULeuven/Protocols

Materials

LB broth
plasmid DNA
cuvettes
Gene Pulse apparatus
incubator at 37°C
LB plates with appropriate antibiotic

Procedure

Prepare eppendorf tubes with 1 ml LB medium.
Gently thaw the cells at room temperature and place them on ice.
In a cold, 1,5ml polypropylene tube, mix 100 µl of the cell suspension with 1 to 2 µl of DNA (DNA should be in a low ionic strength buffer such as TE). Mix well and let sit on ice for 0,5 to 1 minute.
Set the Gene Pulser apparatus at 25 µF and 2,5 kV. Set the Pulse controller to 200 Ohm.
Transfer the mixture of cells and DNA to a cold, 0,2 cm electroporation cuvette (yellow), and shake the suspension to the bottom of the cuvette. Place the cuvette in a chilled safety chamber slide.
Puysh the slide into the chamber until the cuvette is seated between the contacts in the base of the chamber.
Pulse once at the above settings. This should produce a pulse with a time constant of 4 to 5 msec. (The field strength will be 12,5 kV/cm.)
Remove the cuvette from the chamber and immediately add 1ml of LB medium to the cuvette and quickly resuspend the cells with a pasteur pipette. (This rapid addition of LB after the pulse is very important in maximizing the recovery of transformants.)
Transfer the cell suspension to a 17 x 100mm polypropylene tube and incubate at 37°C for 1 hour. (Shaking the tubes at 225 RPM during this incubation may improve the recovery of transformants.)
Plate on selective medium.

TAQ PCR – 2008 – KULeuven

http://2008.igem.org/Team:KULeuven/Protocols

Mix

Prepare this mix sterile and on ice.

2,5µl dNTP's (2mM)
1,25µl Primer$_1$ (20μM) $+$ 1,25μl Primer$_2$ (20μM)
2µl Template
2,5µl Taq buffer
0,2µl Taq DNA polymerase

15,3µl MQ
Cycles
1 x 6min 94°C
30--35 x
1min 94°C
30sec 55-60°C (lowest Tm minus 2°C)
1min/kb 72°C
1 x 6min 72°C
stay 4°C

PFX PCR – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Mix
Prepare this mix sterile and on ice. Afterwards, put in 4 eppendorf tubes 50μl of this mix.

40 µl Amplification Buffer (blue)
6 µl dNTP's (10mM)
4 µl {MgSO_4} (white)
3 µl Primer1 (20µM) + 3µl Primer2 (20µM)
4 µl Template
3,2 µl Pfx DNA polymerase (black)
40 µl Enhancer (red)
96,8 µl MQ (very sterile)
Cycles
1 x 6min 94°C
30--35 x
1min 94°C
30sec 55-60°C (lowest Tm minus 2°C)
1min/kb 72°C
1 x 6min 72°C
stay 4°C

LIGATION – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
vector + insert DNA (digested)
microcentrifuge tubes
heat block (45°C)
Buffer for ligation
T4 DNA ligase
Deionized, sterile water

Procedure
Add 50 ng vector to x ng insert to a microcentrifuge tube. Calculate x using the following formula:
bp V/ bp I = 50 ng V / x ng I
Put the tube in a heat blok at 45°C for 5 minutes.
Add 2 µl Ligation Buffer
Add 1 µl T4 DNA ligase (keep on ice - very sensitive to temperature)
Add sterile water untill you have 10 µl (if necessary).
Incubate them overnight at 16°C.

PCR WITH END FILLING – 2008 – KULeuven
http://2008.igem.org/Team:KULeuven/Protocols
Materials
primers
Klenow buffer
mQ
dNTP
Klenow polymerase
Procedure
Make 6 PCR tubes with 2 µl primer 1 (1 µM), 2 µl primer 2 (1 µM), 2µl klenow buffer and 11µl mQ
3' at 95°C
5' at (annealingt° overlap)
Add 2µl dNTP (2µM) and 1µl Klenow polymerase
30' at 37°C
20' at 75°C
Do a PCR to amplificate

Transformation Protocol 2 – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Transformation Protocol 2
Equipment
Centrifuge
Water bath
Reagents and Materials
-SOC broth, per litre: - 10ml
2% tryptone (20g)
0.5% yeast extract (5g)
10mM NaCl (0.5844g)
2.5mM KCL (0.186375g)
10mM MgCl2 (2.033g)
10mM MgSO4
20mM glucose (3.6g)
-LB broth, per litre:-110ml
1% tryptone,
0.5% yeast extract
0.5% NaCI
-10% PEG 6000-10ml
-One LB agar plate containing streptinomycin 100ug/ml
-Dry Ice
-1.5ml Eppendorf tubes
-50ml Falcon Tubes
Protocol
Preparation of Competent Cells
Day 1
Inoculate 10ml of LB media in a 100ml flask from a single colony of B. subtilis on an LB agar plate and grow overnight at 37oC.
Day 2
Pre-warm 100ml of LB broth (in a 1 litre flask) at 37oC. Remove 1ml of this media and measure the OD600, keeping this cuvette as will later act as a blank.

Collect the overnight culture, remove 0.5ml of the overnight culture and add 1ml of LB media in a curvette and measure the O.D.600. Remove the remaining 9ml of the overnight culture and add to 100ml of LB broth. Grow this culture at 37oC with vigorous aeration (200rpm) to an OD600 of 1.5. Once at an OD600 of 1.5 the cells should be aliquoted into 4x50ml Falcon tubes and centrifugation at 4000 rpm for 15 minutes at RT. After this, remove the supernatant and resuspend the pellets in 10ml of ddH2O. Combine the 4x resuspended pellets so we get 2x50ml falcon tubes and repeat spin. After this remove the supernatant and resuspend in 25ml of ddH2O and repeat the spin.
After the last wash the cells are resuspended in 30% polyethyleneglycol (PEG) 6000 to 1% of the original culture volume (So if our original volume was 100ml then we resuspend in 1ml). To do this pipette 1ml of PEG into one pellet and resuspend, then pipette this suspension into the next pellet and resuspend.
This suspension should then be separated into 100ul samples and pipetted into 1.5ml eppendorf tubes. After pipetting each aliquot place into a tray of dry ice to rapidly freeze samples and store at -80oC.

Electroporation
Remove a 100 µl aliquot and thaw on ice. Add 50µl of cells to a fresh eppendorf tube (50µl = 1 condition). To this add the DNA (Concentrations should range from 50ng-500ng) in a total volume of 10µl (Make up to water if necessary).
Now add to pre-chilled 0.1cm Electroporation gap cuvettes and perform transformation with the following settings:
Field Strength - 12kv/cm (1.2kv)
Capacitamce - 25µF
Resistance - 400 ohms
Pulse Length - 8msec
After transformation add 1ml of SOC broth to the cuvette and pipette into a 15ml falcon tube where a further 1ml of SOC broth should be added.
Place these tubes into the incubator at 37oC with gentle shaking for 90 minutes to allow expression of the antibiotic resistance.
After 90 minutes the cells should be centrifuged at 4000rpm for 15 minutes at room temperature. After spinning the majority of the supernatant should be removed, leaving enough media to allow resuspension ~ typically 100-200µln is ideal.
This suspension should be aliquoted into the centre of an LB plate containing suitable resistance and streaked out on an LB agar plate.

Preparation of XL1-Blue Electrocompetent cells – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Preparation of XL1-Blue Electrocompetent cells
Aims
Preparation of E. coli cells for the cloning of Biobricks and construct construction
Equipment
4,000RPM Centrifuge
Sterile Centrifugation bottles
50ml Tubes
Large Flasks
Eppendorf Tubes
P200 Pipette
Stripettes
Reagents
1 litre of LB medium
Tetracycline

1-2 litres of autoclaved and chilled ddH2O
10% glycerol in ddH2O, autoclaved and chilled
Dry ice bath
Protocol
Keep everything cold where possible - if using a carbon fibre rotor, you may want to put it in a cold room after innoculation.
Set aside an afternoon for this, starting the culture in the morning
Check the culture while growing frequently
Grow up a culture of E. coli XL1-blue cells overnight
Add 40mL of overnight culture to 1 litre of LB medium (containing 20µg/ml Tetracycline)
Test OD immediately after inoculating the litre flask.
Grow cells while mixing at at least 225rpm until the culture reaches an OD600nm of 0.5-0.6 (1.6-1.9×108cells/ml)
First doubling may take 1 hour but doublings after that should be every 20-30 mins, so check often!
When OD is 0.5-0.6, transfer the culture to 2 sterile 500mL centrifugation bottles and cool on ice for a few minutes
Pellet cells in a centrifuge at 4,000g for 15 mins
Quickly but carefully pour off the supernatant then carefully resuspend the cells in 10mL of ice cold ddH2O
Fill both tubes to about 350mL with ice cold ddH2O
Make sure the pellet is fully resuspended!
Repellet the cells (as before) and again discard the supernatant
Resuspend cells again in 10mL of ddH2O, then fill both tubes up to about 150mL with ice cold ddH2O
Repellet the cells (as before)
While repelleting, fill the dry ice bath and set up Eppendorf tubes (approximately 50) in a rack in the dry ice bath
Pour off the supernatant and resuspend the cells in 20mL of 10% glycerol (resuspend one pellet then transfer the solution to the other bottle and resuspend the second pellet)
Transfer the cells and glycerol solution to a sterile 50mL centrifuge tube and pellet for 15 mins at 4,000g
Pour off supernatant and resuspend pellet in 2mL of 10% glycerol
Pipette 50µL aliquots into the Eppendorfs in the dry ice bath
Freeze on dry ice
Depending on pipetting accuracy, between 50 and 60 aliquots should be made
Using a repeating pipette makes this process much faster and reduces risk of contamination
Store at -70°C

PCR – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
PCR
This protocol is desgined for use with the stratagene PfuUltra II Fusion DNA polymerase and is based in part on the PfuUltra II Fusion DNA polymerase usage manual. PfuUltra II Fusion Manual
Aim
To produce clones of two genes from B.subtilis that are too big to have synthesised by GeneArt; for use as an integration site and gene knockout (epsE) or for their original purpose as a transcriptional regulator (xylR).
To produce clones of sequences from vectors; for use as integration sites (amyE), antibiotic resistance (Spectinomycin - aad9) and as a transcriptional repressor (lacI).

A modified protocol for using Taq polymerase can be used if less fidelity is required, to obtainoptimal conditions for use with Pfu DNA polymerase, or to carry out single colony PCR for verification purposes (see below).
Equipment
Heated lid PCR machine
Thin walled PCR tube
Reagents
The reagents required for Pfu and Taq PCR are very similar. The regaents listed here are required for Pfu PCR, the minor changes required for Taq PCR are listed in the Taq PCR secton below. When producing Biobrcik parts, the forward and reverse primers should contain the Biobrick prefix (forward primer) and the complementary sequence to the Biobrick suffix (reverse primer) 5' of the beginning of the annealing sequence.
Distilled H_2O: 18.5µL
10X PfuUltra™II reaction buffer: 2.5µL
dNTP mix (10mM): 0.5µL
B.subtilis genomic DNA (100ng/µL): 1µL
Forward Primer (100ng/µL): 1µL
Reverse Primer (100ng/µL): 1µL
PfuUltra® II fusion HS DNA polymerase: 0.5µL
Total Reaction Volume: 25µL

Note: Template DNA should be diluted to 100ng/µL. If template DNA concentration is below 100ng/µL, 100ng of DNA should be added and the volume of H_2O to be added should be adjusted to maintain a reaction volume of 25µL.
If a vector is used as the template, 5ng of plasmid DNA should be used instead and the volume of H_2O to be added should be adjusted accordingly.
If PCR is proving difficult, particularly for denaturation, DMSO can be added to a final concentration of 10% to increase efficiency
Protocol
Add all the reagents in order (down the list) sequentially to the PCR tube, mxing after each addition.
Place the PCR tubes into the PCR machine and set the programme to the following set-up:
Initial Denaturation: 30 seconds at 95°C (longer for genomic DNA)
10 Cycles of:
30 second denaturation at 95°C
30 second annealing time at Primer Tm - ~3°C (complementary section of primer Tm)
15 seconds (+ 15 second for each additional kb) extending time at 68C (30 seconds for genomic templates)
20 - 30 Cycles of:
30 second denaturation at 95°C
30 second annealing time at Primer Tm - ~3°C (total primer Tm)
15 seconds (+ 15 second for each additional kb)extending time at 68°C (30 seconds for genomic templates)
Final Extension: 5 minutes at 68°C
The resulting solution can then be purified using a PCR purification column, by gel electrophoresis followed by spin purification or can simply be ligated ready for use.

Taq PCR
If using Taq DNA polymerase, the reaction mixture is effectively the same (with Taq buffer rather than Pfu buffer), 0.5µL of Taq should be used and care should be taken to ensure that a suitable

amount(1.5-4 mM) of magnesium (usually as magnesium chloride) is present in the reaction, as some Taq buffers do not contain magnesium.
For Taq PCR, the temperature of the extension step should also be raised to 72°C and more time allowed for extension (60 seconds per kb usually works fine with Taq DNA polymerase)

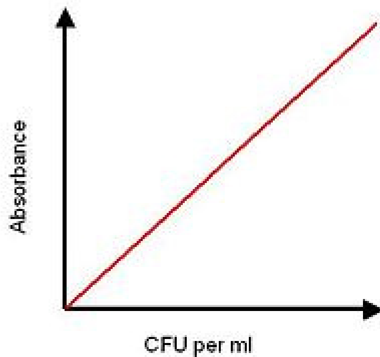
Single Colony PCR
Single colony PCR is a useful tool for rapidly verifying if a colony contains the plasmid with the correct DNA after a ligation. The colony is replica plated, boiled and then used as a PCR template, potenetially with the registry VR and VF2 primers to obtain the approxiamte length of an insert. The reagents are identical to that of a Taq PCR and the method only differs by template preparation and PCR programme.
Preparation of Samples
Further equipment needed: Heating block (or waterbath) capable of eaching 95°C
It is adviasble to replica plate andy samples taken to allow the cells to be grown up after the PCR, as sample preparation kills the cells.
Heat up heating block/waterbath to 95°C
Select distinct colonies from a plate for testing
Pipette 100µL of sterile H2O into eppendorf tubes (1 per colony)
Pick each colony in turn, replica plate the colony on a fresh plate and then mix the loop tip with the water in the eppendorf tube to leave cells as a sample
Be sure to use a fresh tube of water fo reach sample!
Boil the water and cells soltuion for 5 minutes in the heating block/waterbath
Be careful not to leave the sample in the heating block/waterbath too long as this may damage the DNA template
Each sample is now ready to be used as a DNA template in an PCR reaction
PCR
Single colony PCR requires the same set of reagents as standard Taq PCR (see above for reagents and amounts), the DNA template will be 1µL of the sample that was just created.
As with all Taq PCRs, ensure that your PCR reaction contains magnesium as this is vital for Taq DNA polymerase to work correctly.
The programme for the PCR machine is simpler when not producing Biobricks, PCR tubes should be placed into a pre-warmed machine(depending on make and model) and the machine set to;
Initial Denaturation: 30 seconds at 95°C (longer for a genomic DNA template (eg. checking integration))
30 Cycles of:
30 second denaturation at 95°C
30 second annealing time at Primer Tm - ~3°C
30 seconds (+60 seconds for each kb of insert) extending time at 72°C (longer for genomic templates)
Obtaining Results
5µL of each PCR reation should then be mixed with DNA loading buffer and all samples run on a 1% agarose gel with suitable marker and controls to check the length of inserts.

Calibration Curves – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Calibration Curves
The two measurements that will be made for the various devices are fluorescence emission and absorbance at 600nm.
The aim of the characterisation is to use these two measurements in order to create a more generic unit to allow for modular design and easier repetition of experiments. The unit that has been chosen to be used is **GFP molecules synthesized cfu $^{-1}$ sec^{-1}** .

In order to convert our raw data into these units, two sets of calibration curves are needed.
There are broadly two types of calibration curves that we will require,
Absorbance (600nm) vs Colony Forming Units (CFU).
Fluorescence vs intracellular GFP concentration.

Absorbance

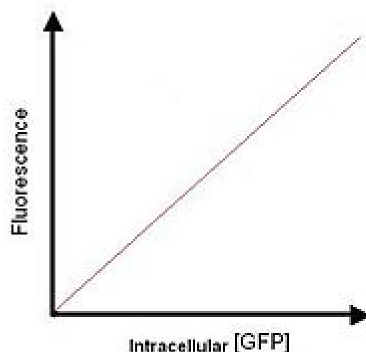
The Absorbance of the cultures in the wells are measured to allow to convert our absorbance raw data into a cell count, so that cell we can relate GFP synthesis per cell.



Example of Absorbance Calibration Curve

Cultures of the E.coli with the relevant vector are grown to various cell densities. A sample of these cultures are taken and a dilution plate is carried out to work out approximate colony forming units per ml of culture.
These various cultures have their absorbance measured at 600nm to give an absorbance measurement for the various cell densities.
This data set is then combined, creating a graph relating the number of colony forming cells per ml to their absorbance measurements.
This curve then allows us to convert absorbance of a known volume of culture to colony forming units within the culture sample.
The same strain will be used throughout the experiments with the various devices. This means that absorbance change is minimal between our devices and so we can apply the same calibration curve.

Fluorescence
Calibration Curve

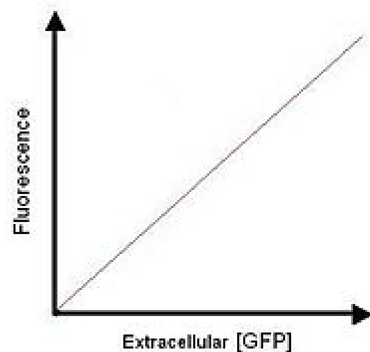


Example of Fluorescence Calibration Curve

In the protocols of the characterisation of the parts, the fluorescence of various wells in a fluorometer plate is measured. These wells correspond to varying conditions i.e. different levels of expression are induced. The fluoresence of these wells is recorded against time.

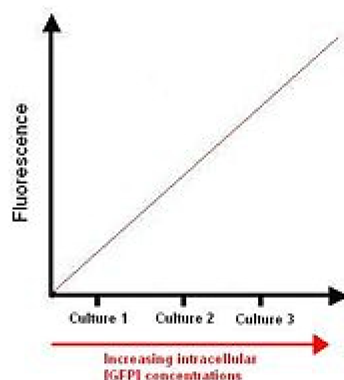
In order to convert the raw data of fluorescence of each well at a specific time point into **GFP molecules synthesized sec^{-1}** we will make use of the **Fluorescent Calibration Curve**.
In order to obtain this calibration curve *(axes shown right)* we need to conduct three experiments. These are described below:

Experiment 1



Fluorescence vs Extracellular [GFP]
The first stage of producing a calibration curve is to produce a graph of **fluorescence vs extracellular [GFP] molecules.**
To do this a known concentration of GFP is diluted into a range of dilutions. The dilutions are carried out in cell lysis of the wild type of the host cells used, e.g. if K12 used as expression cell, then a lystae of K12 cells are used. The reason for this is that the quantum efficieny(ration between absorption of photon to emission of photon) of the GFP may change in the solution of the cell.
With these dilutions we can then measure the amount of fluorescence for various [GFP] in extracellular conditions.
This allows us to plot the graph on the right.

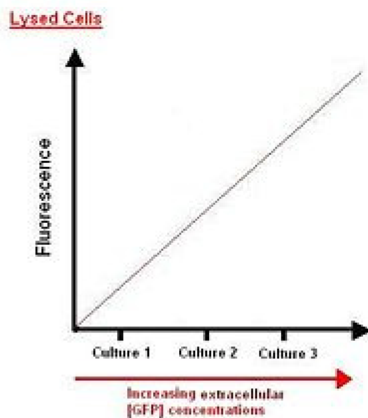
Experiment 2



Fluorescence vs Cultures of varying unknown intracellular [GFP]
From experiment 1 we get fluorescence related to extracellular [GFP], however, with our protocol we are concerned with measuring the intracellular [GFP]. This is because we want to measure fluorescence at various time points for a particular culture of cells, and so by measuring intracellular [GFP] rather than

extracellular [GFP] cells do not have to be lysed and so the same culture can be measured at various times.
The first stage in measuring the intracellular [GFP] is to make cultures that have a range of unknown [GFP] intracellular concentrations.
To do this we take several cultures of cells and expose them to a relevant inducer to induce expression of GFP. However, we stop the reaction at various times so that in the end we have a range of cultures with a range of unknown intracellular [GFP].
For each culture the fluorescence is measured to create a graph of **Fluorescence vs Cultures of varying unknown intracellular [GFP]**.
To the right is an example of what an experiment would look like if 3 cultures were used.

Experiment 3



Fluorescence vs Cultures of varying unknown extracellular [GFP]
The purpose of this experiment is to relate the intracellular florescence of experiment 2 to a known intracellular [GFP].
To do this we need to relate experiment 1 to experiment 2. This means relating the fluorescence of unknown intracellular [GFP] of various cultures(exp2) to the fluorescence of known extraceullar [GFP](exp.1)
To do this the the cultures from experiment 2 are lysed and the fluorescence measured. This fluorescence will represent the fluorescence of unknown extracellular [GFP].
This fluorescence can be related to known [GFP] molecules from experiment 1, by comparison of the fluorescence levels.
With the extracellular [GFP] molecules in the culture known, we can assume that the intracellular [GFP] across the population of the cells is the same. We can then plot a graph of the known intracellular [GFP] vs fluorescence, this is the **Calibration Curve** shown above. This will then mean that if we have a fluorescence measurement we can estimate the intracellular [GFP].

Cell Count v Optical Density Curve Calibration – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Aim
To produce a calibration curve to aid in the normalising of fluorescence values to allow proper characterisation of Promoters and RBSs for B.subitlis. This protocol must give results that are as accurate as possible over a considerable range of Optical Densities.
Equipment
Spectrophotometer
Cuvettes

P20, P200 and P1000 Gilsons
Reagents
20ml of LB in a 250ml flask
B. subtilis transformed with antibiotic selectable marker
LB agar plates with antibiotic
Protocol
Inoculate 20ml of LB broth with B. subtilis containing a selectable marker, we used B. subtilis transformed with spectinomycin. Incubate overnight at 37oC.
Collect culture and measure the O.D.600.
With the O.D.600 calculate how much of the overnight culture is required to give an O.D.600 of 0.5, 1, 1.5, 2, 2.5, 3 in 2ml using the following calculation:
Vol of culture (ml)=(O.D.600 wanted / O.D.600 of Overnight culture) * 2ml
Carry out the dilutions required using LB media to make the volumes up to 2ml.
Remove 1ml of each dilution and then measure the O.D.600 and discard.
Now carry out a series of 10 fold dilutions by removing 100ul of the dilution and adding into to 900ul of LB (this gives 10-1 dilution) carry this on until a suitable dilution is reached. We used the following dilutions:
O.D600 of 0.5 = x10-6 and x10-7
O.D600 of 1 = x10-6 and x10-7
O.D600 of 1.5 = x10-6 and x10-7
O.D600 of 2 = x10-6 and x10-7
O.D600 of 2.5 = x10-6 and x10-7
O.D600 of 3 = x10-7 and x10-8
Plate 100ul of the appropriate dilutions onto an LB agar plate containing the antibiotic selectable marker.
Grow the plates at 37°C overnight
The following day count the number of colonies on the plates and multiply up by the dilution to obtain the colony forming units at each time point. Remember when plating the cells only 100ul of 1000ul was used to plate and so you need to multiply by a further 10.

GFPmut3b Fluorescence vs Molecules of GFPmut3b – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Aims
The aim of this protocol is to produce a calibration curve of fluorescence vs molecules of GFPmut3b. This is to help us convert our fluorescence data in arbitrary units into the normalised units of rate of molecules of GFPmut3b synthesised per cell per min/sec.
Equipment
Fluorometer + PC
Pipette gun
Gilson p20,p200,p1000
Spectrometer
Shaking incubator
Reagents and Materials
1 x 96 Fluorometer Plate (See-through bottom)
Sticky plate lid
Cuvettes
1x10ml of Autoclaved LB media in a 200ml flask Containing suitable antibiotics
ddH2
1mg/ml of GFPmut3b
Protocol
Day 1

Collect 10ml of LB media (containing suitable antibiotics) into a 100ml flask. Inoculate the media with a single colony from a B. subtilis plate (transformed with correct construct) and grow overnight at 37oC.
Day 2
Collect the overnight culture from the incubator, remove 1ml and measure the O.D.600. Calculate the amount of the overnight culture required to return the cells to an O.D.600 of 0.5 in 10ml of LB media, use the following calculation:
Volume of Overnight Culture (X) = (0.5/O.D.600)*10ml
Volume of fresh Culture (Y) = 10-X (Overnight Culture)
Return the cultures to the incubator and grow until an O.D.600 of 1 is reached.
(returning to an O.D.600 of 1 because this gives a suitable cell density for cells in the exponential phase of growth were we are going to characterize the test constructs.)
Now prepare the lysozyme solution by adding 2mg of lysozymes to 1ml of ddH2O, mix thoroughly and store on ice. Add 200µl of this solution, to the 10ml culture drop by drop mixing gently in between drops.
Incubate at 37oC for 15 minutes with occasional mixing - lysis should be indicated by a change in the viscosity of the culture.
Whilst the cells are lysing the GFPmut3b dilutions can be prepared. The stock solution is 1mg/ml (27.8kDa or 37µM), using this the following dilutions can be performed:
[1.] 32.4µl of stock GFPmut3b into 7.6µl o ddH2O = 30µM
[2.] 16µl of [1.] into 10µl of ddH2O = 24µM
[3.] 16µl of [2.] into 10µl of ddH2O = 18µM
[4.] 16µl of [3.] into 10µl of ddH2O = 12µM
[5.] 16µl of [4.] into 10µl of ddH2O = 6µM
Repeat this dilution three times to enable us to average out any errors.
Once the lysis is complete we are ready to carry out the dilutions of GFPmut3b in lysed B. subtilis, Then follow the plate loading schematic below being careful to avoid bubbles and mix solutions thoroughly before adding to the plate.
Place in the plate reader and load up as follows:
GFP plate reader.PNG

Characterization of Constitutive Expression – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Aims
To characterise the constitutive promoter and RBS combinations. This protocol is concerned with characterisation of the steady-state expression of GFPmut3b under the control of the 4 different promoter-RBS combinations. We will be measuring the fluorescence and O.D.600 of the cultures and normalizing our data to the units of rate of molecules of GFPmut3b synthesised per cell per min/sec.
In addition, from comparison of the rate of expression we should be able to determine the relative strength of the RBS.
With the data on the characterisation of the promoter-RBS combinations, we will be able to make an informed decision on which combination we should use for our future test constructs.
The protocol below describes what is needed for one combination of promoter and RBS, we will need to carry this out for all the combinations.
Normally we are only concerned with the steady state expression of a constitutive promoter. However, the conditions under which we measure this could change, for example what media the B.subtilis is grown in. In addition, we may wish to see how the strength of expression changes during the growth of the B.subtilis. This could be achieved by measuring the change in fluorescence between the different growth phases.
Equipment

Fluorometer + PC
Pipette gun
Gilson p20,p200,p1000
Spectrometer
Shaking incubator
Reagents and Materials
1 x 96 well Fluorometer Plate (See through bottom)
Sticky plate lid
Cuvettes
4x20ml of Autoclaved LB media in a 200ml flask Containing suitable antibiotics
Protocol
Day 1
Collect 10ml of LB media (containing suitable antibiotics) into a 100ml flask. Inoculate the media with a single colony from a B.subtilis plate (transformed with correct construct) and grow overnight at 37oC.
Day 2
Collect 3 x 200ml flasks containing 20ml of LB media (containing suitable antibiotics) and remove 1ml of the media and pipette into a blank cuvette (this is to make the LB blank). Remove 1ml of the overnight culture and measure the O.D.600
Dilute the overnight culture 1 in 10 (2ml per culture) in 3 x 20ml of fresh LB media containing suitable antibiotics . Mix thoroughly and remove 1ml of the culture and measure the OD600. Place into a shaking incubator and grow until reaches the exponential phase of growth, the time for this should be determined previously when we do the growth curve but always checked by removing 1ml of each culture and measuring the OD600 using LB media as a blank.
Once the correct OD600 has been reached then pipette 9x200µl of the B. subtilis into a 96 well plate following the plate schematic (in total want 3 repeats for each of the 3 cultures). In addition pipette 200µl of 3xLB media into the 96 well plate. Once the plate has been loaded then carefully place sticky tape onto the top of the plate.
Place into the plate reader and open the protocol for characterisation of constitutive promoters and run protocol.
This needs to be set up to measure the O.D.600 and fluorescence (488nm excitation filter and 525nm emission filter) every 10 minutes for an hour (What do we think of these data collection settings).
Once data is collected dispose of the 96 well plate into the autoclaved rubbish bins (white).

Initial Characterization of pHyperspank Inducible Promote – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Aims
The aim of this protocol is to help us define the dynamic range of the transfer function where the lower and upper threshold response is. This can help us define further concentrations to characterise the transfer function. In addition, we can get an idea of the response time of this inducible promoter. Finally if we need to pursue with cloning these experiments will confirm which of the combinations work and can be pursued for cloning further constructs.
This protocol is to be used to characterise the IPTG inducible Hyper-spank promoter RBS combinations.
Equipment
Fluorometer + PC
Pipette gun
Gilson p20,p200,p1000
Spectrometer
Shaking incubator

Reagents and Materials
1 x 96 well Fluorometer Plate (See-through bottom)
Sticky plate lid
Cuvettes
4x20ml of Autoclaved LB media in a 200ml flask Containing suitable antibiotics
IPTG
Protocol
Day 1
Collect 10ml of LB media (containing suitable antibiotics) into a 100ml flask. Innoculate the media with a single colony from a B. subtilis plate and grow overnight at 37o.
Day 2
Collect 1 x 200ml flasks containing 20ml of LB media (containing suitable antibiotics) and remove 1ml of the media and pipette into a blank cuvette (this is to make the blank for OD measurements).
Remove 1ml of the overnight culture and measure the OD600
Dilute the overnight culture 1 in 10 (2ml in 20 ml) in 1x20ml of fresh LB media containing suitable antibiotics . Mix thoroughly and remove 1ml of the culture and measure the OD600. Place into a shaking incubator and grow until reaches the exponential phase of growth, the time for this should be determined previously when we do the growth curve but always checked by removing 1ml of each culture and measuring the OD600 using LB media as a blank.
Once the correct OD600 has been reached then pipette 27x190µl of the B. subtilis into a 96 well plate following the plate schematic. In addition pipette 200µl of LB media and 190ul of LB media into the 96 well plate.
Now load in 10ul of the inducer into the plate following the schematic (need to be determined) with the concentrations listed below. In addition add 10ul of 10mM IPTG to the LB media alone, this will help us determine whether the IPTG is contributing to the background fluorescence.
Once the plate has been loaded then carefully place sticky tape onto the top of the plate.
Place into the plate reader and open the protocol for characterisation of inducible promoters and run protocol.
This needs to be set up to measure the OD600 and fluorescence (488nm excitation filter and 525nm emission filter) every 10 minutes for 6 hours (What do we think of these data collection settings).
Once data is collected dispose of the 96 well plate into the autoclaved rubbish bins (white).

Concentrations of IPTG:
0x3
1nMx3
10nMx3
100nMx3
1µMx3
10µMx3
100µMx3
1mMx3
10mMx3

Characterization of Light Inducible Expression – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
Aims
The aim of this protocol is to characterise the light induced expression of GFPmut3b under the control of the pctc and pgsbi promoters. In addition to expression of these constructs, we require overexpression of the YtvA receptor protein. The complexity of the characterisation of light inducible promoters means that we need to carry out several levels of testing:
Test the overexpressed YtvA proteins are functional,

Test the exposure time required to induce expression,

Issues to be resolved:
The stationary phase of growth,
How to stop the cultures being exposed to light,
If we Cannot excite the light receptor with the flourometer then what should we use? Could try to filter white light out using filters or cellophane or alternatively we could excite using intense white light.
1. Testing overexpression of YtvA receptor
Equipment
Spectrometer,
Reagents and Materials
2x5ml LB media in 50ml flasks,
2x10ml LB media in 100ml flasks,
pipettes,
cuvettes,
Protocol
Day 1
Pick a colony from a plate of B. subtilis and inoculate 5ml of LB media and grow overnight at 37oC.
Pick a colony from a plate of B. subtilis transformed with construct (define when list is up) and inoculate 5ml of LB media and grow overnight at 37oC.
Both flasks should be totally covered in foil to ensure that they are grown in darkness.
Day 2
Measure the O.D.600 of both the overnight cultures using LB media as a blank. With the O.D.600 calculate the dilution to achieve an O.D.600 of 0.5 in 10ml (does not matter too much what O.D.600 we choose, key is to standardise them) using the following calculation:
Volume of Overnight Culture (X) = (0.5/O.D.600)*10ml
Volume of fresh Culture (Y) = 10-X (Overnight Culture)
To determine whether the YtvA light receptor has been expressed correctly we can measure the absorption of the YtvA within each of the cultures. We should see an increase in absorption within the spectra of YtvA overexpressed culture (Figure 1).
The following wavelengths have been chosen across the absorption spectra of the YtvA:
375nm (An Absorption Peak)
450nm (Absorption Max)
500nm (Not absorbed)
550nm (Not absorbed)
To measure each of these, 1ml of the cultures should be pipetted into a cuvette and measured immediately in a spectrometer, with each wavelength being repeated twice.
What we should observe is an increase in absorption in the B. subtilis expressing YtvA in the absorption spectra and not in the wavelengths out of the spectra.
Figure 1. Spectra of YtvA
2.Testing the exposure time required to induce expression
Equipment
Fluorometer
Reagents and Materials
2x10ml LB media in 100ml flasks
Pipettes
Cuvettes
96 well plate
Plate lids
Protocol

Day 1
Collect 10ml of LB media (containing suitable antibiotics) into a 100ml flask. Inoculate the media with a single colony from a B. subtilis plate and grow overnight at 37o.
Grow this culture overnight in the dark by covering the flask in foil.
Day 2
Collect 1 x 100ml flasks containing 10ml of LB media (containing suitable antibiotics) and remove 1ml of the media and pipette into a blank cuvette (this is to make the blank for OD measurements).
Remove 1ml of the overnight culture and measure the OD600
Mix thoroughly and remove 1ml of the culture and measure the OD600. Now we need to dilute the culture down to a suitable OD600 of 0.2 in 10ml of culture, use the following calculation:
Volume of Overnight Culture (X) = (0.5/O.D.600)*10ml
Volume of fresh Culture (Y) = 10-X (Overnight Culture)
Using the calculated volumes inoculate 1 x 10ml LB media in a 100ml flask that is totally covered in foil. Grow in the shaking incubator at 37oC.
After....hours of growth check the OD600 of the culture using LB as a blank, if the correct OD600 for exponential phase is reached then remove culture from incubator, if it has not then carry on growing for suitable length of time.
Place into a shaking incubator and grow until it reaches the exponential phase of growth, the time for this should be determined previously when we do the growth curve but always checked by removing 1ml of each culture and measuring the OD600 using LB media as a blank.
Once the correct OD600 has been reached then pipette 18x200µl of the B. subtilis into a 96 well plate following the plate schematic. In addition pipette 200µl of LB media into the plate. It is key to minimise the light exposure when loading this plate.
Once the plate has been loaded then carefully place sticky tape onto the top of the plate.
Place into the plate reader and open the protocol for characterisation of light inducible promoters and run protocol.
This protocol needs to be set up to use a suitable emission filter to induce the YtvA receptor. In addition we need to explore how the length of this induction and the intensity of this will affect the activation of the YtvA receptor and so we will need to find out a suitable range of these to test.
After light induction we need to set up the protocol to measure the OD600 and fluorescence (for mRFP1) every 10 minutes for 6 hours.
Once data is collected dispose of the 96 well plate into the autoclaved rubbish bins (white).

Chloramphenicol Resistance Assay – 2008 – Imperial College
http://2008.igem.org/Team:Imperial_College/Resistance
This protocol is designed for use with chloramphenicol and the chloramphenicol acetyltransferase gene, however it may work well for characterising other antibiotic resistance genes, particularly if the antibiotic concentrations are optimised.
Materials
50ml tube
An antibiotic resistant bacterium
Chloramphenicol
LB agar
LB medium
Lots of plates
Spreaders
Method
Chloramphenicol concentrations used in this assay were; 5µg/ml, 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml, 75µg/ml, 100µg/ml, 150µg/ml and 200µg/ml.
Make 4 LB agar plates (depending on how many repeats you want) at each chloramphenicol concentration.

Transfer approxiamtely 10ml of LB into the 50ml tube and chloramphenicol to a concentration of 5µg/ml.
Innoculate the flask with resistant bacterial strain and grow overnight
The following morning, plate 50µl of the bacterial solution onto 2 plates per concentration, 100µl onto the third plate at each concentration and 150µl onto the final plates.
Grow plates in the incubator or on the bench overnight.
Check plates for growth after 24 hours and note the approximate precentage of the plate covered in cells.
Results
This was used to test chloraphemicol acetyltransferase activity and resistance infered.
These results can be accessed here.

Competent Bacteria Solutions – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Competent_Bacteria
KCM 5x
100ml

0.5M KCl	3.7 g	KCl 74,56 g/mol
0.15M CaCl2	2.2 g	CaCl2 2H2O 147,02 g/mol
0.25M MgCl2	5 g	MgCl2 6H2O 203,30 g/mol
autoclave		

TSB
95 ml

LB	about 80 ml
10% PEG (MW 3.350)	10 g
20 mM Mg++	10 ml of 200mM
autoclave	
5% DMSO	5 ml

Transformation Barbara – 2008 - ESBS-Strasbourg
DNA in small volume (maximal 10µl)
+ 20µl of KCM 5X
qsp 100µl H2O
Add 100µl of competent bacteria
Leave 20 min on ice, then 5 min at room temperature
It is possible to let them grow again for 1 hour at 37°C, or plate them immediately
No thermic shock

Transformation Heat shock – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Transformation_Heat_shock
Protocol is a mixture of the IGEM standard protocol and the protocol used in the local lab
ligation or 10 ng plasmid

add 50-100 µL of competent bacteria
take on ice for 30 min
heat shock at 42 °C for 2 min
add 300 µL LB and incubated at 37°C for 45 min
plate

PCR – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/PCR
I: Classic PCR

a) Remarks

-Use of the Taq polymerase
-Te=72°C
-Tm should be comprised between 55°C and 65°C. There is some exception, but in any case the Tm should inferior to the Te
-Tm of primers should be very similar (a difference of about 2 degrees is accepted)
-Gently vortex and briefly centrifuge all solutions after thawing
-Adapt the protocol function of the mother concentration we have
-We have to calculate the different volumes for a final volume of 50µL

b) Protocol

Mother solution	**Final concentration**	**Quantity, for 50 µl** of reaction mixture
10X Taq buffer	1X 5 µl	5µL
2 mM dNTP mix	0.2 mM of each	5µL
Primer I (10µM)	0.1-1 µM	2,5 µL
Primer II (10µM	0.1-1 µM	2,5 µL
Taq DNA Polymerase	1.25 u / 50 µl	
25 mM MgCl2 (we have it)	1-4 mM	5µL
Template DNA	10ng/µL	
Water	_	qsp

With the pFusion polymerase:
primers for(10µM) 2,5µL
primers rev(10µM) 2,5µL
DNA template 10ng/µL
Buffer + MgSO4 5µL
dNTP(2mM) 5µL
pFusion 1µL
qsp water 50µL

PCR conditions:
See the conditions commonly used, usually its a programm which is already saved on the machine
Of course, just adjust the annealing temperature ;-)

II: Site directed mutagenesis

a) Remarks
-Use of the Fusion polymerase, for higher fidelity/processitivity
-Tm higher, about 78°C (see primers table)
-If the mother solutions own a different concentrations than those described below, then adapt the corresponding volume for the mix
-Precisions could be add after the firsts experiments (e.g: for the PCR conditions, depends of how much it works)
-Gently vortex and briefly centrifuge all solutions after thawing

b) Protocol
If we start with this solutions
-5x Phusion HF Buffer
-Phusion DNA polymerse (2u/µl)
-Primer 1 (5 µM)
-Primer 2 (5 µM)
-dNTPs
10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP
Then we have this protocol:
Mix:
10 µl tampon enzyme 5x concentré
1 µl dNTPs à 10 mM (200 µM de chaque)
x µl de plasmide (1 pg-10 ng)
5 µl du primer 1 (5 pmol/µl) (0.5 µM final)
5 µl du primer 2 (5 pmol/µl) (0.5 µM final)
0,5 µl de Phusion DNA polymearse (0.02 u/µl final)
H20 (q.s.p. 50 µl)

PCR conditions:
Step 1 : denaturation 30 s at 98°C
Step 2 : denaturation 10 s at 98°C
Step 3 : hybridation 30 s at Tm-2°C
Step 4 : elongation x (15-30 s/Kb) mn at 72°C
Step 5 : back to step 2, x time
Step 6 : elongation 5-10 mn at 72°C
Step 7 : maintain at 4°C

Keep the resulting solution at -20°C until to use it

Purification of PCR product – 2008 - ESBS-Strasbourg
I: Purification of classic PCR product
a) Remarks
In this case we obtain linear DNA which could be purified after a Gel migration or directly after the PCR.

	Purification's yield
Directly after PCR	~90%
After gel migration	~75%

b) Protocol

See the Quiagen kit instruction for both types of purification.

II: Purification of mutagenesis PCR product

a) Remarks
Here we get the product in a plasmid form. It's more difficult to purify the new plasmid (which own the mutation) from the original plasmid. There are several possibilities:
-Use of the Dpn1 enzyme, which recognize the methyl Dna, in other words the original plasmid would be degraded. In this case, just add Dpn1 in the PCR product, and after the degradation the solution can be directly employ for transformation
-Purification after gel migration: difficult it will be very hard to distinguish plasmids which own the mutation and the other ones. One possibility could be to use Agarose Low melting point, or to decrease the agarose concentration (0,5 or 0,3). Then be careful cause the gel would be more difficult to handle (fragile)
see Agarose gel preparation

b) Protocol
-see Restriction digestion part for a Dpn1 digestion
-Follow the Quigen kit instruction for traditionnal purification
Take max. 400mg of gel, no more

Miniprep: plasmid amplification – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Miniprep:_plasmid_amplification
a) Remarks

Antibiotic	**Working concentration**
Ampicillin	50-100µg/mL
Chloramphenicol	25-170µg/mL
Kanamycin	10-50µg/mL
Streptomycin	10-50µg/mL
Tetracyclin	10-50µg/mL

b) Protocol
Take one colony and put it in 5 to 7mL of LB medium containing the antibiotic
12 to 16h over night at 37°C, 200-250rpm
Notice that you will need about 3mL of culture to perform the plasmid purification

You can either take the quick purification kit and follow the instruction of the kit discription.
Or if quality is not so important you may use the following methode
b.1) MiniPrepProtocol of the A.P. Sibler course
Spin down 1.5 mL of the Overnight culture (1min at 10 000rpm)
Take off the supernatant leaving around 100 µL medium
Resuspend the cells by vortexing
Add 300µL TENS; vortex 2-5 sec
Add 150µL NaACo(3M pH5)fastly; vortex 2-5 sec
Spin down 5 min at max. speed
Pipette the supernatant in a new tube without getting contamination of the pellet
Add 900µL ice cold ethanol (100% -20°C) and vortex
Spin down 5 min at max. speed, take note of the orientation of the tubes

Take off the supernatant being careful to not detach the pellet
Wash with 1 mL (25% TE and 75%EtOH); DO NOT RESOLVE THE PELLET
Spin 2 min at max. speed
Take off the supernatant and let dry the pellet completely
Resovle in 40µL RB (0,6 mL TE + 10 µL 1% gélatine + 2 µL RNase à 10 mg/mL)

10µL may contain 1µg of plasmid

Plasmid Purification – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Plasmid_urification
a) Remarks
We can estimate the concentration of our final product, by using a spectrophotometer. The wavelength to enter is 260 nm, knowing that 1 unit correspond of a 50µg/mL concentration. Be aware that there is a Nanodrop at the Pharma faculty, and maria proposed us to use it if we need. The advantages are that we can get our plasmid concentration by wasting only 1µL, and we also get the purety of it.

b) Protocol
Follow the Nucleospin kit instructions

Restriction digestion – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Restriction_digestion
a) Remarks

Name	**Digestion time**	**Reaction temperature**	**Buffer to use (100%)**	**Heat inactivation**	**Unit number**
EcoR1	2h	37°C	NEBuffer 1/2/3/4	65°C, 20 min	0,13
Spe1	2h	37°C	NEBuffer2+100µg/mL of BSA	65°C, 20 min	0,5
Xba1	2h	37°C	NEBuffer2+100µg/mL of BSA	65°C, 20 min	0,13
Not1	2h	37°C	NEBuffer3+100µg/mL of BSA NEBuffer2 (only 50%)	65°C, 20 min	0,25
Pst1	2h	37°C	NEBuffer3+100µg/mL NEBuffer 1/2 (only 75%)	80°C, 20 min	0,5
Dpn1	1h	37°C	NEBuffer 1/2/4	80°C, 20 min	0,13

NB motto: the less unit number an enzyme has, the stronger activity she own

b) Protocol
Two different strategies could be considerate: -In the case of a step-by-step digestion process, always begin to digest with the enzyme the less efficient
-In a double digestion case, check of course the buffer to use, and the activity of the two enzymes must be very similar. Maria don't support this strategy so much, however we can try it and check if we have good result (it could be usefull for the Spe1 and Xba1 digestion of course)

Furthermore, Maria use to do digestion during 2h, then she adds once again 1µL of enzymes for another digestion time of 2 hours. She says its very efficient, but I find it quite long (four hours…) Discussion to elaborate the best strategy has to be hold

standard method:
-30µg DNA
-20 enzyme unit (5u/µL), of each enzymes
-10µL tampon RE 10X
-10µL BSA 10X -qsp H2O 100µL
->digestion (see table)

double digestion protocol used for plasmid verification 17/07/08 (KK):
10µL of preped plasmid (around 1µg)
2µL Tango buffer (10x)
0.2 µL BcuI (SpeI) - two times the quantity which is necessary
0.2 µL XbaI - two times the quantity which is necessary
7.6 µL H20
This protocol has to be up scaled, if you want to go on working if the digested fragement

Agarose gel preparation – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Agarose_gel_preparation
a) Remarks
-For an ultrapure agarose gel preparation, see Paul he has some reference. I dunnow if we have to buy it or if some lab workers work with it here

b) Protocol

Agarose (g/100mL)	Dna resolution (kb)
0,5	1-30
0,7	0,8-12
1	0,5-10
1,2	0,4-7
1,5	0,3-3

Buffer: TAE

Dephosphorylation – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Dephosphorylation
a) Remarks
Do the reaction directly in the RE mix

b) Protocol
-Add 2 unit of CIP for 2µg of vector
-Digestion 1h max. at 37°C

Ligation – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Ligation

1)Remarks:

2)Protocol:
For insert/vector ligation:
see iGEM protocol
For intra vector ligation:
5 T4 DNA ligase unit, for 25-50ng of DNA
2c) Protocol according to Fermantas
50-400 ng vector
1 to 3 times the molar amount of the insert
2 µl ligation buffer
1 µl ligase
to 20 µl water
-> vortex and spin down
-> 1h at 22°C water bath (in 4°C room)
-> enzyme inactivation at 65°C for 10 min
-> directly usable for the transformation

Concentrations Measurements – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/Concentrations_Measurements
1)by spectrophotometer
step1: Prepare your sample:
2µL of the solution
400µL Water

step2: put lambda=260nm

step3: do the blank with the same water you used for dilution

step4: Do the conversion absorbance->concentrations
1DO = 50µgDNA/mL = 50ng/µL
=>Multiply your DO per 50, and then per 200 for the dilution factor. Then you have your DNA concentrations in ng/µL.

2)By nanodrop
Ask Maria to use it at the Pharma faculty. You only need 1µL of your solution.

TENS – 2008 - ESBS-Strasbourg
http://2008.igem.org/Team:ESBS-Strasbourg/TENS
Tris-HCl 10 mM pH8
EDTA 1 mM pH8
NaOH 0.1 M
SDS 0.5%
Make a Sterilfiltration of the solution or use steril stock solutions for its preparation

For 40 mL of TENS:
0.8 mL of 0.5M Tris-HCL pH8
0.1 mL of 0.4M EDTA

0.4 mL of 10 M NaOH
1 mL of 20% SDS
fill up to 40 mL with milliQ water

LB : Luria Browth medium – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
We used a dehydrated culture medium from Pronadisa containing :
Triptone 10.0 g/L
Yeast Extract 5.0 g/L
Sodium chloride 10.0 g/L
Dissolve 25g of the mix in 1L of distilled water. For plates pouring, add 15g agar for 1 L of solution.
Shake and close, then autoclave it to sterilize.

SOC – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
Super Original Catabolite Repressor
Bactotryptone 20g.
Bacto-yeast extract 5g.
NaCl 0.5g.
1M KCl 2.5ml
ddH2O to 1000 ml
Total Volume 1000ml
Adjust pH to 7, with 10N NaOH. Autoclave to sterilize Add 20 ml of 1M glucose before use.

TE – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
10xTE for 1 liter from stock solutions 10 ml 1M Tris-HCl pH 8.0 2 ml 0.5M EDTA pH 8.0 988 ml ddH2O
→ 10xTE is 10 mM Tris-HCl and 1 mM EDTA
For the Tris-HCl use Tris base and adjust to desired pH using HCl.

SOB: Super Original Broth – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
Used in growing bacteria for preparing chemically competent cells Ingredients
0.5% (w/v) yeast extract
2% (w/v) tryptone
10 mM NaCl
2.5 mM KCl
20 mM MgSO4
Per liter:
5 g yeast extract
20 g tryptone
0.584 g NaCl
0.186 g KCl
2.4 g MgSO4
!!! Adjust to pH 7.5 prior to use. This requires approximately 25 ml of 1M NaOH per liter.

CCMB80 buffer – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
For 1L

10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
80 mM CaCl2.2H2O (11.8 g/L)
20 mM MnCl2.4H2O (4.0 g/L)
10 mM MgCl2.6H2O (2.0 g/L)
10% glycerol (100 ml/L)
adjust pH DOWN to 6.4 with 0.1N HCl if necessary
adjusting pH up will precipitate manganese dioxide from Mn containing solutions.
sterile filter and store at 4°C
slight dark precipitate appears not to affect its function

Antibiotics – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
For each antibiotic, solutions of 5 ml have been done and stored at -20°C (09.07.08)

Cloning – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook

Competent cells preparation – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
We followed the protocol coming from OpenWetWare.

Transformation – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook

Thaw competent cells on ice for 30 minutes.
Mix cells and DNA, put back on ice for 20 minutes.
Heat shock: 45 seconds at 42° C.
Add 200 µl of LB broth (without antibiotics).
Incubate at 37° C, 230 rpm for 1 hour.
Plate the cell culture on agar containing the correct antibiotic.
Incubate at 37° C overnight.

Plasmid Purification – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
We use the QIAprep Miniprep Spin Kit.
To get a higher DNA concentration, use only 30 ml Elution Buffer.

Ligation – *2008 – EPF-Lausanne*
http://2008.igem.org/Team:EPF-Lausanne/Notebook
Preparation:
Digest insert and vector.
Example:
700 ng DNA
1 µl NEB Buffer 2 (or depending on enzymes used)
1 µl BSA 10X Buffer
0.1 µl Enzyme 1
0.1 µl Enzyme 2
Complete with water to final volume of 10 µl.
Incubate at 37° C for 1h30 - 2h.
If DNA concentration is low, complete to e.g. 20 µl instead and adjust Buffer volumes accordingly.

After digestion, treat the vector with Antarctic Phosphatase (1 µl AP, 1 µl AP Buffer) to prevent autoligation. Incubate for 20 minutes at 37° C.
Run digested sample on an agarose gel.
Purify the fragments of interest using a Gel Extraction Kit.
Ligation:
For correct reaction calculations, follow DNA Ligation Protocol on OWW
The following simplified protocol worked for inserts up to a couple of hundreds basepairs:
6 µl Insert
1.5 µl Vector
1 µl Ligase Buffer with ATP
0.5 µl T4 DNA Ligase
1 µl Water
Autoligation Control: Prepare the same reaction again but replace Insert with water.
Incubate 30 minutes at room temperature.

Bacillobricks: Introduction of BioBricks™ into *Bacillus subtilis – 2008 – Edinburgh*
http://2008.igem.org/Team:Edinburgh/Protocols/Bacillobricks
Bacillus subtilis is potentially superior to *E. coli* as a host for some projects, for several reasons:
It is much more effective at secreting proteins into the medium, as *E. coli* lacks the Main Terminal Branch of the General Secretory Pathway.
As a Gram positive bacterium, it lacks the toxic lipopolysaccharide (endotoxin) of Gram negative bacteria such as *E. coli*
The cells are considerably larger, making it easier to visualise intracellular components.
B. subtilis forms endospores, a highly stable, heat and dessication resistant resting state which can be stored dry for years or decades, and will then germinate in less than 30 minutes when added to a suitable growth medium.
B. subtilis is not pathogenic and has even been used as a probiotic organism in human foods.
However, standard BioBrick™ vectors do not allow introduction of BioBricks™ into *B. subtilis*. We felt that *B. subtilis* was potentially a suitable host for our 'Edinburgh Process' of conversion of cellulose to starch, due mainly to its ability to secrete enzymes such as cellulases. We therefore investigated two processes to allow introduction of BioBricks™ to *B. subtilis*, either on a plasmid or by integration into the genome. These experiments were carried out mainly by C. French (instructor) and Nimisha Joshi (advisor).
Introduction of BioBricks™ into *B. subtilis* on a plasmid
A standard method of transferring BioBricks™ between *E. coli* and *B. subtilis* would be the use of a shuttle vector which could replicate in both organisms, and in fact the Edinburgh iGEM 2007 team did demonstrate this using the *Lactobacillus* plasmid **pTG262**. pTG262 replicates in both *E. coli* and *B. subtilis* from the same versatile origin of replication, and has a multi-cloning site with EcoRI and PstI sites allowing convenient incorporation of BioBricks™. pTG262 was submitted to the Registry as BBa_I742103, but our understanding, from one team that tried to acquire it from the Registry, was that it is not currently available as the transformation failed. Which brings us to the problem with pTG262 - it does not transform lab strains of *E. coli* with high efficiency, at least not in our hands. (For more information about pTG262 and its ancestors **pWV01** and **pSH71**, see deVos and Simons (1994), and this summary.
To get around this problem, we decided to check whether we could ligate a BioBrick™ into pTG262 and then transform *B. subtilis* directly with the ligation mixture, rather than initially preparing DNA from *E. coli*. Initially we tested this idea using BioBrick™ **BBa_J33204**, bearing the reporter gene *xylE* encoding **catechol-2,3-dioxygenase** (which converts catechol, a cheap, colourless substrate, to bright yellow 2-hydroxy-*cis,cis*-muconic semialdehyde). We have previously demonstrated that this reporter gene works well in *B. subtilis*. The insert was cut out with EcoRI and PstI and ligated with

pTG262 cut with the same two enzymes. The ligation was then used to transform *B. subtilis* using a standard procedure and cells were plated on LB with chloramphenicol (10 mg/l). Chloramphenicol-resistant colonies were obtained, and the presence of *xylE* was confirmed by PCR, but no XylE activity was detected (ie, no visible yellow colour on addition of a drop of 10 mM catechol to the colonies). It was therefore concluded that BioBricks^{TM} can be introduced into *B. subtilis* by this method, and that expression does not occur in the absence of a promoter.
The experiment was repeated using a composite BioBrick^{TM} consisting of **BBa_J33207** (*lac* promoter from *E. coli*) with BBa_J33204 (*xylE* reporter gene). Again, chloramphenicol-resistant colonies were obtained but no XylE expression was observed, suggesting that this promoter was not active in this context, even though the *lacI* repressor gene was not present.
To check that expression could be achieved, we finally turned to the only BioBricked native *B. subtilis* promoter in our freezer, **BBa_J33206** (*B. subtilis ars* promoter, induced by sodium arsenate, cloned as part of the Edinburgh iGEM 2006 arsenic biosensor project). We made a composite BioBrick^{TM} consisting of J33206+J33204, ligated this with pTG262 and transformed *B. subtilis* as above. Since the pSB1A2 vector band could not be separated from the Pars+xylE BioBrick^{TM} on a gel (as they were the same size), it was expected that half of the colonies would contain the correct insert. In fact, one of four clones tested showed evidence of XylE activity (ie, a yellow pigment produced when a drop of 10 mM catechol was added to a colony) and only this clone showed the presence of *xylE* by PCR.
A plasmid of the expected size was detected in plasmid DNA preps from this clone, but since the construct contains a significant amount of native *B. subtilis* sequence, we cannot at present exclude the possibility that the construct may have integrated into the genome at the *ars* locus. This can be tested by PCR to check the size of this locus.
Interestingly, this clone showed **highly sensitive arsenic-dependent induction of XylE activity** and was capable of detecting arsenic at the WHO recommended threshold level of 10 ppb, unlike our previous attempts at non-BioBrick^{TM}-based *B. subtilis* arsenic biosensors. This makes it potentially a useful biosensor for use in developing countries such as Bangladesh, where arsenic in groundwater is a major public health problem, since the biosensor can be stored and distributed in the form of dried endospores, which is not possible with *E. coli*-based biosensors. Here are some sample data from an experiment in which the 'Bacillosensor' clone was incubated overnight in 50% v/v LB, 50% v/v water with 10 mg/l chloramphenicol and various concentrations of arsenic (as sodium arsenate, the most environmentally relevant form of this toxin). Incubation was at 37 C with shaking. The following morning, catechol was added to a final concentration of 0.5 mM and the samples were incubated at room temperature without shaking for several hours. Cells were then removed by centrifugation, and the absorbance at 377 nm (peak absorbance of the yellow product) of a 1/10 dilution was measured against a water blank.
sterile growth medium: 0.027
no arsenic: 0.048
10 ppb arsenic (WHO safety limit): 0.090
25 ppb arsenic: 0.117
50 ppb arsenic (Bangladesh safety limit): 0.184
75 ppb arsenic: 0.228
100 ppb arsenic: 0.309
The yellow colour was clearly visible by eye, making this potentially a useful addition to our range of arsenic biosensors, especially for use in developing countries, where the ability to store and distribute the organism in a dry form as spores will be especially useful.
Integration of BioBricks^{TM} onto the *B. subtilis* chromosome
B. subtilis becomes naturally competent at certain stages of its life cycle, and will readily take up linear DNA from the medium and incorporate it into the chromosome by homologous recombination (Sonenshein *et al*, 1993). We therefore sought to develop a method to prepare linear DNA fragments bearing a BioBrick^{TM} and chloramphenicol resistance gene flanked by upstream and downstream DNA. The first step was to choose a locus of integration. The *amyE* locus, encoding amylase, is commonly used for this purpose, but since we hoped to test cellulose degradation genes, we were reluctant to disrupt a

polysaccharide degradation locus (even though this might have been appropriate for this particular project, since starch hydrolysis would be undesirable in a starch-producing organism). Westers *et al* (2003) have presented a list of 332 genes which are dispensable in *B. subtilis*, and after examination of this list, we decided to incorporate our genes into a locus associated with lytic genes of the prophage PBSX. Our upstream DNA site would be 1 kb of DNA preceding the *xepA* region, and our downstream DNA would be 1 kb of DNA in the *xlyB-spoIISB* region. The inserted DNA would replace the lytic genes *xepA, xhlA, xhlB*, and the 5' end of *xlyB*, but *spoIISB* would be left intact. The following PCR primers were designed:

upstreamF: ggactt ggatcc gccattgg (BamHI)
upstreamR: ggt gaattc tttatactggtcagc (EcoRI)
downstreamF: gaaaaaccc gagctc tatcc (SacI)
downstreamR: gaac ggatcc atgtttattatgg (BamHI)

Additionally, the following primers were designed to clone the chloramphenicol resistance gene of pTG262:

catF: atg ctgcag tctatcccggcaatag (PstI) weak no
catR: ttt gagctc tttccggcgaggc (SacI)

PCR was performed to obtain the three products: upstream DNA with BamHI-EcoRI sites; chloramphenicol resistance gene with PstI-SacI sites; downstream DNA with PstI-SacI sites. These three fragments were then digested: 'upstream' with BamHI; 'cat' with SacI; and 'downstream' with SacI and BamHI. The products were mixed together and ligated in a triple ligation, which was used as template for a PCR reaction using primers catF and upstreamR. This generated a single PCR product of about 4 kb with this structure:

PstI - CML RESISTANCE - SacI - DOWNSTREAM DNA - BamHI - UPSTREAM DNA - EcoRI

This was then stored as **Bacillobrick linear vector 1**. The following protocol was then used to generate linear BioBrick™-containing DNA for transformation of *B. subtilis*:

Digest BioBrick™ (in pSB1A2 or similar vector with EcoRI and PstI, excise the BioBrick™ band from a gel and purify.
Digest linear vector DNA with EcoRI and PstI.
Ligate the two pieces of DNA.
Use a small amount of ligation reaction as template for PCR with primers upstreamF and downstreamR. This should generate a single product of size 4 kb plus the size of the BioBrick™. The product should have this structure:

UPSTREAM DNA - EcoRI - BIOBRICK™ - PstI - CML RESISTANCE - SacI - DOWNSTREAM DNA

Check this product on a gel, and if it looks OK, use it to transform *B. subtilis* according to a standard procedure.

Initially we tested this protocol using BBa_J33204 which carries the promoterless reporter gene *xylE*, as described above. Chloramphenicol (10 mg/l)-resistant colonies were obtained, whereas control transformations without DNA did not yield colonies. XylE activity was not detected, but the presence of *xylE* was confirmed by colony PCR. We then repeated the procedure using a composite BioBrick™ BBa_J33207+BBa_J33204, which has the *xylE* reporter gene under control of a *lac* promoter. The result was the same: transformants were obtained, and the presence of*xylE* was confirmed by PCR, but no XylE activity was detected. Finally, we used composite BioBrick™ BBa_J33206+BBa_J33204, which, as noted above, includes the native *B. subtilis ars*promoter with the *xylE* reporter gene. The result was again the same: chloramphenicol-resistant clones carrying *xylE*, but no XylE activity detected, even with induction by arsenate, which, as noted above, led to detectable XylE activity in the plasmid version of this experiment. We therefore conclude that this method works well for introducing BioBricks™ into the chromosome of *B. subtilis*, but that we have yet to demonstrate expression of genes introduced in this way (although the chloramphenicol resistance gene is clearly expressed). We plan to test the use of stronger *B. subtilis* promoters and alternative loci of integration.

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BABEL: BioBrick™ Assembly with Blunt-Ended Ligation – 2008 – Edinburgh
http://2008.igem.org/Team:Edinburgh/Protocols/BABEL
BABEL is an alternative, restriction enzyme-free method for generating BioBricks™. It is fully compatible with the BioBrick™ 1.0 standard and also with all of the proposed successor standards, and is itself a potential successor to the BioBrick™ 1.0 standard in that it can be used for fully scarless assembly. Babel is based on blunt-ended ligation of PCR products followed by fusion PCR and self-ligation. Here we describe the use of BABEL to generate fully compliant BioBricks™ in pSB1A2, a procedure referred to in our laboratory as 'SOB', or 'Son Of Babel'. Possible extensions of this concept to scarless assembly should be obvious. The 'Son of Babel' procedure is as follows:
Generate a blunt-ended linear version of pSB1A2 by PCR using appropriate primers which contain the entire BioBrick™ prefix and suffix. Primers we have used for this are: sobf2 tactagtagcggccgctgcag and sobr1 ctagaagcggccgcgaattc. Since these primers lie entirely within the prefix and suffix regions, they should work for any BioBrick™ vector. Be sure to use a proof-reading polymerase which will generate a blunt-ended product. We use Kod (Invitrogen), which is as accurate as Pfu and 4 to 5 times faster. Purify your product and store it in the freezer, as it can be used for all of your BABEL cloning experiments.
Design primers for your insert. These should include only the insert, no non-complementary tails (apart from the extra TAA that may be added to the end of a coding sequence BioBrick™), no prefix or suffix. For example, for a coding sequence BioBrick™, the forward primer should start with ATG... and the reverse primer with TTATTA.. (giving the double TAATAA stop codon). Since there is no requirement for prefix or suffix sequence, the primers can be shorter and cheaper than those for standard BioBrick™ cloning.
Perform PCR to obtain your blunt-ended insert PCR product (again, be sure to use a proof-reading enzyme which will give a blunt-ended product - not Taq, which usually leaves an A overhang). Check the product on a gel and purify it.
Ligate your insert PCR product to your linear vector PCR product in a reaction mixture which includes both T4 DNA ligase and T4 Polynucleotide Kinase (PNK). PNK is required to add a 5'-phosphate to the DNA to allow ligation, since standard oligonucleotide primers have a free 5'-hydroxyl group. Fortunately, PNK works well in T4 DNA ligase buffer, which also provides the necessary ATP.
You can now transform with this primary ligation, but since the linear vector PCR product can self-ligate, most of the colonies will probably just be vector, so unless there is an easy screen or selection for those containing the insert, we recommend doing a second PCR as described below (we did make a vector allowing blue-white selection of insert-containing colonies, but it did not generate a standard BioBrick™ vector so would not have allowed direct submission of our products to the Registry).
Perform a secondary PCR reaction using the insert forward primer and vector reverse primer, using a small amount of the primary ligation as a template. This should give you a single PCR product consisting of vector and insert fused together. At this stage, you can also add a ribosome binding site to a coding sequence BioBrick™ by using a variant reverse primer which contains the ribosome binding site sequence. We used primer sobrbs1, ctagtacctcctctagaagcggccgcaattc, which adds ribosome binding site BBa_K1180012, but found that this was much more likely than the standard reverse primer to generate multiple PCR products.
Check the secondary PCR product on a gel, and purify it if it looks OK. Then self-ligate it in a reaction mixture containing T4 DNA ligase and T4 PNK as described above.

Transform *E. coli* with the ligation mixture. Since self-ligation is highly efficient, even with blunt ends, the great majority of colonies should contain the desired construct.
Miniprep a few colonies, check insert size by restriction digest, and sequence to be sure that the ends are intact, with no missing bases.
Advantages and disadvantages of BABEL
Since this method does not use restriction digests, any gene can be cloned straight into a BioBrick™ vector, even if it contains multiple forbidden restriction sites which would prevent cloning using standard BioBrick™ prefix and suffix cloning sites. MABEL (qv) can then be performed to remove the offending restriction sites.
By choice of primer in the secondary PCR, it is possible to recover the insert in both forward and reverse orientations, which is not possible using standard BioBrick™ cloning methods. There are situations where it might be advantageous to recover a promoter or a coding sequence in both the forward and reverse orientation; for example, in the Edinburgh 2007 cell-division-detector project.
Prefix and suffix are in the vector PCR product, and are not associated with the insert. This means that the same procedure can be used for scarless assembly, obviating the problems with fusion proteins which are the main impetus for discussions as to a replacement standard for BioBrick™ 1.0 (see the BioBrick™ Foundation Technical Standards Working Group discussion for July archived here.
One potential disadvantage of BABEL is that multiple rounds of PCR are required, increasing the risk of PCR induced mutations. However, with highly accurate proof-reading polymerases this does not seem to be such a big issue: we have genes which have been through BABEL plus several rounds of MABEL mutagenesis (which works in a similar way) and have detected no undesired mutation events.
User Experience
We used Son-Of-Babel in this project to clone *glgC*, *crtE*, and the maize isoamylase genes in pSB1A2. In all cases we were ultimately successful in recovering the desired clones, but in some cases the process was not as smooth as we might have hoped. In particular, use of the alternate reverse primer with the ribosome binding site pre-added had a strong tendency to generate incorrect PCR products, suggesting that this primer might also be binding spuriously to the other end of the vector sequence. Although we do not have good evidence at this stage, we suspect the GC-rich NotI sites at both ends of the vector may play a part in this problem. Since the NotI sites are no longer used, we understand that they may be removed from the next specification for BioBricks™ (see discussion site link above). Also, in the case of the maize genes, which for some reason were unexpectedly hard to amplify in any case (requiring an extended denaturation time and the presence of 10% v/v glycerol for efficient amplification), a subset of the clones had missing bases at the end suggesting some exonuclease activity, though we only had to sequence three clones in each case to get the correct product.

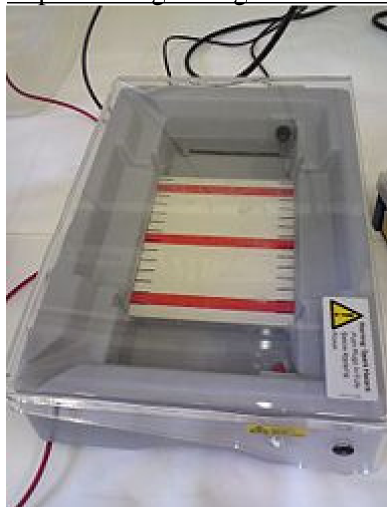
Site-drected mutagenesis using the MABEL protocol – 2008 – Edinburgh
http://2008.igem.org/Team:Edinburgh/Protocols/MABEL
MABEL (Mutagenesis with Blunt-Ended Ligation) is a fast, cheap, simple and reliable alternative to QuickChange for site-directed mutagenesis to remove undesired restriction sites or to add specific mutations. In our project we used MABEL to remove two PstI sites from *crtI* (this was performed by Mr. Douglas Armstrong prior to the official start of the iGEM project), two EcoRI sites from *glgC*, and a PstI site from *cex*, and also to introduce the G336D mutation into *glgC* to abolish feedback inhibition. The MABEL procedure is as follows:
Design two non-overlapping divergent primers around the target site for the mutagenesis. The base to be altered should be at the 5' end of one of the two primers, and the other primer should start at the next base along in the sequence (see example below). Since the primers do not contain non-complementary tails and do not overlap, they can be quite short: 18 base pairs or so should usually be sufficient.
Perform PCR using a very small amount of plasmid DNA as the template, using a fast and highly accurate proof-reading polymerase. We recommend Kod polymerase (Invitrogen) for this, as it is at least as

accurate as Pfu and 4 to 5 times faster. Note that proof-reading polymerases such as Kod will generate a blunt-ended PCR product.
Check the PCR product on a gel and purify it.
Self-ligate the blunt-ended PCR product in a reaction which includes both T4 DNA Ligase and T4 Polynucleotide Kinase (PNK). The PNK is required to add 5'-phosphate groups to the PCR product to allow ligation, since oligonucleotide primers generally have a free 5'-hydroxyl group rather than a phosphate. Fortunately PNK works well in ligase buffer, which also provides the necessary ATP. A typical ligation mixture using Promega reagents might contain 3.5 microlitres PCR product 3.5 microlitres water, 1 microlitre 10 x T4 DNA ligase buffer, 1 microlitre T4 DNA ligase, and 1 microlitre T4 Polynucleotide Kinase, incubated overnight at 16 C.
Transform *E. coli* with the ligation. Since self-ligation is more efficient than intermolecular ligation, the colonies should predominantly contain the desired mutation.
Check a few minipreps for loss of the restriction site. Sequence to confirm that the mutation has been made cleanly with no bases added or deleted. This is a recommended safety precaution, but in all our mutagenesis experiments, we never detected any such undesired changes.
Example
MABEL was used to remove this PstI site at codons 261 and 262 of the *cex* gene encoding exoglucanase of *Cellulomonas fimi*:
gac ttc cgg cag aac **ctg cag** cgg ttc gcg gac ctg ggc gtg gac
Since a silent change was wanted, the desired new sequence was:
gac ttc cgg cag aac **ctg caa** cgg ttc gcg gac ctg ggc gtg gac
Therefore, mutagenic primers were designed with the new A base being at the 5' end of the forward primer, and with the reverse primer starting with the complement of the rest of this codon, so that the two primer ends abut but do not overlap:
Forward A CGG TTC GCG GAC CTG G (anneal 58°C)
Reverse TG CAG GTT CTG CCG GAA G (anneal 58°C)

Use of Edinbrick1 in preparation of BioBricks™ from PCR products – 2008 – Edinburgh
http://2008.igem.org/Team:Edinburgh/Protocols/Edinbrick1
Edinbrick1 was developed by the Edinburgh 2006 iGEM team and was used extensively in both 2007 and 2008 to facilitate BioBrick™ construction. Although it was not developed by the 2008 team, we describe it here to increase awareness of it in case anyone else might find it useful.
Edinbrick1 consists of the standard BioBrick™ vector pSB1A2 with insert BBa_J33207 which includes a *lac* promoter and *lacZ'* minigene encoding the N-terminal 77 amino acids of the beta-galactosidase gene from *E. coli* B. A SacI site, GAGCTC, has also been added at the start of this insert overlapping the XbaI site (TCTAGA) of the prefix. Edinbrick1 is designed to facilitate cloning of PCR products in pSB1A2 to make BioBricks™. The standard protocol we use is as follows:
Design primers for the PCR. One primer should contain a full prefix or suffix, the other a partial prefix or suffix. Thus the PCR product can be cloned as an EcoRI/SpeI or XbaI/PstI fragment. Alternatively, if there is no internal SacI site in the insert, and if you do not have a precise requirement for the first few bases of the insert (as with coding sequence BioBricks™), shorter primers can be used with a SacI site in the forward primer and an SpeI site in the reverse primer.
Perform PCR, test the reaction product on a gel, and purify it.
Set up a single-tube digestion reaction which contains both Edinbrick1 vector and the PCR product with the appropriate set of restriction enzymes: either EcoRI/SpeI, XbaI/PstI, or SacI/SpeI.
When the digestion is complete, purify the DNA using your favorite method (we use glass beads and sodium iodide) and add ligase buffer and T4 DNA ligase.
Transform *E. coli* with the ligation mixture and plate on LB plates with ampicillin, Xgal and IPTG. Blue colonies should be Edinbrick1, white colonies should be pSB1A2 with the desired insert replacing the BBa_J33207 insert of Edinbrick1.

In our hands this procedure is quick and reliable and does not require purification of bands from a gel. One problem with this procedure is that it cannot be used if the BioBrick™ being made includes *lacZ*, as in this case the desired colonies would also be blue. To get around this, Edinbrick2 (BBa_J33204 in pSB1A2) can be used. This includes *xylE* from *Pseudomonas putida* S13, which encodes catechol-2,3-dioxygenase, a reporter gene which converts catechol (a cheap, colourless, soluble subsrate) to bright yellow 2-hydroxy-*cis,cis*-muconic semialdehyde. BBa_J33204 does not include a promoter but we have found that in pSB1A2 (which lacks the flanking terminators of pSB1A3) there is sufficient expression in colonies to detect the activity - just add a drop of 10 mM catechol in water to each colony, and those which are XylE+ will go bright yellow within a few minutes. Of course, if you are confident that *lacZ'* will be expressed in your construct, you can just plate on Xgal and choose the blue colonies.

Agarose Gel – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols



Agarose Gel is made by adding the appropriate mass of Ultrapure Agarose (usually 0.8g) to a duran bottle, and then adding 100ml of TAE buffer to the bottle. 10µL of SyBr Green (10000x) can be added if necessary.
The whole is then microwaved at high for 1 minute, stood for 3 minutes and again heated for 1 minute. Boiling should be avoided and the powder should fully dissolve. It should then be left to cool.
The ends of the gel tray must be taped with autoclave tape and firmly sealed.
When the solution is hand hot the gel can be poured. Pipette around 1ml of the solution up each of the taped ends of the gel tray to prevent leakage. Insert the comb and pour the gel to around 6ml.
Leave to set and remove the comb and tape.

Loading the Gel – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
Mix around 17µL of DNA sample with 3µL of loading dye. The exact amount of DNA varies according to solution strength, but should be around 80ng
We've come to discover that adding 19µL DNA with 1µL loading dye yields crisper bands.
Place the gel tray with gel into the running bay (wells at black end) and add enough TAE buffer to just cover the gel.
Carefully load around 20µL of sample into wells, including some ladder as reference. Normal Agarose gels do not need to have every well filled - there is TAE in them already.

Running a Gel – 2008 – Cambridge

http://2008.igem.org/Team:Cambridge/Protocols
Connect up the red lead to the red terminals and the black lead to the black terminals of the power supply and the running bay.
Make sure the wells are at the black end.
Set the power supply to 80 volts and switch it on.
Check the gel every 20 mins or so to make sure that the gel is running the right way and that the yellow dye (equivalent to 60bp) has not run to the end of the gel.
When the front dye has reached about 3/4 of the way to the end of the gel turn the power supply off and disconnect the leads.

Gel Visualization – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
If no SyBr green has been added the gel needs to be placed in the EtBr solution for 20 mins.
Drain the gel and place in the UV visualiser.
If extracting make sure the prep UV button is pressed, and take a picture of the gel.
To extract the gel slide the light tray out, **ensure shielding for all who are involved**, and carefully cut out the gel fragment, usind prep UV.
SyBr Green gels can also be visualised using special visualisers.

Gel Extraction – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
Link to list of extracted parts
We found we could not extract enough DNA from the registry, so we are upgrading the extraction to the "bigger, better, faster, stronger" method.
Warm 50µL of EB in Eppendorf tubes at 50°C and add 4 punched spots.
Keep it at 50°C for 20mins
Spin down for 3 minutes at 13,000 g.
Warm for 10mins
Spin down again for 3mins.
Pipette off the supernatant which should contain DNA.
We then confirmed with PCR.

Competent Cells Stocks – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
Chemically competent cells are made by growing overnight in 200ml LB in a shaking incubator at 37°C. 200ml are spun down at 3800rpm for 8 mins, resuspended in 20ml $CaCl_2$, spun down again and resuspended in 4ml $CaCl_2$. Again spun down (6500 rpm for 5 mins)and resuspended in 4ml 60%glycerol, spun down and resuspended in 2ml 60%glycerol. Finally they are aliquoted (100µL) into stock tubes and flash frozen in liquid nitrogen.
To recover cells thaw the tubes on ice, spin down and resucpend in 100µL ice cold $CaCl_2$.
Electroporation competent TOP10 and DH5α cells were made by growing overnight in 200ml LB in a shaking incubater at 37°C. 100ml were spun down at 3800rpm for 8 mins, resuspended in 25ml SDW, spun down again and resuspended in 2ml SDW. Again they were spun down (6500 rpm for 5 mins)and resuspended in 2ml 60%glycerol, spun down and resuspended in 2ml 60%glycerol. Finally they were aliquoted (100µL) into stock tubes and flash frozen in liquid nitrogen.
To recover cells thaw the tubes on ice, spin down and resucpend in 100µL ice cold SDW.

Dephosphorylation – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
Add appropriate volume of 10x SAP buffer to restricted fragments.

Add 1ul of Shrimp Alkaline Phosphatase for every pmol of DNA ends. For example, the cut plasmid pSB4C5, which is about 3kb, requires 1 uL of SAP for every ug of vector.
Incubate for 1 hour at 37°C.
Heatkill enzyme for 15 mins at 80°C

E-Gel – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols

Our E-gel machine from invitrogen
E-Gel is used to confirm size of products, but not for extraction.
The E-Gel must be prepared by placing inside the Gel Dock and running the Pre-run for 2 minutes.
The Gel can then be loaded. 19μL of DNA, made up with SDW as necessary, is added to each well, and 1μL of loading dye is also added. Adding less dye allows a more sharply defined band.
All unloaded lanes must be filled with SDW, to ensure correct running.
The gel is then run, using the Run program, for 26 mins.
The gel can then be visualised in the UV visualiser, for EtBr, or using the E-Gel visualiser, for SyBr Green.

Fast Digestion – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
The following are added to a 1.5μL eppendorf:
2μL plasmid DNA
2μL 10x fast digest buffer
enough water to make up to 19μL (18μL if double digest)
1μL of each fast digest enzyme
If using PCR product the whole is made up to 30μL, using 10μL DNA and 3μL buffer.
The tubes are then incubated at 37°C for 5mins and then the enzyme inactivated at 80°C for 15mins
Fast Ligation
To calculate the amount of insert you need, use the equation:
2CB/AD
A = the length of the vector backbone
B = the length of the insert
C = ng of the backbone you will use (concentration*volume) -> usually between 500ng to 1ug
D = concentration of the insert
The result will be the volume of insert you should add to achieve a 2:1 molar ratio of insert to backbone.

The following are added to a 1.5μL eppendorf:
xμL vector DNA
xμL gene DNA

1.5µL 10x fast digest buffer
1.5µL 10mM ATP
enough water to make up to 15µL
1µL of fast ligase enzyme
The tubes are then incubated at room temperature for 5mins and then the enzyme inactivated at 75°C for 15mins
We have now got a different fast ligation kit.
Now the following are added to a 1.5µL eppendorf:
xµL vector DNA
xµL gene DNA
4µL 15x fast digest buffer
enough water to make up to 19µL
1µL of T4 fast ligase enzyme
The tubes are then incubated at room temperature for 5mins.

Flame Photometry – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols

Chris attempts to unravel the secrets of the Flame Photometer

Set Up

Make sure discharge pipe (on right hand side) is placed in something to collect discharge, and valve is not completely shut.
Check clear tube is connected to compressor OUTLET, and securely attached.
Turn on compressor.
Gauge on back of photometer should read 12psi.
Check gas pipe is attached at tap.
Turn on gas.
Wait 1 minute.
Turn on photometer. It should click, and orange "flame on" light should come on.
Place beaker of distilled water in the photometer tray, and make sure thin tube reaches water.
Wait 15 minutes before taking any readings.
Use "blank" dial to adjust reading to 0 for distilled water.

Taking readings

Place thin tube into sample, far in, as the liquid will be taken up quite rapidly.

Wait for reading to stabilise (reading may continue to fluctuate at high values) and take reading.
Place tube in distilled water beaker between each sample, waiting until reading returns to 0.

In case of compressor blow-out

Turn off gas at tap.
Turn off photometer.
Turn off compressor.
Redo set up steps, with 15 minute waiting time reduced to 5, as the equipment will already be almost up to temperature.

In case of wildly fluctuating "zero" readings

Tap digital display.
Allow tube to dry (take out of SDW) before loading next sample.

Gel DNA Recovery (Zymoclean) – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
Add 3 parts ADB Buffer to 1 part Gel volume
Incubate 55°C for 5-10 minutes, until gel melts
Place solution into Zymo-Spin column and then into a collection tube
Spin for 30 seconds. Empty collection tube when necessary
Add 200µL Wash Buffer. Spin for 30 seconds. Repeat wash step.
Place column into Eppendorf. Add 6-10µL SDW to elute DNA. Add more SDW to elute more DNA - normally 20µL.

Glycerol Stock (Modified Version) – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
Inoculate single colony growing on plate into 10 ml LB with appropriate selection. Grow overnight.
Add 100µL culture to 500µL sterile 80% glycerol stock (need to sterilize using sterile .2µm filter and syringe).
Vortex briefly
Freeze at -80 degrees.
Media Preparation
NOK 2.0 (No potassium nutrient broth)
Measure out:
6.0g Na_2HPO_4
3.0g NaH_2PO_4
0.5g NaCl
1.0g NH_4Cl
0.0147g $CaCl_2$
0.246g $MgSO_4.7H_2O$
2.0g Glucose
1.0g NOK Amino Acid Powder (see below)
Add to 1dm3 (1l) of SDW and shake well. Note heating 40-50°C may be necessary to achieve full dissolution of solutes. There may be a small amount of indeterminate crud remaining at the end of this process; this is entirely normal and can be ignored. This mixture should then be filter sterilised into 50ml separate tubes (reduces contamination risk of whole batch). Keep refrigerated or frozen until needed.

NOK Amino Acid Powder composition

This is a solid homogenized mixture of all solid amino acids (except possible GluR0 channel agonists alanine, threonine, serine, glycine, glutamine & glutamate.)
Current batch is stored at room temperature on the chemical shelf near the gel area. To make a new batch, measure out 0.5g of each of the following (L-forms ONLY):
Arginine
Asparagine

Aspartic Acid
Cystein
Histidine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Proline
Tryptophan
Tyrosine
Valine

Bacillus media – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols
10X Medium A base:
Yeast extract 10g
Casamino acids 2g
Distilled water to 900mL
Autoclave, then add :
50% glucose, filter sterilized 100mL
10X Bacillus salts:
$(NH_4)_2SO_4$ 20g
Anhydrous K_2HPO_4 139.7g
KH_2PO_4 60g
Tri-sodium citrate 10g
$MgSO_4 \cdot 7H_2O$ 2g
Water to 1000mL
Medium A
Sterile distilled water 81mL
10X Medium A base 10mL
10X Bacillus salts 9mL
L-Tryptophan (11mg/mL) 0.1mL
Medium B
Medium A 10mL
50mM $CaCl_2 \cdot 2H_2O$ 0.1mL
250nM $MgCl_2 \cdot 6H_2O$ 0.1mL

Important: Autoclave Medium A base before adding glucose, and autoclave Bacillus salts. Store aliquots of 10X Medium A base 10mL and 10X Bacillus salts 9mL and keep them in the fridge, never use them twice to avoid contamination
Oxygen Electrode
For the protocol see http://www.rankbrothers.co.uk/download/digioxy.pdf

Plasmid Miniprep (Zyppy) – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Protocols

Plasmid Miniprep kit
Add 100µL 7x Lysis Buffer to 600µL Cell Culture. Invert 4 to 6 times
Add 350µL cold Neutralization Buffer. Invert 4 to 6 times
Centrifuge 13,000g for 2 minutes
Transfer supernatant to Zymo-Spin column
Place column in a collection tube and spin for 15 seconds. Discard flow through
Add 200µL Endo-Wash Buffer. Spin for 15 seconds
Add 400µL Zyppy Wash. Spin for 30 seconds. Repeat this step one more time for better purity.
Transfer column to clean Eppendorf. Add 30 to 100µL Elution Buffer to the column and spin for 15 seconds to elute DNA
Primer Preparation and Storage
Sigma Primers arrived as tubes of dried oligos
Suspend in sterile distilled water (SDW) to form a 100µM stock solution - stored at -20°C
10µM aliquots taken using 10µL stock and 90µL SDW - stored at 4°C
PCR

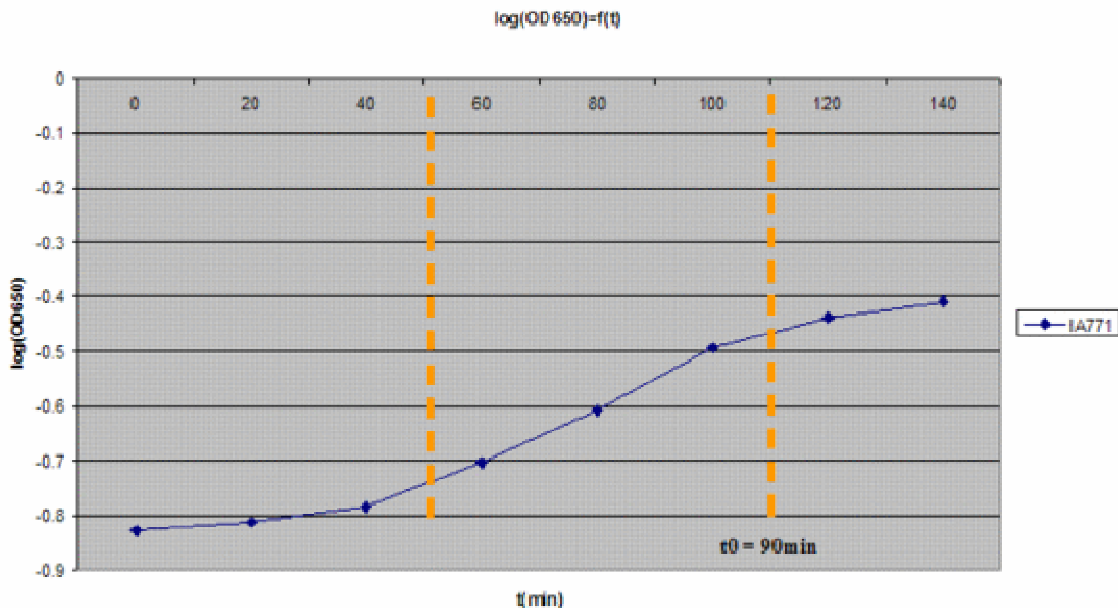
Our Piko PCR machine provided by Finnzymes
To make 50µL of PCR mix the following were added to Eppendorf tubes:
5µL of DNA in EB buffer
2.5µL of each primer
25µL of 2x Finnzymes mastermix
15µL of sterile distilled water
These values can be adjusted for any volumes of final mixture.
The total final volume is transferred to thin walled tubes (may need to be spun down) and then placed in a PCR machine and the appropriate program run. When using primers designed to produce longer product than original gene touchdown cycles should be run with annealing at the initial binding Tm and then normal cycles at the full length Tm.
Making your own 4x Master Mix
Per 20µl Reaction, Add in this order:

Sterile Distilled Water
20μL - 5.2μL - volume of template and primer DNA (12.8 μL if 2μL of DNA)
4μL of 5x HF buffer
0.4μL of 10mM dNTPs (0.1μL of each)
0.6μL of 100% DMSO (3% rxn volume)
0.2μL of Phusion Polymerase
E.coli transformation

Plates with transformed cells
First chemically competent cells are made
Cells are grown for 1-2 hours in LB and pelleted (at 4000rpm for 8 min.)
Pellet is resuspended in 1mL 50mM CaCl
Again cells are pelleted.
Pellet is resuspended in 200μL 50mM CaCl
To transform cells
To chilled tubes add 5μL of plasmid DNA + EB solution to 50μL of Cells (Chemically competent cells)
Chill on ice for 30 minutes
Heat shock at 42°C for 60 seconds
Chill on ice for 2 minutes
Add 200μL of SOC
Incubate at 37°C for 2 hours
After transformation cells need to be grown under selection conditions.
Qiagen QIAquick PCR Purification Kit
(from Qiagen QIAquickspin Handbook)
This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the new MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge. Notes:
Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
All centrifuge steps are at 13,000 rpm (~17,900 x g) in a conventional
tabletop microcentrifuge.

Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.
For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
Place a QIAquick spin column in a provided 2 ml collection tube.
To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.
Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Making Bacillus Subtillis competent – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Bacillus_subtilis_transformation
Grow one blank plate of *Bacillus subtilis* (or several if you want to transform different strains) for 20 hours at 37ºC (plate been kept on the bench for several days would be better)
Inoculate about 12mL of medium with several colonies. Mix the contents of the tube. Check with OD650. Start OD should be between 0.1 and 0.2. Be careful to pipette 0.8mL of this mixture into the cuvette to measure and dispose of it after measurement to avoid contamination in the main mixture.
Incubate at 37ºC with vigorous shaking. Read the OD650 every 20min (never keep the solution you used for measuring!)
Plot log(OD650) in function of time. After a brief lag, you should observe a exponential increase. After awhile, it will leave the exponential growth; the moment at which it leaves the exponential path is denoted as t0 (3 on the graph). It should take about 100min and the OD should be between 0.35 and 0.55.



At t0, incubate for 90 minutes at 37ºC with vigorous shaking.
Transfer 0.05mL of this culture into 0.45mL of pre-warmed Medium B in an Eppendorf tube. You have to prepare one tube for each transformation, plus an extra tube for a DNA-less control.
Incubate the diluted cultures at 37ºC with shaking for 90min. At this moment, the cells are HIGHLY COMPETENT.
To check for competency, you can look at cells under the microscope; competent cells are very motile.
Transforming Bacillus Subtillis – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Bacillus_subtilis_transformation
Spin Eppendorf tubes containing cells. Remove 400µL of liquid to keep only 100µL of the culture (to concentrate cells). Re-suspend the cell pellet in the remaining culture.
To transform from competent glycerol stocks, spin the tube at about 1600rpm for 20min, remove the supernatant (glycerol), and add 100µL of pre-warmed medium B.
Mix the cells thoroughly.
Add 0.6µg of DNA to the competent cells.
Incubate for 30min at 37ºC with shaking.
Plate 100µL of transformed cells onto selective agar.
Glycerol Stocks for Bacillus Subtillis – 2008 – Cambridge
http://2008.igem.org/Team:Cambridge/Bacillus_subtilis_transformation
To freeze competent Bacillus cells, spin down the fresh competent cells to obtain a pellet.
Remove all supernatant.
Re-suspend cells in 500µL 60% glycerol.
Freeze tubes at -80ºC.

Plates preparation – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Autoclave LB medium with 2% agar.
Cool (at about 50°C, to prevent agar polymerization).
Before pouring the plates add antibiotic (Ampicillin 1000x [ ] or Kanamicin 200x [ ]).
Put about 20ml of medium per plate.
Leave it solidify and store at 4°C.

Up
Biobricks amplification – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Puncture a hole through the foil with a pipette tip (wash it everytime with bleach-distilled water-EtOH 95%) into the spot that corresponds to the Biobrick™-standard part that you want.
Soak the paper in 5µl of TE buffer.
Rest for 20 minutes at 50°C.

Up
Transformation – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Thaw the competent cells in ice (do not refreeze).
Dispense 80µl of cells into microfuge tubes on ice.
Add 0.1-0.3µg of plasmidic DNA or the respective amount of the ligation reaction.
Keep on ice for 30min.
HeatShock at 42°C for 60sec without agitation.
Keep on ice for 2min.
Add 0.8ml of LB medium at room temperature.
Incubate at 37°C for 1hr with agitation.
Pellet the cells and discard most of supernatant, leaving about 100µl.
Streak on plates containing appropriate antibiotics.
Incubate the plates overnight at 37°C.

Up
Inoculation – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Put 5 ml of LB media in a 50ml tube.
Add the appropriate antibiotic.
Pick one colony from the plate with the inoculation loop
Put cells in solution.
Incubate the plates overnight (12 hours) at 37°C.

Up
Miniprep – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Pellet for 10 mins at 4400 rpm and discard most of supernatant.
Resuspend pelleted bacterial cells in 250µl of Buffer P1 and tranfer to a microcentrifuge tube.
Add 250µl of Buffer P2 and mix thoruoghly by inverting the tube 4-6 times.
Add 350µl of Buffer N3 and mix immediately and thoruoghly by inverting the tube 4-6 times.
Centrifuge for 10 min at ~18,000 x g in a table-top microcentrifuge.
Apply the supernatant (from step 4) to the QIAprep spin column by decanting or pipetting.
Centrifuge for 30-60 s. Discard the flow-though.
Recommended: Wash the QIAprep spin column by adding 0.5 ml of Buffer PB and centrifuging for 30-60 s. Discard the flow through.
Wash QIAprep spin column by adding 0.75ml of Buffer PE and centrifuge for 30-60 s.
Discard the flow through and centrifuge for an additional 1 min to remove residual wash buffer.
To elute DNA, place the QIAprep column in a clean 1.5 ml microcentrifuge tube. Add 30µl of Buffer EB (or water) to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

Up
Digestion reaction – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

E/S cut
enzyme 1 --> ECO R1
enzyme 2 --> SPE
buffer --> ECO R1
X/P cut
enzyme 1 --> Xba
enzyme 2 --> Pst 1
buffer --> 3
S/P cut
enzyme 1 --> SPE
enzyme 2 --> Pst 1
buffer --> 2
E/P cut
enzyme 1 --> ECO R1
enzyme 2 --> Pst 1
buffer --> ECO R1

Mix:
0.5 µl of BSA
0.5µl of each enzyme
3µl of buffer
5µl of DNA
20.5µl of H2O(most pure)
Spinning down.
1h at 37 °C.
20 min at 80 °C to block the enzymatic action.
Put in ice.
Start run preparation.

Up
Gel preparation – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 2

150ml of Buffer TBE 1x.
Add the appropriate quantity of Agarose for the desired thickness
0.7%= 1g of agarose
0.7-1% general(from 200 bases to 3Kb)
0.5% big pieces (6-7 Kb)
2-3% small pieces (100 bases)
Microwaves for 2 min.
Cool down under flowing water.
Add 10µl of EtBr.
Prepare the gel tray and set in place the wide-thoot comb.
Pour the gel in the gel tray (work in the hood for safety purpose)
Get rid of bubbles and let solidify.

Up
Electrophoretic run – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 2

Put the solidified gel in the apposite run container.
Check that TBE fully covers the gel.
Gently extract the comb.
Add the Loading buffer in the digested DNA.
Loading: substance that loads the sample with dye so that we can see the evolution of race and weight the DNA so that deposits in the well; concentrated at 6x.
Make the deposit in the wells of the samples and loading of reference (a part of loading should be prepared to scale by reference).
Close the container.
Start the run:
50/100 V --> until separate bands
120 V --> until half run
140 V--> until end of run.

Up
Gel extraction – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 2

Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).
Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.

After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).
Add 1 gel volume of isopropanol to the sample and mix.
Place a QIAquick spin column in a provided 2 ml collection tube or into a vacuum manifold.
To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min or apply vacuum to the manifold until all samples have passed through the column. Discard flow-through and place the QIAquick column back into the same tube.
Recommended: add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min or apply vacuum. Discard flow-through and place the QIAquick column back into the same tube.
Centrifuge the column in a 2 ml collection tube (provided) for 1 min at 18,000 x g.
Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
To elute DNA, add 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl of elution buffer to the center of the QIAquick membrane, let the column stand for 1 min and then centrifuge for 1 min.
If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Up
Ligation reaction – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Ligation with one insert and one vector --> f.v.=20µl
8µl of insert
4µl of vector
1µl of T4 ligase
4µl of Buffer 5X
3µl of H_2O mQ
Ligation with two insert and one vector --> f.v.=30µl
4µl of vector
8µl of insert 1
8µl of insert 2
1µl of T4 ligase
6µl of Buffer 5X
3µl of H_2O mQ
Conservation:
40 min at Tamb, or
3 hours at 15°C

Up
Chemiocompetent cells– 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Take some colonies of DH5α cells from a fresh streaked plate and inoculate into 125ml of Soc medium.

Grow the cells overnight at 25°C (it is advised to grow them slowly in order to have them better synchronized). It takes approximately 20 hours. It is advisable to grow the cells at 25°C overnight and then to shift them at 37°C. Bacteria are ready for harvesting when OD600 is between 0.37 and 0.4. Higher OD will lead to less competent cells (it is important to harvest the bacteria when they are still in the logarithmic phase of growth).
Spin down the cells (at maximum speed) at 4°C for 10 min.
Re-dissolve the pellet in 40ml of Transformation buffer.
Incubate on ice for 10 min.
Spin down the cells (at maximum speed) at 4°C for 10 min.
Re-dissolve the pellet in 10ml of Transformation buffer.
Add 700µl of DMSO.
Aliquot (200µl) and freeze at -80°C.

Up
Mediums and buffers – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Soc medium
To prepare 1l of SOC medium dissolve in ultrapure water:
Tryptone 4g
Yeast extract 1g
1M NaCl 2ml
1M KCl 0.5ml
5M NaOH 200µl to adjust the pH to 6.8
After autoclaving add 2ml each of 2M Mg-salt (1M MgSO4 and 1M MgCl) and 2M glucose.

LB medium
To prepare 1L of LB medium dissolve in ultrapure water:
Tryptone 10g
Yeast extract 5g
NaCl 10g
5M NaOH 200µl
Autoclave and store at room temperature.

Transformation Buffer
(always made fresh)
To prepare 100ml dissolve in ultrapure water:
(15mM) CaCl2 0.2205g
(250mM) KCl 1.864g
(10mM) Pipes 0.302g
Adjust pH with KOH to 6.7.
Add (55mM) MnCl2 (0.89g of MnCl2x2H2O).
Filter with 0.22µm filter.

M9 medium
Dissolve 56.4g in 1l of distilled water.
Autoclave for 15 min at 121°C.
This convenient 5x concentrate can be stored and diluted as needed to prepare 5l of 1x M9 minimal salts.
For M9 minimal medium:

Aseptically dilute 200ml of M9 minimal salts, 5x[ ] with 800mL of sterile water, if necessary, cool to 45-50°C.
Aseptically add 20ml of sterile 1M glucose and 2mL of sterile 1M magnesium solfate to prepare 1l of M9 minimal medium.
If desired aseptically add 0.1ml of 1M sterile calcium chloride to the M9 minimal medium. M9 minimal medium may also be supplemented with the appropriate amino acids.

Up
Antibiotics stocks preparation – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Ampicillin
Dissolve ampicillin in ultrapure water 100mg/mL (stock 1000x [ ], working concentration100 µg/mL). Aliquot and store at -20°C.

Kanamicin
Dissolve Kanamicin in ultrapure water 10mg/mL (stock 200x [ ], working concentration 50µg/mL). Aliquot and store at -20°C.

Up
IPTG stocks preparation – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

IPTG (Isopropyl β-D-1-thiogalactopyranoside) 100mM stocks preparation for Plac induction (IPTG MW=238.31):
0.476g in 20ml of ultrapure water. Working concentration 1mM

Up
Fluorescence test – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

Growth O/N for 15h in LB (5ml) of:
transformed E. coli with appropriate antibiotic;
E. coli.
All measures must be done in M9 with OD=1.2.
The day after, in the morning, measure OD.
To obtain OD=1.2:
centrifuge 4ml of bacterial culture at 4400rpm for 3min at 25°C;
discard the supernatant;
resuspend the cell pellet in 6ml of M9 medium with glucose and appropriate antibiotic (1000x [ ]).
Measure OD: adjust OD to 1.2 through further dilution or cell growth.
Adjust PMT offset with untransformed E. Coli.
Test culture fluorescence before IPTG induction.
IPTG induction:
centrifugate bacteria culture at 4400rpm for 3min at 25°C;

discard the supernatant;
resuspend cell pellet in M9 medium and 2mM IPTG with appropriate antibiotic (1000x [ ]);
Incubate at 37°C.
Test fluorescence after 10min, 20min, 30min, 1h, 2h.

M9 supplemented media – 2008 – Bologna
http://2008.igem.org/Team:Bologna/Biosafety#Protocols

Biosafety level: 1

For 1L of 1X media
200 mL 5X M9 minimal salts
Dissolve 56.4 g Bacto M9 minimal salts, 5X from Difco in 1L H2O
Separate into 200 mL aliquots
Autoclave to sterilize. 121°C for 15 minutes.
34 mL 10 mg/mL thiamine
Dissolve 10 mg per mL of H2O
Use a 0.22 µm filter to filter-sterilize
10 mL 40% glycerol
Add 80 mL glyerol to 120 mL of H2O
Autoclave to sterilize
20 mL 10% Casamino acids
Dissolve 50 g Bacto Casamino acids from Difco in 500 mL H2O
Autoclave to sterilize
2 mL 1M MgSO4
Dissolve 24.65 g MgSO4·7H2O in 100 mL H2O
Autoclave to sterilize
100 µL 1M CaCl2
Dissolve 14.7 g CaCl2·2H2O in 100 mL H2O
Autoclave to sterilize
733.9 mL H2O
Sterilize deionized water in autoclave
Combine above solutions using sterile technique. (May notice some precipitation during preparation but precipitate should go back into solution once volume is brought up to 1L with sterile H2O.) Add antibiotic as appropriate and store at 4°C

Testing the activity of biobrick T9002
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Testing_the_activity_of_biobrick_T9002
T9002 is a luxR based receiver construct with a GFP reporter. The ability of DH5α transformed with T9002 to detect AHL and produce GFP was tested.

Grew up two culures (DH5α transformed with T9002 and untransformed DH5α)in LB and incubated overnight (37°C, shaking) to log phase
Made stock solution of AHL: Ethyl acetate acidified with glacial acetic acid (0.01% v/v)(1µl acetic acid in 10ml ethyl acetate)AHL added to this solution to give a concentration of 10^{-1}M (0.0021g AHL in 100µl)
From stock solution of AHL , made up the following dilutions:
10^{-4}M (1µl stock AHL in 1ml dionised H_2O)
10^{-6}M (1µl stock AHL in 100ml dionised H_2O)
Overnight cultures were OD'd, and diluted in LB to an OD_{600} of ~0.15

In 0.5ml eppendorfs:
10^{-6}M T9002 ---> 2µl 10^{-4}M in 200µl T9002 cell suspension
10^{-6}M DH5α ---> 2µl 10^{-4}M in 200µl DH5α cell suspension
10^{-8}M T9002 ---> 2µl 10^{-6}M in 200µl T9002 cell suspension
10^{-8}M DH5α ---> 2µl 10^{-6}M in 200µl DH5α cell suspension
Eppedorfs were vortexed gently to mix
Tubes then incubated at 37°C, shaking for 5 minutes
10µl of cell suspension was put on a microscope slide and covered with a coverslip.
Bright Field and GFP images were taken of each slide.

Transformation using electroporation
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Transformation_using_electroporation
Sterilization of the cuvettes:
Open the cuvette and put both (lid and cuvette) into a UV light machine (CL-1000 Ultraviolet Crosslinker from UPV)
Give 1200 "energy" (press "start" and wait for the end of the countdown)
Electroporation
Prepare 950 µl sterile LB broth in a 1.5 ml tube and put in on ice
Put the cuvettes at least 2 mins before adding the bacteria on ice
Thaw the electric competent cells (*E. coli* DH5α) on ice (here 50 µl cells)
Add the DNA to the cells (for good working DNA in high concentration like pUC19 with 10 pg/µl use 1 µl; for BioBrick DNA use 5 µl if it was prepared with 10 µl of water)
Choose program Ec1 (BIORAD MicroPulser) and change the view to "ms" time
Dry the wet cuvette with a paper towel and put it into the machine
Give the pulse and notice the time: A pulse between 5-6 ms is a good value
Add immediately 950 µl ice cold LB broth and transfer everything back into the tube
Incubate for 1 h at 37°C 225 rpm
Plate a part of the solution or spin the cells down, remove most of the supernatant (800-900 µl), resuspend them and plate all on a LB plate containing antibiotics
Incubate the plates overnight at 37°C

Transformation with heat shock
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Transformation_with_heat_shock
Thaw the chemical competent bacteria (50 µl *E. coli* DH5α) on ice
Add the DNA by pipetting it directly in the middle of the bacteria solution und mix by stirring gently with the pipette tip
Incubate 20 min on ice
Heat shock: incubate 60 s at 42°C in a water bath
Incubate 2 min on ice
Add 250 µl SOC broth (stored in the freezer; thaw before and check for no contamination!)
Incubate for 1 h at 37°C 225 rpm
Plate 50 µl and 100 µl on two LB plates with antibiotics
Incubate the plates at 37°C overnight. Store the remaining bacteria in the fridge.

Resuspending Primers
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Resuspending_Primers

Before resuspending the primers, they need to be ordered. Giving a name to the primer, start with "iGEM" (e.g. iGEM_VF2). Collect the data about the primer (sequence, T_m , concentration…) in the lab book and on the N-drive in the "primers"-folder.
The primers were lyophilized before sending. To resuspend the primers, distilled water is added to a concentration of 100 µM. The right amount of water to be added is denoted on the sheet that was send with the primers. Keep these sheets in the black folder.
After adding the water, vortex the tube and let it incubate for 5 minutes at room temperature to dissolve the DNA. Vortex again and decant several µl in another tube for normal usage. Never use the original tube for preparing PCRs!!! Store the primers in the freezer.

Media
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Media

LB broth or agar (200 ml)

Component	Concentration [%]	Amount for 200 ml
Yeast Extract	0.5	1 g
Bactotryptone	1	2 g
NaCl	1	2 g
Agar	For plates 2	For plates 4 g

Swimming agar medium

Component	Concentration [%]	Amount for 200 ml
Bactotryptone	1	2 g
NaCl	0.5	2 g
Agar	various	

SOC medium

Component	Concentration [%]	Amount for 95 (100) ml
Bactotryptone	2	2 g
Bacto yeast extract	0.5	0.5 g
NaCl	0.05	0.05 g

Mix the three components with 95 ml distilled water and autoclave it. Just before using, add (filter sterilized):
0.5 ml 2 M MgCl2
2 ml 1 M Glucose

Preparation of electro competent cells
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Preparation_of_electro_competent_cells
5 ml LB were inoculated with a colony from a freshly streaked *E. coli* DH5α and incubated overnight at 37°C and 225 rpm
4x 1 l flasks, each containing 100 ml LB, were inoculated with 1 ml overnight culture per flask, then incubated as before until the OD_{600} reached 0.3 to 0.4 (ca. 3-4 h)
Cell tubes were decanted into 8x 50 ml falcon tubes and centrifuged at 2500 g in a Beckmann CS-6R bench top centrifuge for 10 min at 4°C. All manipulations henceforth were carried out with the cells on ice.
After discarding the supernatants, each cell pellet was resuspended in 50 ml ice cold sdw (sterile distilled water) and centrifuged as before.

After discarding the supernatants, the cells were resuspended in the remaining liquid and pooled into 4 tubes, then resuspended in 50 ml ice cold sdw and centrifuged as before.
After discarding the supernatants, the cells were resuspended in the remaining liquid and pooled into one tube, then resuspended in 50 ml ice cold sdw and centrifuged as before.
The single cell pellet was resuspended in 1.2 ml ice cold 20 % glycerol and divided into 50 µl aliquots, which were rapid frozen in liquid nitrogen and stored at -80°C.

DMSO stocks
http://2008.igem.org/Team:BCCS-Bristol/Protocols-DMSO_stocks
Make an overnight culture of the bacteria in LB broth (If the strain carries a resistance gene on a plasmid, you have to use LB with antibiotics)
Next morning, put 70 µl DMSO into a cryotube that you labelled with the strain's name and the date
Add 930 µl overnight culture
Incubate immediately for 30 min on ice
Store the DMSO culture in the -80°C freezer
In the evening (or in one of the following days), make a streak out of the stock to see whether the bacteria are ok

Colony PCR
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Colony_PCR
Components of the PCR Mix:
Template DNA: Colonies of a transformation plate
Primers: for BioBricks the primers VF2 (forward) and VR (reverse) are used
Master Mix (MM): "Taq 2x Master Mix" from NEB # M0270L
PCR-Mix for one reaction (20 µl):

Volume	Component
10 µl	MM
9.6 µl	sterile water
0.2 µl	forward primer (100 µM)
0.2 µl	reverse primer (100 µM)

Prepare additionally to the number of colonies you want to test one reaction for the negative control and one reaction as reserve since it might be not enough solution in the end!
PCR instructions:
Touch with a pipette tip the colony on the plate and try to get as much bacteria as possible
Touch now the bottom of a PCR reaction tube and turn the tip several times
Take this tip and make a small streak out on a plate (LB + antibiotics) and incubate the plate at 37°C during a day or overnight
Mix primers and water
When everything is ready, take the MM out of the fridge, add it to the primers and the water and bring the MM as soon as possible back into the fridge
Mix the PCR mix and give 20 µl in each PCR reaction tube (either using a new tip or ensure that you don't get in contact with any DNA)
Close the tubes properly, mix them gently with the vortexer and spin them shortly down
Start the PCR
PCR programme:

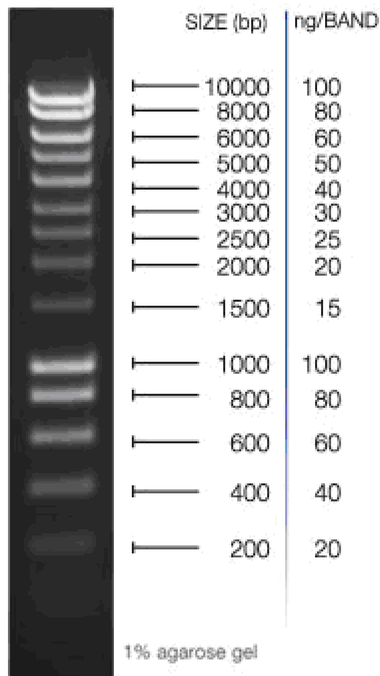
Initial denaturation	95°C	5 min (if it's not a colony PCR, take only 2 min)
Denaturation	95°C	30 s
Annealing	55°C*	30s

Extension	68°C	1 min/1 kb fragment length
Final extension	68°C	2 min

* or the appropriate temperature for the primers

Agarose Gel Electrophoresis – 2008 – BCCS Bristol
http://2008.igem.org/Team:BCCS-Bristol/Protocols-Agarose_Gel_Electrophoresis
Gel preparation:
For a thick gel in a small chamber, boil 50 ml 0.5x TBE in the microwave (for a thinner gel to insert only 5 µl sample in each well 40 ml are sufficient)
Cool the solution until you can touch it with your hands for a longer time
Add 0.5 µl ethidium bromide per 10 ml TBE and mix while avoiding air bubbles
Pour the gel into a chamber with a comb that you prepared before
Let the gel cool down and become solid (15-20 min)
Sample preparation:
Add 10 % "Sample Loading Buffer" (BIORAD) to the sample
Mix gently and spin shortly down
Loading the gel:
Remove the comb and put the gel with the slide into a chamber (the wells need to be on the end with the black pole!!!)
Fill the chamber with 0.5x TBE until the gel is covered
Use 5 µl HyperLadderI (BIOLINE)
For colony PCR, put 5 µl of each reaction in the well
Close the chamber with the lid (black pole to black=negatively charged and red to red=positively charged…)
Run the gel with 80-100 V

The Sample Loading Buffer contains two dyes: Bromophenol blue runs at ~300 bp and Xylene cyanol FF runs at ~4 kb.



Recipe for Competent Cells – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Materials
Frozen stock DH5a
250mL Erlenmeyer Flask (w/ caps)
LB tubes
Sterile LB
Instructions
1. From frozen stock DH5a, inoculate 5mL LB tubes and grow overnight at 37˚C
2. Autoclave 250 mL Erlenmeyer flask with 50 mL LB broth for tomorrow. Don't forget to loosen to cap if you are not using tin foil.
3. Next morning: add culture into the autoclaved 250mL Erlenmeyer Flask with 50 mL LB broth.
4. Grow at 37 ˚C for 2-3 h to OD 0.4-0.6 i.e. log phase. Ask a lab leader to look at it to save time, or otherwise use a spectrophotometer to read it at absorbance at 600nm.
5. Transfer cultures to a falcon tube and spin at 4000 rpm for 15 min at 4˚C
6. Resuspend in 20 mL of 0.1 M $MgCl_2$ (preferred) or $CaCl_2$ (note: swirl until cells are completely resuspended, do not vortex.)
7. Spin again.
8. Resuspend in 10mL of 0.1 M $CaCl_2$

9. Incubate on ice for 4 hours
10. Spin again and prepare $CaCl_2$-glycerol solution.
___ Culture tubes
(___+1) x 0.86mL $CaCl_2$
(___+1) x 0.14mL 100% glycerol
11. Do the following in 4˚C walk-in:
- Resuspend a culture tube with 2mL of the $CaCl_2$-glycerol solution, and then resuspend a second culture tube with this.
- Aliquot into 200 µL or 500µL

Transformation – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Before you start
Cells lose their competence once thawed, so be sure to:
a) keep them on ice at all times.
b) use cells immediately after thawing.
c) discard any unused thawed cells.
Materials
Competent cells
DNA
Ice + Ice bucket
1.5mL microfuge tube
Glass Spreader
Ethanol
Agar Plate with desired antibiotic resistance
Alcohol burner
Instructions
1. Thaw competent cells on ice. Include 2 extra ones for a Negative (no DNA) and Positive control.
2. In a 1.5 mL microfuge tube, add 50 µL of cells and 1 µL of DNA, and pipette up and down to mix.
3. Incubate on ice for 30 min.
4. Transfer directly to 42C heat block for 45 s to "heat shock".
5. Transfer to ice for 2 min.
6. Add 500 µL of LB.
7. Incubate at 37C for 1 h on the shaker.
8. Centrifuge tubes at 13,000rpm for 2 mins.
9. Decant most of the supernatant. Leave approximately 50 µL.
10. Prepare and label plates with date and construct names. Let the plates dry in the flow hood if necessary.
For each sample, the following steps must be carried out quickly in the laminar flow hood to ensure proper spreading of cells.
11. In the laminar flow hood, resuspend pellet in remaining liquid by pipetting up and down.
12. Transfer to center of plate.
13. Dip glass spreader in ethanol and flame.
14. Let the glass spreader cool by gently touching the agar.
15. Spread liquid around plate until it appears dry.
16. Seal plates with Parafilm or put in a bag.
17. Incubate inverted in 37C incubator overnight.

Inoculation Protocol – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols

Materials
DNA Samples
Ampicillin (2µL/sample)
Kanamycin (2.5µL/sample)
Liquid LB Media (1 tube/sample)
Instructions
1. Label all tubes.
2. Do the following in the flow hood:
- Pipette the correct amount of antibiotic into the corresponding DNA tubes. 2 µL for ApR, 2.5 µL KmR.
- Dip the sterile stick into the DNA and into the corresponding media tube.
3. Incubate at 37 degrees celcius overnight on the agitator.

Glycerol Stock Preparation – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Before you start
- It is important to use aseptic technique when carrying out the procedures
Materials
5 mL culture tube
60% Glycerol
Sterile screw-top cryotube
Instructions
1. Inoculate a 5 mL culture tube.
2. Incubate overnight on the shaker.
3. Pipette 500 µL of 60% glycerol culture into a sterile screw-top cryotube.
4. Piptette 500 µL of culture into the cryotube.
5. Label and date cryotube.
6. Vortex.
7. Cover the label with clear tape.
8. Store in -80°C freezer.
9. Add strain to database.

Ligation (10µL recipe with 5 insert : 1 vector ratio) – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Materials
MQ water
Ligase buffer
Ligase
Eppendorf tube
Vector DNA
Insert DNA
Instructions
1. Calculate ligation volumes. Total volume should be 10 µL and insert to vector ratio between 3:1 –5:1.
2. In one tube mix
- Vector DNA
- Insert DNA
- 10× ligase buffer
- Ligase
- MQ if needed
3. Leave on the bench for 1 hour (or even overnight)

Gel Electrophoresis – 2008 – Waterloo

http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Materials
Agarose
1X TAE buffer
Gel Red
1:10 diluted 1kb DNA ladder
Loading Dye
Gel Rig
Instructions for 0.8% Agarose 100 mL gel
Prepare the Gel
1. Measure 0.8 g of agarose on weigh paper.
2. Measure 100mL of TAE buffer using a graduated cylinder into a flask.
3. Add agarose to flask. Swirl to dissolve as much as possible.
4. Microwave for 20 seconds, swirl. Repeat until agarose is dissolved. Allow argrose to cool down to room temperature.
5. Add 2 µL of Gel Red into the gel.
6. Cover the ends of the gel tray with masking tape to seal them.
7. Pour the gel into the tray.
8. Drop the combs into the slots to form the wells.
9. Allow the gel to set.
10. Remove the combs and rinse with distilled water.
Preparing samples
1. Pipette 10µL each digested sample into a labeled microfuge tube (for gel extraction use 20 µL).
2. Add 2µL of loading dye to each sample (for gel extraction use 4 µL)
3. Find the diluted 1kb DNA ladder.
4. Organise the tubes in loading order.
5. Record loading order
Running the Gel
1. Place the tray in the rig with the wells closest to the far side.
2. Add TAE buffer until the surface of the gel is just covered.
3. Pipette 10 µL of the digestion mixture and diluted ladders into the appropriate wells.
4. Put the cover on. Black at the back and red at the head.
5. Connect the wires to an available power supply.
6. Set the power supply to 120V and start it.
7. Check that bubbles are forming near the electrodes.
8. Wait about 45 minutes. The dye front should be 3/4 of the way down the gel.

DNA Extraction – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Materials
All required materials provided in the Fermentas DNA Extraction Kit (K0513)
Instructions
1. Follow the instructions given in DNA Extraction Kit.
2. Nanodrop the construct.

Digestion of Plasmid DNA – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Before you start
- Know what is the total digestion volume mix. From that you can determine the enzyme concentration which will have to be at or below 10%. You can also calculate the buffer volume based on the concentration of the buffer needed

Materials
MQ water (V_{total} - V_{buffer} - V_{DNA} - $V_{enzymes}$)
Buffer (depends on required concentration: For 10x buffer, add 1/10 V_{total}
DNA (1 µL)
Enzymes (0.5 µL each/digest)
Microfuge tube (1/digest)
Instructions
1. In one tube, mix the above materials in the given order.
- Keep the enzymes cold as much as possible. Do not leave them out for longer than necessary.
2. Vortex briefly.
3. Centrifuge 2-3 seconds.
4. Digest 1 hour at 37ºC.

Colony PCR) – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Materials
- 20 µL of Master Mix
Calculations for Master Mix
___ number of colonies (and control)
(___+1) x 0.5µL dNTPs
(___+1) x 15µL PCR H2O MQ
(___+1) x 2.5µL Taq Buffer
(___+1) x 0.5µL fwd primer
(___+1) x 0.5µL rev primer
Instructions
1. Prepare agar plate with the correct antibiotic. Draw a grid with as many squares as the colonies you're screening.
2. Label PCR tubes for each colony and one control (no DNA or colony)
3. Add 6µL of PCR H2O MQ to each PCR tube
4. Pick a colony from the agar plate with a pipette tip and resuspend in 6 µL PCR H2O. (if inoculating LB, resuspend in 7 µL PCR H2O)
5. Add 1 µL of resuspended DNA to a grid square. Incubate overnight at 37C.
6. Optional: Add 1 µL of resuspended DNA in corresponding LB tube and inoculate overnight
7. Prepare master mix. Vortex master mix before adding Taq. Once you added Taq mix gently.
8. Add 20 µL of Master Mix to each PCR tube.
9. Centrifuge briefly in the PCR machine. Use the pulse option.
10. Start the PCR machine on the desired cycle.

1-2-3 Miniprep (Resuspension, Lysis, Neutralization) – 2008 – Waterloo
http://2008.igem.org/Team:Waterloo/Notebook/Protocols
Before you start
Prepare solutions 1, 2, and 3. Only solution 2 needs to be made fresh. Solutions 1 and 3 can be made in excess and stored.
Solution 1
1. Combine
- 11 mL of 50 mM glucose
- 8.33 mL of 25mM Tris·Cl adjusted to pH 8.0
- 6.67 mL of 10mM EDTA adjusted to pH 8.0
2. Autoclave
3. Add RNase to make the concentration 450 µg/mL
4. Store in 4°C fridge

Solution 2
160 µL MQ water/sample
20 µL 10% SDS/sample
20 µL 2N NaOH/sample
Solution 3
1. Combine
- 60 mL of 5M KAc
- 11.4 mL glacial acetic acid
- 28.5 mL MQ water
2. Store in 4°C fridge
Materials
- Solution 1 (100 µL/sample)
- Solution 2 (200 µL/sample)
- Solution 3 (150 µL/sample)
- 1.5 mL microfuge tube (3/sample)
- Chloroform (150 µL/sample)
- 95% ethanol (2 mL/sample)
- 70% ethanol (150 µL/sample)
Instructions
1. Label microfuge tubes.
2. Pour ~1.5mL cell culture in a microfuge tube till it's almost full.
3. Centrifuge for 30 seconds at 13,000 rpm.
4. Decant the supernatant.
5. Repeat steps 2-4 about 3 times.
6. Resuspend well in 100µL of solution 1.
7. Add 200µL of solution 2.
8. Mix gently by inverting 6 to 8 times.
9. Add 150µL of solution 3.
10. Mix gently by inverting 4 to 6 times.
11. Centrifuge for 5 min at 13,000 rpm. While waiting, label new sets of tubes.
12. Transfer supernatant to a new 1.5 mL tube. Discard old tubes.
13. Add 150µL chloroform.
14. Vortex until well mixed.
15. Spin for 3 min at 13,000 rpm. Label new sets of tubes.
16. Transfer top aqueous layer to a new tube. Discard old tubes.
17. Add 2× (~800µL) of ice-cold 95% ethanol.
18. Vortex.
19. Centrifuge at 13,000 rpm for 20 minutes.
20. Do not disrupt pellet. Carefully draw off the supernatant with a pipette and discard to liquid waste.
21. Rinse with 150 µL ice-cold 70% ethanol.
22. Dry tubes open in the 37ºC for 10 minutes.
23. Resuspend DNA in 50 µL MQ water.
25. Check concentration with Nanodrop.

Transformation of Competent Cells – 2008 – University of Ottowa
http://2008.igem.org/Team:University_of_Ottawa/Polymerase_Chain_Reaction
This protocol allows for fast and relatively efficient transformation of competent E. coli cells stored in TSS. All steps performed at the workbench should be accompanied by a flame for sterility. An alternate protocol that includes a heat shock treatment is also provided, but has not been shown to improve efficiency.
Thaw competent cells (DH5α, XL10, etc) stored at –80$deg;C on ice.

Set water bath or heat block to 42°C, remove appropriate number of agar plates with correct antibiotic selection from the cold room to adjust to room temperature. If plate is wet, incubate at 37°C incubator.
Add a maximum of 20 µl of ligation mix (~50 ng of vector with insert) to a labeled tube containing the aliquoted competent cells (maintain cells on ice). An effort should be made to minimize the volume of DNA.
Add 100 µL (lab grown) or 50 µL (commercial) of competent cells to each tube. Mix by gently pipetting up and down with a wide bore pipet tip.
Set tubes on ice for 20 minutes.
Place tubes in the 42°C water bath or heat block for 45 to 90s to allow entry of DNA by "heat shock".
Set tubes back on ice for at least 5 min.
Add 900 µl of LB broth to each tube of competent cells.
Incubate competent cells in shaker for 45 min at 37°C and 200 RPM.
Spin tubes for about 2 minute at 6,000 RPM (max setting) to pellet cells.
Discard all but 100µl of supernatant.
Resuspend cells in remaining supernatant by gently pipetting the mixture up and down, while trying not to create bubbles.
Pipette cell suspension onto LB agar plate with appropriate antibiotic selection conditions. Immerse spreader in 100% ethanol, pass it through flame and allow ethanol to burn off. Allow spreader to cool by making contact with the LB/agar away from the cell suspension with a back and forth movement for 1-2s.
Spread cells evenly around with sterile spreader.
Allow cells to absorb into gel at room temperature on bench top for at least 30 min.
Incubate plates upside down at 37°C overnight to prevent condensation from settling on the agar gel.

Polymerase Chain Reaction (PCR) – 2008 – University of Ottowa
http://2008.igem.org/Team:University_of_Ottawa/Polymerase_Chain_Reaction
This protocol serves as a guideline for setting-up a Polymerase Chain Reaction (PCR) for the amplification and manipulation of specific DNA templates. Optimization can be achieved by varying the composition and concentration of the reaction components, as well as altering the temperature and duration of the different thermal cycling steps. Consult the product information sheet specific to the polymerase employed. For an overview of the theoretical basis and the various applications of PCR consult: Short Protocols in Molecular Biology, Chapter 15 and Molecular Cloning: A Laboratory Manual, Chapter 8. Successful production of the desired DNA fragment by PCR is sensitive to contamination. Ensure that the work area is clean and use pipette tips and tubes reserved for PCR. To prevent cross-contamination of reagents, "double-dip" pipetting should be avoided.
Here are two examples that have resulted in successful amplification of yeast genomic DNA and plasmid DNA achieved using Taq polymerase (NEB) and Phusion High Fidelity Polymerase (NEB).

Taq polymerase
1. On ice, mix the following PCR reaction components with the polymerase added last. For convenience, a master
mix can be made.

Reaction components	1x Vol (µl)
10X Reaction buffer	2.5
10mM each dNTP	0.5
Forward primer (10pmol/µl)	1.25
Reverse primer (10pmol/µl)	1.25
DNA template	2

Taq polymerase	0.5
Filter sterile ddH2O	17
Total	25

2. Place tubes in the Thermal cycler and execute the following program:
94°C for 5 min
94°C for 1 min
50-60°C for 45 s
72°C for 1 min / 1kb
Go to step 2, repeat 29 times
72°C for 10 min
4°C hold

Phusion High Fidelity Polymerase

1. On ice, mix the following PCR reaction components with the polymerase added last. For convenience, a master mix can be made.

Reaction components	1x Vol (µl)
5X Reaction buffer	10
10mM each dNTP	1
Forward primer (10pmol/µl)	2.5
Reverse primer (10pmol/µl)	2.5
DNA template	4
Phusion polymerase	0.5
Filter sterile ddH2O	29.5
Total	50

2. Place tubes in the Thermal cycler and execute the following program:
98°C for 30 s
98°C for 10 s
50-60°C for 20 s
72°C for 30 s / 1kb
Go to step 2, repeat 29 times
72°C for 10 min
4°C hold

Note: Typically, 20 ng and 100 ng of plasmid or genomic DNA respectively has been used as template.

PCR protocols – 2008 – University of Lethbridge
http://2008.igem.org/Team:University_of_Lethbridge/Notebook/Protocols
CheZ (Taq pol)
From September 28, 2008 in the "Chemotaxis" lab book:
- Tempate DNA 1uL
- 10X Econo Taq Buffer 2.5uL
- dNTP mix 1uL

- Primer 1 5uL
- Primer 2 5uL
- Econo Taq 0.25uL
- ddH2O 19.75uL

Cycling Conditions:

- Incubate PCR Reactions 2 min at 94 C
- Denature 30 sec at 94 C
- Anneal 30 sec at 47 C
- Extend 1 min/kb at 72 C
- Final extension 7 min at 72 C
- Hold indefinitely at 4 C

CheZ (Phusion pol)

Conditions for one reaction (25 uL):

-1x HF buffer (5 uL 5x buffer)
-Forward primer (1.5 uL)
-Reverse primer (1.5 uL)
-dNTPs (0.5 uL)
-Phusion (0.25 uL)
-mQH2O (15.25 uL)
-Template (1 uL)

Cycling protocol ("cheZ"):

1. Initial denaturation @ 98C for 4 mins (1 cycle)
2a. Denaturation @ 98 C for 30 sec (35 cycles for step #2)
b. Annealing 47.0C for 30 seconds
c. Extension @ 72C for 30 sec
3. Final extension @ 72C for 7 mins (1 cycle)
4. Hold at 4C

Riboswitch (Taq pol)

From October 14, 2008 in the "Riboswitch" notebook: Objective: PCR of the riboswitch in a 50 uL x 9 reactions.

Master Mix (1x):

-10x Buffer: 5 uL
-10 mM dNTP: 1 uL
- 50 mM MgCl2: 1.5 uL
- 10 mM reverse primer: 1 uL
-10 mM forward primer: 1 uL
-Taq polymerase: 0.2 uL
-d2H2O: 39.3 uL

rspA TIR (Taq pol)

From September 30, 2008 in "rpsa TIR" notebook

Reaction Conditions:

- 1.0 uL Template DNA
- 5.0 uL 10x Buffer
- 2.0 uL 10 mM dNTPs
- 1.0 uL Forward Primer
- 1.0 uL Reverse Primer
- 0.5 uL Econo taq polymerase
- 39.5 uL Optima H2O

Cycling Conditions:

1. 94 C 2 min
2. a. 94 C 15 sec

b. 47 C 15 sec
c. 72 C 15 sec
3. Repeat step 2 for 25 cycles
4.72 C 5 min
xylE (Phusion pol.)
Master Mix for 1 reaction (50 uL):
- 10x Buffer: 5 uL
- 10 mM dNTPs: 1 uL
- 50 mM Mg2+: 1.5 uL
- 10 uM RF: 1 uL
- 10 uM RR: 1 uL
- Phusion polymerase: 0.2 uL
- H20: 39.3 uL
- template: 1 uL
Cycle conditions "xylE":
1. Denaturation: 94 C for 1 min
2. a. Denaturation: 94 C for 30 seconds
b. Annealing: 52 C for 30 seconds
c. Extension: 70 C for 30 seconds
Repeat Step 2 for 30 cycles
3. Final extension: 72 C for 10 minutes, then hold at 4 C.

Squeeze 'n Freze Gel Extraction – 2008 – University of Lethbridge
http://2008.igem.org/Team:University_of_Lethbridge/Notebook/Protocols
-Run the sample you wish to extract on a TAE-Agarose Gel

-Cut out the band you wish to purify

-Incubate in 3 gel volume 0.3 M NaAc (pH 7.0) at room temperature for 30 minutes.

-Make your own spin column from a small microfuge tube with a hole cut out of the bottom, involves flamage
and a wire, stuff with glass wool. This tube should be inserted inside a 1.5 mL microfuge tube.

-Transfer the solution to the spin column.

-Freeze the tube in liquid Nitrogen for 1 minute, then spin at full speed for 15 minutes.

-Precipitate the DNA in 95% Ethanol. Remove the supernatant.

-Wash in 75% Ethanol. Remove as much ethanol as possible.

-Centrifige for 5 minutes then, remove the rest of the ethanol.

-Let pellet air dry for 10 minutes, this allows all ethanol to evaporate off.

-Resuspend in TE Buffer (pH 8.0), 10 uL.

-Quantify either by Gel or UV Spec.

-Proceed with ligation.

DNA Extraction – 2008 – Montreal
http://2008.igem.org/Team:Montreal/Notebook
Midiprep
1. Set up 50ml of 1xLB with 100 ug/ml antibiotic of your choice.
Pen-strep should be at 100mg/ml stock so use 50ml per 50 ml.
Kanamycin should be at 10mg/ml (it's poorly soluble) so use 500 ul per ml NOTE - for low copy number plasmids, increase total culture volume to 100ml.
2. Distribute the LB/antibiotic mix into 50ml-tubes (25ml in each). This gives room to aerate.
3. Add 250ul overnight culture (from a 5ml prep) to each tube, incubate on the shaker at 37C overnight.
4. Pellet the bacteria in a desktop clinical centrifuge, 10-15 min. at medium setting should do it. (You should have that kind of centrifuge on the C-floor, it might take either 50ml tubes or 15ml tubes seem to be more efficient.
Hint: if you don't see a pellet, the centrifugation was too short or too slow. These will settle out at room temperature eventually, so don't worry, just add more time.
5. Decant supernatant, drain well, pool all pellets of the same type into 5ml buffer P1. Make sure RNAse A and LyseBlue (included) were added to P1. Distribute the mix along 10 microfuge tubes, 500ul each.
6. To each microfuge tube, add 500ul buffer P2, mix by inverting several times (do not vortex). Do one tube at a time (add, mix, go to next tube, add mix...). You know it's mixed when it turns from white/beige to uniformly blue - no white streaks allowed.
7. Leave at room temperature 5 minutes. By the time you get to tube 10, it may be close for the time to start the next step 8 for tube 1.
8. Add 500ul buffer P3, mix by inverting as above. Do one tube at a time, same order as above. A white precipitate should form and all the blue should disappear.
9. Leave tubes on ice for 10 min.
10. Centrifuge tubes in a microfuge at max speed for 10 min.
11. Pool all supernatants into a 15ml tube. Pipet with P1000; take liquid only, no white flakes.
12. If there are flakes, re-aliquot into clean microfuge tubes. There will be fewer tubes than before. Re-centrifuge, pool supernatants into another clean 15ml tube. If there are no chunks, go on to step 13.
13. Pre-equilibrate a midiprep column with 5ml QBT buffer.
14. When all the buffer has gone through, pour on your cleared bacterial lysate from step 12.
15. When all the sample has gone through, pour the column full of QC Buffer (no need to measure, just fill right to the top). Allow to drain, repeat once.
16. When all the QC buffer has gone through, move the column to a clean 50 mL tube, then apply 5mL buffer QF. This step elutes the DNA
17. Add 3.5ml isopropanol to the eluted DNA, distribute among six microfuge tubes, centrifuge at max speed for 10 min.
18. Decant supernatant, pool all pellets into one ml of Ethanol. You should see little white flakes floating around.
19. Centrifuge 10 minutes at max speed - do NOT forget the balance tube. Ethanol weighs less than water, if you use a balance, you may need a smaller volume.
20. Decant supernatant, pipet off any remaining liquid with a p-10 tip, air dry 10 min.
21. Resuspend in EB or TE or in 10mM Tris pH8.0, recommended volumes: tiny pellet (barely visible) 25ul, medium pellet 50ul, huge pellet 100uL.
Maxiprep
NB: To measure the O.D. 260/280, usually 4ul in 400ul water is acceptable.

Seeding – 2008 – Montreal
http://2008.igem.org/Team:Montreal/Notebook
Seeding is a method for amplifying a single colony of genetically identical bacteria.
1. Pipet 5.0 mL of LB broth into two 10 mL culture tube, under sterile conditions.

2. Add the applicable antibiotic (amp+, 1 µL/mL LB; kan+, 5 µL/mL)
3. Identify an area on the transformation plate with single colonies. "Pick" one colony by picking it up with a sterile P200 pipet tip, and eject the tip into the media of the culture tube.
4. Leave one tube with only LB and antibiotic as a control
5. Fix the caps on the tubes loosely, to allow air circulation and subsequent bacterial growth.
6. Place the tubes in a shaking incubator at 37ºC for 16-20 hours.

Transformation – 2008 – Montreal
http://2008.igem.org/Team:Montreal/Notebook
1. Remove vial of pre-prepared TOP10 chemically competent cells from -80˚C freezer and thaw in ice.
2. Add between 10-100ng of DNA in solution (varies with cell competency).
3. Mix the solution gently by tapping – anything more vigorous will result in potential damage to the competent cells.
4. Incubate on ice for 30 minutes; meanwhile, ensure that a water bath is set to 42˚C and pre-heat a vial of SOC medium.
5. Heat shock the transformation tube in a 42˚C water bath for exactly 30s.
6. Remove the transformation tube from the water bath and add 250µL of SOC medium, then place on ice for 60s.
7. Shake and incubate at 37˚C for one hour.
8. Plate on appropriate media and antibiotics for approximately 20 hours, then check for colonies

Gel Preparation – 2008 – Montreal
http://2008.igem.org/Team:Montreal/Notebook
1. Measure 50mL of TAE buffer and mix with 0.5g of Agarose powder.
2. Thoroughly mix the two in the flask and microwave the mixture for roughly about 1 minute (or until you see bubbling).
3. Add 1.5µL of Ethidium Bromide (light sensitive) and swirl until a fine mixture is seen.
4. Pour the mixture onto the casting tray (make sure that each end is taped), with a comb inserted in one of the ends.
5. Wait for the gel to solidify over time. Optional: Loosely cover the gel with aluminum foil to reduce EtBr breakdown in light.
6. Once hardened, remove the comb and load the gel into the electrophoresis box.
7. Fill the reservoir with enough buffer to submerge the gel entirely.

Running the Gel
1. To a DNA sample (15µL volume), add 2µL of loading dye. The loading dye acts to increase specific gravity and visibility of the DNA samples.
2. Fill this entire volume (17µL) into the wells in the agarose gel using a P20 micropipette. Record the order of samples and their respective wells.
3. Add between 2-5µL of DNA ladder to one empty well. It is sometimes preferable to add ladder to the two wells relatively far apart, to help line up the ladder with DNA bands.
4. Apply a voltage of 100V across the gel for 45-60 minutes. Exceeding this voltage would excessively heat the gel and yield irregular DNA migration. Optional: Cover the reservoir with aluminum foil to reduce EtBr breakdown in light.
5. When finished, remove the gel from the reservoir and photograph under UV light to confirm the presence and size of the DNA bands.

Bacterial Transformation – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Thaw 100 µL of competent cells (per transformation) on ice just before they are needed
Add DNA (max 20ul) thawed cells and mix by flicking the side of the tube. Leave on ice for 30 minutes

Heat shock for 2 minutes at 42 degrees Celsius or 5 minutes at 37 degrees Celsius
Place on Ice for 5 minutes
Add 250ul SOC medium to each tube
Incubate for 30 to 60 minutes with shaking at 37 degrees Celsius. (Note that for Kanamycin containing plasmides always use one hour)
Spin down to remove all supernatant except approximately 100 µL
Plate approximately 30 µL on each of two antibiotic plates
Grow overnight at 37 degrees Celsius
For this protocol we used a couple of controls
Positive Control - pBluescript in TOP10 cells on ampicillin plates
Negative Control - TOP10 cells grown on ampcillin plates

Rehydration – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Biobrick parts are shipped from the registry in a dehydrated from. As such they must be rehydrated before they can be used.
Puncture a hole through the foil with a pipette tip into the well that corresponds to the Biobrick - standard part that you want
Add 15 µL of diH20 (deionized water)
Let the water sit for 5 minutes
Take 2 µL DNA and transform into your desired competent cells, plate out onto a plate with the correct antibiotic and grow overnight. Your goal here is to obtain single colonies

Taq PCR Protocol – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Reagent	Volume (1x)	Volume (3x)	Volume (5x)	Volume (15x)
Sterile H2O	36 µL	108 µL	180 µL	540 µL
10X Taq Buffer	5 µL	15 µL	25 µL	75 µL
2mM dNTPs	5 µL	15 µL	25 µL	75 µL
Forward Primer (100 ug/ul)	1 µL	3 µL	5 µL	15 µL
Reverse Primer (100 ug/ul)	1 µL	3 µL	5 µL	15 µL
50mM MgCl2	1.5 µL	4.5 µL	7.5 µL	22.5 µL
Taq Polymerase (50 ug/ul)	0.5 µL	1.5 µL	2.5 µL	7.5 µL

Thermocycler Conditions
1 Cycle - 6 minutes at 95 degrees Celsius
36 cycles of:
1 minute at 95 degrees Celsius
1 minute at 58 degrees Celsius (this step done at 65 degrees Celsius for higher GC content)
1 minute at 72 degrees Celsius
1 Cycle - 10 minutes at 72 degrees Celsius then HOLD at 4 degrees Celsius
Conditions were varied as needed. For example in cases of longer products all 1 minute times were increased to 1.5 or even 3 minutes

Plasmid Preparation Protocol - from GenElute Plasmid Miniprep Kit – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Harvest Cells
Pellet 5 mL of an overnight culture.
Resuspend Cells

Completely resuspend the bacterial pellet with 200 uL of resuspension solution. Pipette up and down to throroughly resuspend cells until homogenous. Incomplete suspensions will result in poor recovery. Another rapid way to resuspend the cell pellet is to scrape teh bottoms of the microcentrifuge tubes back and forth five times across the surface of a polpropylene microcentrifuge tube storage rack with 5 X 16 holes .
Lyse Cells
Lyse resuspended cells by adding 200 µL of the lysis solution. Immediately mix the contents by gentle inversion (6-8 times) until the mixture becomes clear and viscous. Do Not Vortex . The lysis was allowed to proceed for 5 minutes before neutralization.
Neutralize
Precipitate the cell debris by adding 350 µL of the Neutralization/Binding solution. Gently invert the tube 4-6 times. Pellet the cell debris by centrifuging at maximum speed for 10 minutes.
Prepare Column
Insert a GenElute Miniprep Binding Column into a provided microcentrifuge tube. Add 500 µL of the Column Preparation Solution to each miniprep column and centrifuge at max speed for 60 seconds. Discard the flow through liquid.
Load Cleared Lysate
Transfer 600 µL of the cleared lysate from step 4 to the column prepared in step 5 and centrifuge at max speed for 60 seconds. Discard the flow through.
Wash Column
Add 750 µL of the diluted Wash Solution to the column. Centrifuge at max speed for 60 seconds. The column wash step removes residual salt and other contaminants introduced during the column load. Discard the flow through liquid and centrifuge again at maximum speed 2 minutes without any additional wash solution to remove excess alcohol.
Elute DNA
Transfer the column to a fresh collection tube. Add 100 µL of molecular biology reagent water to the column. Centrifuge at Max speed for 2 minutes. DNA is now present in the eluate and is ready for immediate use or storage at -20 degrees Celsius.

Construction Technique – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Determine the order of the two parts you will be putting together; the one in front will be referred to as the insert, while the one behind will be referred to as the vector. Both the vector and the insert need to have their own separate tube, at least in the beginning.
Restriction Digest Protocol
In the Insert Tube...
600 ng of DNA (To figure out the volume, the calculation is 600 / concentration of plasmid. This gives you volume in µL).
Water, so that the volume of both DNA and water in the tube is 35 µL total
4 µL of React 1 Buffer
0.5 µL of EcoR1
0.5 µL of Spe1
In the vector Tube...
250ng of DNA (To figure out the volume, the calculation is 250 / concentration of plasmid. This gives you volume in µL).
Water, so that the volume of both DNA and water in the tube is 35 µL total
4 µL of React 2 Buffer
0.5 µL of EcoR1
0.5 µL of Xba1

Put both tubes into the 37°C water bath for one hour. After, place them into the 65°C heating block for 10 minutes. This deactivates any enzymes in the tube (which is ok, because by now they've done all they need to). Take the insert out, and put it in a -20°C freezer.

Antarctic Phosphatase Protocol

To the vector tube, add 5 µL of 10x Antarctic Phosphatase Buffer, 4 µL of water, and 1 µL of Antarctic Phosphatase. We do this to prevent the vector from closing up again without any insert. Put the tube into the 37°C water bath for 30 mins. After, place it in the 65°C heating block for 10 minutes.

Ligation Protocol

Take the insert out of the freezer, and add 5 µL of insert and 5 µL of vector to a new tube. Label the rest of each tube as Unligated, put the date on the tube, and stick it in the -20°C freezer incase your ligation/transformation doesn't work. To the single tube of 10 µL mix, add 10 µL of 2x Quick Ligase Buffer, and 1 µL of Quick Ligase. Let this sit at room temperature for 5 minutes.

You are now done. If you are going to transform this construction product, add all 21µL to a tube of whichever competent bacteria you're using.

LB - Agar Plate Preparation Protocol – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Weigh 35g of LB-Agar powder mix per litre of media desired. One litre makes 40-50 plates

Select an appropriate flask; the lab autoclave will cause flasks half full and above to boil over! Use a 2L flasks for up to .5 L of media, a 4 litre flask for up to 1.5L, etc

Disolve LB-Agar, using water from one of the wall mounted nanopure filters. Add a stir bar and use a magnetic stirrer to speed things up

Cover the flask with aluminum foil, and secure the foil with autoclave tape. The foil should be somewhat loose (to avoid building pressure in the flask while sterilizing and blowing the foil off), but not so loose that lots of liquid can escape

Put the flask in a plastic autoclave tray, load into the autoclave, and sterilize using the 20 minute liquid program

Once the autoclave finishes venting (which can take twice as long as the sterilization proper), check that the foil covering is still in place. If it is not, the media is contaminated! Unload using the insulated oven gloves

Allow the media to cool until it can be handled without the oven mits. The cold room can be used to speed this up. Alternatively, if a large batch of media is prepared flasks may be kept hot in the prep lab water bath, to avoid all of them cooling at once. Agar polymerization cannot be reversed once it starts (and if it begins to set in the flask you're in trouble!), but media can be kept from setting further by keeping it hot.

Once media is cool, add other desired ingredients. Use the magnetic stirrer to mix, but do NOT add a stir bar now, or the media will be contaminated. (If one wasn't added before, you must do without.) Common additions include:

ampicillin (stock 100mg/ml, final 100ug/ml)
kanamycin (stock 50mg/ml, final 50ug/ml)
chloramphenicol (stock 50mg/ml, final 10ug/ml)
x-gal (stock 40mg/ml, final 40ug/ml)

To achieve final concentrations, add 1mL of stock per 1L of media, except for chloramphenicol, where 0.6mL per 1L of media is added instead

Pour directly from the flask into sterile petri plates. Use a quick pass with a bunsen burner flame to snuff out bubbles that form during pouring. Do not subject the plate to continuous heat or the plate will melt, and the heat sensitive ingredients added in the previous step will be destroyed. Bubbles can allow cells to access nutrients without being exposed to the plate's antibiotic, and should be blown out immediately before the gel can set. It's a good idea for one person to pour while another flames bubbles.

Allow the plates to stand right side up overnight, or until the gel sets if they are needed sooner. Plates should be stored upside down to keep condensation from falling on the media. Store petri plates in the plastic bags they ship in, in the 4 degree cold room.

Over Night Growth – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Adapted from Butanerds Protocols from the University of Alberta iGEM Protocols pdf
What you will need
10mL culture tube. Use 16mm x 160mm or 16mm x 125mm
5 mL LB
5 uL 1000X antibiotiecs
Single colonies on a plate (best not to start an over night from a glycerol stock)
Protocol
Pipet 5uL 1000X antibiotic into culture tube
Add 5mL non-contaminated LB. Do this first. Then add antibiotic
Select a single colony using a sterile toothpick or flamed loop that has been cooled
Place toothpick or loop in culture tube and stir
Remove toothpick or loop and place culture tube in incubator at 37 C overnight shaking vigorously (250 RPM)

Glycerol Stock Preparation – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Adapted from Butanerds Protocols from the University of Alberta iGEM Protocols pdf
What you will need
Overnight bacterial growth
screw captubes
glycerol
Protocol
Pipet 0.5mL of 50% glycerol into 3 1.5 screw cap tubes
Add 0.5mL of overnight culture to each tube
Pipet up and down to gently mix
Flash freeze in liquid N2 or dry ice/ethanol bath
Place in -80 C freezer when frozen

Agarose Gel Electrophoresis Protocol – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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Adapted from Butanerds Protocols from the University of Alberta iGEM Protocols pdf
What you will need
1X TAE
Graduated Cylinder
125 mL flask
Agarose
Gel Pouring Tray
Tape
Gel rig
Ethidium Bromide
Protocol
Measure out 120mL of buffer
Transfer buffer to 125 mL flask
Weigh out enough agarose to make a 1% gel (in our case 1.2 g of agarose was the right amount)
Transfer agarose to 125mL flask
Melt agarose in microwave until solution is almose boiling, stirring every 15-20 seconds (should be around 2 minutes)

Allow agarose to cool (do not let it cool to the point where it is hard)
Add 3 uL of Ethidium Bromide to the cooling agarose
Assemble the gel pouring apparatus by inserting gate into slots. Use a pastuer pipet to run a bead of molton agarose along the edges of the gates to seal the box and prevent leaks
Allow gel to cool until flask can be handled comfortabley
Place comb in the gel rig
Pour agarose into gel tray
Allow to solidify. While the gel is solidifying prepare the samples. Add your sample and 1 uL 10x Loading Dye, 4 uL of DNA and 5 uL of water
Pour 1X TAE over gel so that gel is covered by a 3-5mm buffer
Load samples into lane (Don't forget to load a 1kb+ ladder into one of the lanes)
Hook electrodes to gel apparatus
Run the apparatus at 100V for 30 - 45 minutes (make sure to watch that the dye does not run off the gel)
Visualize the gel and record the results

Restriction Digest – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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This protocol is also described as a part of our Construction Technique. Start by selecting the order of the two parts you will be putting together; the one in front will be referred to as the insert, while the one behind will be referred to as the vector. Both the vector and the insert need to have their own separate tube, at least in the beginning. This is important because it allows for clean addition new parts to a the circut
In the Insert Tube...
600ng of DNA (To figure out the volume, the calculation is 600 / concentration of plasmid. This gives you volume in µL).
Water, so that the volume of DNA and water in the tube is 35 µL
4 µL of React 1 Buffer
0.5 µL of EcoR1
0.5 µL of Spe1
In the vector Tube...
250ng of DNA (To figure out the volume, the calculation is 250 / concentration of plasmid. This gives you volume in µL).
Water, so that the volume of DNA and water in the tube is 35 µL
4 µL of React 2 Buffer
0.5 µL of EcoR1
0.5 µL of Xba1
Put both tubes into the 37°C water bath for one hour. After, place them into the 65°C heating block for 10 minutes. This destroys any enzymes in the tube (which is ok, because by now they've done all they need to). Take the insert out, and put it in a -20°C freezer.

Ligation Protocol – 2008 – Calgary Wetware
http://2008.igem.org/Team:Calgary_Wetware/Protocols

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This protocol is also described as a part of our Construction Technique. Start by selecting the order of the two parts you will be putting together; the one in front will be referred to as the insert, while the one behind will be referred to as the vector. Both the vector and the insert need to have their own separate tube, at least in the beginning. This is important because it allows for clean addition new parts to a the circut
Take insert out of the freezer and ad 5 uL of insert and 5 uL f vector to a new tube
Clearly label the remaining tubes of each (insert and vector) as Unligated, put the date on the tube and place in -20 C freezer in case the transformation does not work
To the single tube containing both insert and vector add 10 uL of 2x Quick Ligase Buffer and 1 uL of Quick Ligase.

Let this sit at room temperature for 5 minutes

Overnights – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols
Inoculating LB culture tubes:
Add 5ul Amp50 or Amp100 to 5ml LB broth in tube.
Using sterile inoculating loop, touch an isolated colony.
Swirl loop through LB + amp broth.
Incubate overnight at 37 C.
Minipreps – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols
QIAprep Spin Miniprep Protocol
Isolating DNA from LB culture tubes:
Add 2X 750ul broth from overnights into a 1.5ml tube. Centrifuge for 1 min to pellet and aspirate liquid.
Add 250ul Buffer P1 * and resuspend pelleted cells.
Add 250ul Buffer P2 ** and invert tube 4-6 times.
Add 350ul Buffer N3 and invert tube immediately 4-6 times.
Centrifuge for 10 min.
Apply supernatant to QIAprep spin column.
Centrifuge for 1 min and discard flow through.
Add 500ul Buffer PB, centrifuge for 1 min and discard flow through (only if high nuclease activity).
Add 750ul Buffer PE, centrifuge for 1 min and discard flow through.
Centrifuge for an additional 1 min.
Place spin column in a clean 1.5ml tube. Add 50ul Buffer EB, let stand for 1 min and centrifuge for 1 min.
These may be stored at -20 C.
*stored at 4 C
**heat first in 42 C waterbath for 5 min
Digests – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols
Reaction mix:
DNA 5-15ul
10X NEB 2 buffer 10% of total volume
10X BSA 10% of total volume
Enzyme 1 0.5ul ALWAYS KEEP ENZYMES ON ICE!
Enzyme 2 0.5ul
MQH20 as need to bring up to total volume
Add to PCR tubes and incubate at 37 C for 1-2 hrs.
Liquify 2% agarose gel (for fragments <2 kbp) by heating in microwave.
Pour into gel mould - don't forget the well-maker!
Add dye to PCR tubes such that 1/6 of total volume is dye.
(ie. 20ul digest mix + 4ul dye = 24ul total volume. 4/24 = 6)
Load 10ul of DNA ladder into first well.
Load 10-30ul of DNA digest (+dye) into the remaining wells. NEVER MAKE YOUR GEL SYMMETRICAL!
Add fresh TEB if DNA fragments in the gel will be excised. Use "used once" TEB if not.
Run at 110V for small gels or 140V for large gels for ~50 min. KEEP AN EYE ON YOUR GEL!
Soak the gel in ethidium bromide for 10-15 min.
If you are excising a fragment: view the gel under low frequency UV light.
If you are simply viewing the gel: view the gel under high frequency UV light.
Save a copy of the gel and print off a picture.

To excise a gel fragment:
ENSURE YOU ARE WEARING A UV FACE SHIELD, GLOVES AND LONG SLEEVES.
Using a razor blade, cut out the desired gel fragments. Avoid excess agarose.
Place into labeled 1.5ml tubes.
These may be stored at -20 C.

PCR – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols

Reaction mixture:
10X PCR 20 buffer 2.5ul
10mM dNTP 0.5ul
primer 1 1.0ul
primer 2 1.0ul
50:1 Taq:Vent 0.5ul ALWAYS KEEP ENZYMES ON ICE!
DNA 1.0ul
MQH20 18.5ul
Total Volume: 25ul
Run in thermocycler PCR program.

Gel Extraction – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols

Exracting DNA from gel purified samples - QIAquick gel extraction protocol
Measure ng/ul concentration and A260/280 for the DNA sample by placing 1.5ul of DNA on the nanodrop.

Ligation – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols

Reaction mixture:
vector DNA equal amounts of vector and insert are desired
insert DNA compare concentrations C1V1 = C2V2
10X NEB T4 ligase buffer 1.5ul
T4 ligase 1.0ul ALWAYS KEEP ENZYMES ON ICE!
MQH2O as needed to bring up to total volume
Incubate at 16 C for 30 min in thermocycler.
May be stored at 4 C.

Transformation – 2008 - NINThttp://2008.igem.org/Team:Alberta_NINT/Protocols

Thaw competent (XL1-B) cells on ice - ~15 min.
Add 1.5ul DNA into each tube of cells.
Cool on ice for 30 to 60 min.
Heatshock cells at 42 C for 1 min.
Cool on ice for 2 min.
Add 900ul SOC media to each tube of cells.
Incubate at 37 C, shaking, for 1 hr.
Incubate LB + amp100 plates at 30 C to warm them.
Plate 150ul of cells on LB + amp100 plates
Incubate at 37 C overnight.
Plating:
Add 150ul of cells in SOC media to a sterile LB plate.
Dip spreader in ethanol and flame to burn off the ethanol.
Touch spreader to media, avoiding the cells on the plate, to cool it.
Keeping spreader level, spin the plate so that the cells are evenly distributed around the plate.
Let plate dry before inverting and incubating.

Colony PCR – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols

Reaction mixture:
PCR 20 buffer 2.5ul
10mM dNTP 0.5ul
primer 1 (5mM) 1.0ul
primer 2 (5mM) 1.0ul
50:1 Taq:Vent 0.5ul ALWAYS KEEP ENZYMES ON ICE!
DNA (cells: 1 colony in 25ul MQH2O) 1.0ul
MQH2O 18.5ul
Colony PCR thermocycler program:
[96 C for 2 min
96 C for 20 sec
54 C for 20 sec
68 C for 1 min 30 sec] (35X)
4 C hold

Sequencing – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols

Reaction mixture:
DNA 1.0ul
primer 1.0ul THAW IN 42 C WATERBATH
Big Dye Mix 2.0ul KEEP ON ICE - THAW FOR 1 MIN ON BENCH BEFORE USE
Sequencing buffer 2.0ul
Pellet Paint 1.0ul THAW IN 42 C WATERBATH, VORTEX 10 SEC
MQH2O 3.0ul
Sequencing thermocycler program:
[96 C 10 sec
50 C 20 sec
60 C 2 min 30 sec] (30X)
4 C hold
Sequencing:
Add 10ul 25mM EDTA to 1.5ml tube.
Add 10ul sequencing reaction to tube. Pipette to mix.
Add 32ul 95% ETOH and vortex for 5 sec.
Centrifuge for 3 min. to pellet.
Aspirate liquid. BE CAREFUL NOT TO SUCK UP YOUR PELLET!
Add 200ul 70% ETOH and vortex for 5 sec.
Centrifuge for 1 min.
Aspirate liquid. BE CAREFUL NOT TO SUCK UP YOUR PELLET!
Dry with open lid in fume hood or in dark for 15 min.
Take to MBSU for sequencing.

Annealing – 2008 – NINT
http://2008.igem.org/Team:Alberta_NINT/Protocols

Resuspend DNA by centrifuging dry DNA tubes for 2 min. and adding 200ul Buffer EB.
Leave at room temperature for 10 min and then vortex for 30 sec.

Reaction mixture:
10X PCR 20 buffer 2.5ul
DNA strand 1 5.0ul
DNA strand 2 5.0ul
MQH2O 12.5ul
Anneal thermocycler program:
90 C 1 sec

90 C 10 sec
-0.6 C / cycle (100X)
4 C hold

Making a Glycerol Stock – 2008 – NINT

http://2008.igem.org/Team:Alberta_NINT/Protocols

From fresh overnights, take 750ul of culture broth and add it to a stock tube.
Add 750ul of 60% glycerol.
Vortex for 30 seconds.
Store at -80 C.

LacZ Assay – 2008 – NINT

http://2008.igem.org/Team:Alberta_NINT/Protocols

LacZ Assay PDF file

Point mutation Quick-Change method – 2008 – USTC

http://2008.igem.org/Team:USTC/Notebook

Primer design: both of the mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. The desired mutation should be in the middle of the primer with ~10-15bases of correct sequence on both sides. On each side of the desired mutations of my primers, 4*(the number of Gor C)+2*(the number of A or T)>45. It is better that the primers terminate in one or more C or G bases. Polymerase Chain Reaction: we use PrimeSTAR (TaKaRa) as the polymerase.

system:	concentration	volume
5*PS Buffer	5*	4.0ul
dNTP mixture	2.5mM each	2.0ul
primer1	25uM	0.2ul
primer2	25uM	0.2ul
template	changeable	~
PrimeSTAR	2.5U/ul	0.2ul
ddH2O	~	add to 20ul

process program

Predenaturing	94℃	5 min	
Denaturing	94℃	30sec	
Annealing	follow the Tm	30sec	30cycles
Extension	72℃	theoretically 1kb/min	
Last extention	72℃	10min	
Hold	10℃		

DpnI digestion of the amplification products: DpnI will digest parental methylated and hemimethylated DNA .
Transformation: follow the standard protocol. Nick of the mutated molecule will be repaired in cells.
Retrieved from "http://2008.igem.org/USTC/Notebook/Point_mutation_Quick-Change_method"

Regular Transformation protocol for Top10/DH5α cells – 2008 – USTC
http://2008.igem.org/Team:USTC/Notebook
Take out an appropriate aliquot of Top10/DH5α ChemComp cells from -80 freezer
Let cells thaw on ice! for ~5-10min until heat shock!
Aliquots are either 50µl (small PCR tubes) or 100µl (bigger tubes).
Transform 50 µl of cells with 3-5µl of ligated DNA
Keep on ice 30min. This step is to let the salt from the ligation equilibrate over the cells.
Heat shock 90 sec at 42C. You can set a thermocycler to 42*C instead of making a water bath.
Put cells back on ice for 5min.
Add your 50µl of cells to 200 µl LB media in a 1.5ml epp tube.

Incubate at 37 C for 1 hour. You can just place the 1.5ml epp tubes in a little plastic cup and put them on the *shaker in the 37*C room.
Using 1.5ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
Ampicillin and kanamycin appear to do fine with 1 hour growth.
Plate 200 µl on the appropriate antibiotic plates. Use sterilized glass stick to spread.

Preparing Antibiotic Stocks – 2008 – USTC
http://2008.igem.org/Team:USTC/Notebook
Ampicillin
Stocks & Usage
Stock Concentration - 50mg/ml in H_2O
Aliquots - 100µl and 500µl
Working Concentration - 50µg/ml
Kanamycin
Stocks & Usage
Stock Concentration - 10mg/ml in H_2O
Aliquots - 200µl and 1ml
Working Concentration - 20µg/ml
Chloramphenicol
Stocks & Usage
Stock concentration - 34mg/ml in 100% Ethanol
Aliquots - 1ml
Working concentration = 25µ/ml (Stringent), 170µ/ml (relaxed)

DNA Ligation – 2008 – USTC
http://2008.igem.org/Team:USTC/Notebook
DNA ligases are used with restriction enzymes to insert genes into plasmids.

Materials
DNA plasmids double digested previously(eg.XbaI and PstI); . Measure concentration in Nanodrop beforehand.
Biobricks double digested previously(eg.SpeI and PstI)
equal amount of T4 ligase.

Procedure
The following volumes apply to a 20µl DNA ligation.
add the following components to PCR tube:
9µl DNA plasmids
1µl biobricks
10ul Solution I (T4 ligase and buffer)
MIX the reaction by stiring
Incubate the reaction at 16*C for 2hrs or overnight to ensure complete ligation.

Restriction Endonuleases Double Digestion – 2008 – USTC
http://2008.igem.org/Team:USTC/Notebook
Materials
DNA; the thing you want to cut. Usually plasmid or PCR product. Measure concentration in Nanodrop beforehand.
Appropriate NEB 10x Buffer (check the NEB enzyme chart or catalogue to find compatible buffers).
Appropriate enzymes.

ddH$_2$O
BSA (100x from NEB)
Procedure
The following volumes apply to a 20µl analytical digest; for larger, preparative digests, simply scale up (eg. for a 30µl digest, use 3µl of 10x buffer, etc)
add the following components to PCR tube:
2µl BSA (diluted to 10X beforehand)
2µl 10x Buffer
appropriate amount of DNA
0.5µl of each enzyme
appropriate amount of ddH$_2$O to PCR tube. (20µlTotalVolume-(BSA + Buffer + DNA + Enzyme)µl
eg. 20µl-(5µlDNA+2µlBuffer+2µlBSA+0.5µlEnzymeA+0.5µlEnzymeB)=10µl ddH$_2$O)
MIX the reaction by stiring
Incubate the reaction at 37*C for 2-4hrs to ensure complete digestion.
Store digest at -20*C or run immediately on gel.

Agarose Gel Electrophoresis – 2008 – USTC
http://2008.igem.org/Team:USTC/Notebook
Materials
DNA, the thing you want to analyze/cut out. Measure concentration in nanodrop beforehand.
1xTAE buffer.
Gel-red dye, 10,000x.
DNA Marker DL2000 and DL15000.
Melted Agarose in 1xTAE.
melting appropriate amount of Agarose mixed in TAE in the microwave. (eg. to prepare 100ml of melted 1% agarose, mix 1g of agarose in ~99ml 1xTAE in a foil-covered flask and microwave for 4+min; keep an eye on it every couple of minutes when you microwave so it doesn't boil over; be careful to wear gloves when handling hot liquid containers). Can leave on bench and re-melt every time you need it.
Preparation
Make a 1% agarose solution in 100ml TAE, for typical DNA fragments. A solution of up to 2-4% can be used if you analyze small DNA molecules, and for large molecules, a solution as low as 0.7% can be used.
Carefully bring the solution just to the boil to dissolve the agarose, preferably in a microwave oven.
Let the solution cool down to about 60 °C at room temperature, or water bath. Stir or swirl the solution while cooling, Wearing gloves from here on.
Add 10 µl Gel-Red(10000X) per 100 ml gel solution for a final concentration of 0.5 ug/ml. Be very careful when handling the concentrated stock.
Stir the solution to disperse the gel-red, then pour it into the gel rack.
Insert the comb at one side of the gel, about 5-10 mm from the end of the gel.
When the gel has cooled down and become solid, carefully remove the comb. The holes that remain in the gel are the wells or slots.
Put the gel, together with the rack, into a tank with TAE. The gel must be completely covered with TAE, with the slots at the end electrode that will have the negative current.
Procedure
After the gel has been prepared, use a micropipette to inject about 2.5 µl of stained DNA.
Close the lid of the electrophoresis chamber and apply current (typically 100 V for 30 minutes with 15 ml of gel).
The colored dye in the DNA ladder and DNA samples acts as a "front wave" that runs faster than the DNA itself. When the "front wave" approaches the end of the gel, the current is stopped.
The DNA is stained with gel-red, and is then visible under ultraviolet light.

PCR – 2008 – USTC
http://2008.igem.org/Team:USTC/Notebook
Briefly, a typical reaction is set up as follows:

1. set up pre-labeled reaction tubes on ice
2. add the following components:
2µL PCR buffer (rock gently after thawing, quick spin before use)
1.2uL $MgCl_2$
0.4uL dNTPs
200nM final concentration of each primer VF2 and VR (0.2uL)
0.2uL Taq enzyme
template DNA
(note: add ddH_2O to 20µL, the total volume of PCR is 20µL)
3. make sure reaction tubes are properly capped before placing in thermocycler
4. perform PCR with an initial heating step at 94C for 5 minutes followed by 25-35 cycles of 30sec at 94C, 30sec at 55C and 1Kb/min at 72C

colony PCR – 2008 - USTChttp://2008.igem.org/Team:USTC/Notebook
0.2 uL primer #1 (to 25uM)
0.2 uL primer #2
0.4 uL dNTPs
0.4 uL $MgCl_2$
2 uL PCR Buffer
2 uL colony culture
0.2 uL Taq enzyme
15.8 uL H_20
perform PCR with an initial heating step at 94C for 5 minutes followed by 25-35 cycles of 30sec at 94C, 30sec at 55C and 1Kb/min at 72C,and 72C for 10min

1. PCR – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook

PCR System			
Reagent	Concentration/Activity	Volume (50uL System)	Volume (100uL System)
10x Pyrobest buffer II	10x	5	10
Pyrobest		0.3	0.5
dNTPmix	10mM each	1	2
Primer 1	10uM	1	2
Primer 2	10um	1	2
Template DNA	changeable	0.5	1
MgCl2(Deletable)	0.2M	0.5	1
ddH2O		40.5	81

(Pyrobest DNA polymerase from Takara Co.Ltd.)

PCR Program		
Step	Condition	Time
1	95℃	5min

2	95℃	30sec
3	[Tm(fu)-4]℃	30sec
4	72℃	DNA length/kb/min
5	RETURN TO STEP 2	30-35 cycles
6	72℃	10min
7	4℃	HOLD

2. Fusion PCR – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook

The basic system is similar to common PCR. There are some notes to raise the fusion efficiency:
a. Complementary region length: 15-20bp
b. Raise the annealing temperature in the fusion step.

Fusion PCR Program		
Step	Condition	Time
1	95℃	5min
2	95℃	30-50sec
3	{Tm(fu)+[(-2)~5]}℃	40-80sec
4	72℃	DNA length/kb/min
5	RETURN TO STEP 2	10-15 cycles
6	72℃	5min
7	Add amplification Primers	
8	95℃	2-5min
9	95℃	30sec
10	[Tm(fu)-4]℃	30sec
11	72℃	DNA length/kb/min
12	RETURN TO STEP 2	25-30 cycles
13	72℃	10min
14	4℃	HOLD

3. Restriction Digestion – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook

Restriction Digestion System		
Reagent	Concentration/Activity	Volume(50uL system)
DNA		<1ug
Restriction Enzyme buffer	10x	5uL
Enzyme 1		1uL
Enzyme 2		1uL

ddH2O		to 50uL

Incubate at 37℃, 1.5 hrs or longer (Enzymes from Takara Co.Ltd or NEB).

4. Ligation – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook

Ligation System	
Reagent	Volume(10uL system)
Solution I	5uL
DNA fragment	3.5uL(changeable)
Vector	1.5uL(changeable)

Incubate at 16-18℃,1hr or longer (Ligation Kit from Takara Co.Ltd).

5. Transformation – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook
1. Place TOP10 cells (Transgen, 100uL per well) onto ice for 10 min.
2. Add 10uL ligation mixture to the cells.
3. Place the cells on ice for 20min.
4. Heat shock at 42℃ for 1min, then place on ice for 2min.
5. Add 900uL LB without antibiotics, and shake at 37℃ for 1h.
6. Centrifuge at 8,000rpm for 1min.
7. Decant the supernatant, resuspend the cell pellet in 200uL LB, and spread the cells on LB plates of corresponding antibiotics.

1. Advanced Protocol for Parts Extraction – 2008 - Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook
We have revised the Parts Extraction Protocol and obtained a higher efficiency of transformation. Here are some details:
1. Dissolve the plasmid in Elution Buffer for 30min or longer to get a higher concentration of plasmids.
2. Shake cells at 37℃ for 2h or longer before spread. This would help the cells recover from heat shock.
By Yilong Zou

2. BioBrick Parts Making Protocol – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook
1. get desired sequences through NCBI or other sources and check for restriction sites
FOR no (Xba1 or Spe1)
GOTO STEP2
ELSE
GOTO STEP9
2. Design primers with half-prefix (Xba1) and half-suffix (Spe1)
3. PCR from according genome/plasmid
4. Purify PCR product using Gel Extraction Kit (Transgen)
5. Digest with Xba1+Spe1 (Takara)
6. Ligation with pSB1AC3, which was digest with Xba1+Spe1 (Takara) and treated with CIAP
7. Transform to TOP10 cells
8. Identify clones with colony PCR
GOTO STEP20
9. Design primers with full-prefix and full-suffix

10. PCR from according genome/plasmid
11. Purify PCR product using Gel Extraction Kit (Transgen)
FOR EcoR1
GOTO STEP12
ELSE
GOTO STEP14
12. Digest with Xba1+Pst1 (Takara)
13. Ligation with pSB1AC3, which was digest with Xba1+Pst1 (Takara) and treated with CIAP
GOTO STEP16
14. Digest with EcoR1+Spe1 (Takara)
15. Ligation with pSB1AC3, which was digest with EcoR1+Spe1 (Takara) and treated with CIAP
16. Transform to TOP10 cells
17. Identify clones with colony PCR
18. Extract plasmid and site-directed mutate by fusion PCR
19. Transform to TOP10 cells
20. Extract plasmid and send sequencing
END ^^
By Qi Liu

3. Quick & Easy Knock-out Protocol – 2008 – Tsinghua
http://2008.igem.org/Team:Tsinghua/Notebook
(Reference 1)
1. Transform BW25113 with pKD46, incubate at 30℃ on LB plate with Amp+100.
2. Incubate BW25113-pKD46 in 100mL LB (Amp+100) at 30℃ until OD600=0.5~0.1. Add 1mL L-arabinose and incubate until OD600=0.6.
3. Place the cells on ice for 1h, wash with cooled water twice and 10% glycerol once, resuspend with 100uL 10% glycerol to make the competent cells of BW25113-pKD46.
4. Transform BW25113-pKD46 with 10uL PCR product of the DNA segment (for short, S) to be knock-out(30ng/uL), incubate at 30℃ on LB plate with Kan+50.
5. Identify clones using clony PCR with S-upstream + Kan, S-downstream + Kan, and S-upstream and S-downstream.
6. Incubate at 37℃ without antibiotics to throw out pKD46.
7. Select clones with Amp+100 (the cells would die) and Kan+50 (the cells would survive) LB plates.
8. Treat the selected cells as described in Step 2&3, then transform with pCP20, and incubate at 30℃ on LB plate with Amp+100.
9. Pick out several clones and incubate them at 42℃ for 24h.
10. Select clones with Amp+100 and Kan+50 LB plates(the cells would die on both plate).
11. Further identify clones using clony PCR with S-upstream and S-downstream.
12. Extract plasmid and send sequencing.
By Yicheng Long

Digestion and Ligation - 2008 - NTU-Singapore
http://2008.igem.org/Team:NTU-Singapore/Wetlab/Protocols
To ligate 2 Biobrick parts together, firstly we identify one part to be "vector" and the second part to be "insert". There are 2 possibility of ligation:
Case I: Insert in front – Vector behind
Case II: Vector in front – Insert behind
Case I: Insert in front – Vector behind
1.Digest Insert gene with EcoRI & SpeI and Vector gene with SpeI & PstI.
Purify the digested products with PCR Purification Kit and test for concentration and purity using

NanoDrop.

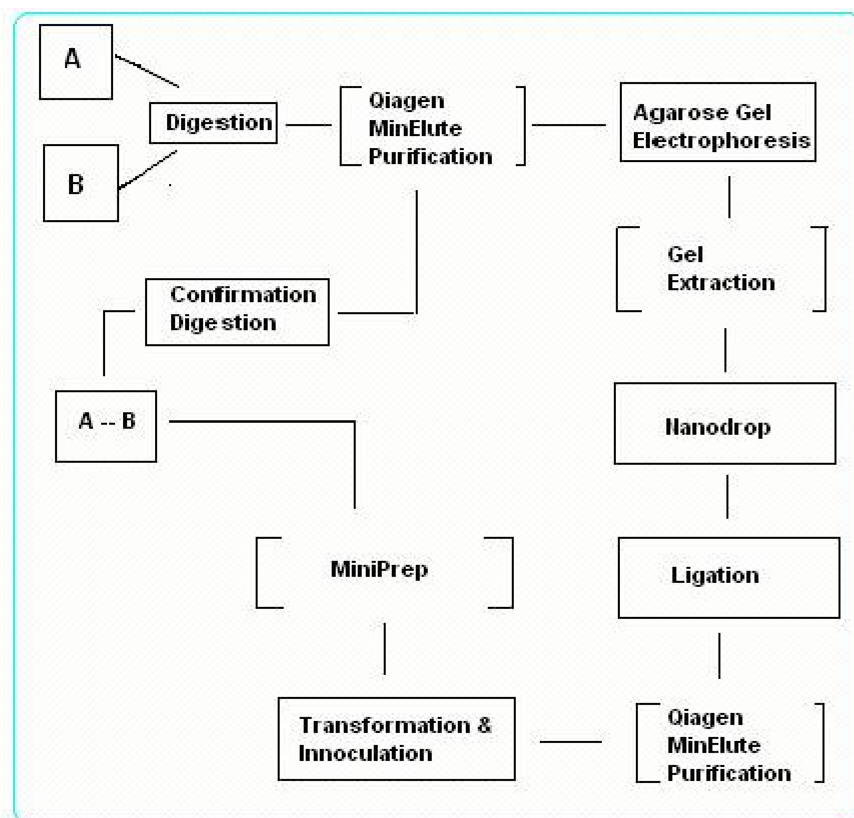
2.Perform a gel electrophoresis of the purified products, followed by a gel extraction using PCR Gel Extraction Kit.

3.Fuse the Insert gene to the Vector gene to form a circular plasmid DNA using T4 DNA Quick Ligase. Transform the DNA plasmid into Top 10 Competent Cells and select using appropriate antibiotic treatment.
Vector gene contain antibiotic resistant gene that confers resistance to the cells that are successfully transformed.

4.Extract the ligated DNA using MiniPrep Kit and digest the resultant DNA using EcoRI & PstI.

5.Lastly, perform a gel electrophoresis for confirmation. Check if the nucleotide length from the gel run matches the hypothetical nucleotide length of the digested product. If the result matches, ligation is successful.
Case II: Vector in front – Insert behind
1. Digest Insert gene with XbaI & PstI and Vector gene with EcoRI & XbaI. Purify the digested products with PCR Purification Kit and test for concentration and purity using NanoDrop.

2. Repeat Step 2 ~ 5 as from Case I



Summary of Digestion and Ligation Procedures
OD600 measurement of pLsrA-Lysis-containing LuxS mutant upon addition of AI-2 - 2008 - NTU-Singapore

http://2008.igem.org/Team:NTU-Singapore/Wetlab/Protocols
1) One colony of LuxS(-) W3110 strain containing pLsrA-lysis plasmid was inoculated overnight in 50ml LB inside 250ml flask at 37 degrees C and 225rpm.

2) After 16 hour inoculation, 0.5 ml of cell sample was diluted in 50ml fresh LB.

3) Diluted cell sample was distributed into the wells of the black-96-well microplate with an amount of 200 µL per well.

4) 50µl AI-2-containing supernatant was added into corresponding wells initially (set 1) and 1 hour after incubated inside absorbance-meter (set 2).

5) Five different supernatant solutions used, which correspond to the time points when they had been obtained during AI-2 preparation: 3, 4, 5, 6, 8 hours.

6) Control samples included
i) cell suspension alone
ii) cell suspension with 50µl LB added initially (set 1)
iii) cell suspension with 50µl LB after 1 hour incubation (set 2)
iv) cell suspension with 50µl water added initially (set 1)
v) cell suspension with 50µl water added after 1 hour incubation (set 2)

Transformation – Chiba - 2008
http://2008.igem.org/Team:Chiba/protocol/transformation
From 2008.igem.org
>Protocol
Day 1 morning
100 ml SOB medium in 1L or 500 mL flask and sterilize
E.coli culture grown in 2 mL of fresh LB medium.
Day 1 night
Inoculate preculture (100 µL-1 mL) to sterile SOB medium.Shake culture vigoruoussly at 20-25 ℃ until OD is 0.4-0.6.
Day 2
Transfer the culture to ice 10min.
Prepare Wash buffer and Competent buffer by adding 3 mL Dilution buffer to 3 mL of Wash buffer(x2) and to 2.5 mL of Competent buffer(2x), respectively.(on ice)
Pellet the cells by centrifugarion at 2500rpm for 6 min.
Remove supernatant and genlly resupend the cells in 6 mL ice-cold Wash buffer(1x).
Pellet the cells by centrifugarion at 2500rpm for 6 min.
Completely remove the supernatant and gently resuspend the cells in 6 mL ice-cold Competent buffer(1x).
Aliquot (on ice) 100µL of cell suspension into sterile 1.5 mL microtube and store in deep freezer.

Simple Hot Start PCR (& Extraction, Digestion, Ligation) - 2008 - Beijing Normal
http://2008.igem.org/Team:Beijing_Normal
Special protocol
To obtain some genes, we often have to use bacteria chromosome or large size plasmid as template in which high GC% content and complex secondary structures are seriously hampering PCR and thus

leading to complete failure. To solve this problem, we have developed a special protocol-- hot start method combined with additive(sole or mixed)-- which is most helpful .

Simplified Hot Start PCR

Hot start method is to prevent primer dimer and low specified product yield. In our experiment, We use common taq polymerase to replace commercial high-priced hot-start polymerase.

After a 5min 95℃ pre-heat step, DNA polymerase is added to each PCR tube before the PCR cycling begin. After that, the regular PCR cycling could begin.

Explanation

When reaction components are mixed at room temperature, reaction set up below the optimal primer annealing temperature, which permits nonspecific primer annealing and extention.Undesired, non-specific primer extetion products formed this way may be amplified in the PCR, resulting in misprimed products and primer ologomers.In hot start PCR, DNA polymerase is withheld from the mixture until the system has reached a temperature that favours specific primer annealing. As a result hot start PCR can greatly improve specificity, sensitivity and yield in a PCR.

Effective Additives

The most simple additive in our PCR is DMSO(>=99.9% purity) at a concentration of 5%(V/V). It works well in most 'problematic' PCR, however, when DMSO fails in some case, we have to turn to a magic mixed additive (2.7 M betaine, 6.7 mM DTT, 6.7% DMSO, and 55 ug/ml BSA) for help. This excellent additive has solved several PCR where even 5% DMSO fails.

In certain PCR there is no yield without this additives, for example the ones by which we amplify bphA1A2A3A4 and bphBC.

Explanation

One major factor limiting the output of PCR routines is that a number of DNA sequences are poorly or not amplifiable under standard reaction conditions, either because of their high GC-content or/and their instrict propertoties to form secondary structures. This protocol is intended for GC-rich DNA sequences. This is a concentration dependent combination of betaine, dithiothreitol, and dimethyl suloxide. According to the references the concentration ratio is: a mixture containing 2.7 M betaine, 6.7 mM DTT, 6.7% DMSO, and 55 ug/ml BSA. It is stable at -20℃ for at least 3 months.

References for special protocol:

1. David E. Birch et al., Simplified hot start PCR, Nature 381:445-446 (1996)

2. S.Kaijalainen et al., An alternative hot start technique for PCR in small volumes using beads of wax-embedded reaction components dried in trehalose, Nucleic Acids Research 21:2959-2960 (1993)

3. Yukihiro Kitade et al., Effect of DMSO on PCR of Porphyra yezoensis(Rhodophyta) gene, Journal of Applied Phycology 15:555-557 (2003)

4. Markus Ralser et al., An efficient and economic enhancer mix for PCR, Biochemical and Biophysical Research Communications 347:747–751 (2006)

PROTOCOL-1 Pattern formation – 2008 – iHKU

http://2008.igem.org/Team:iHKU/protocol

Day1--Inoculation

Inoculate single colony into a 5ml LB with antibiotics into a Sterilin 20cm3 tube. Incubate in 37oC rotor at medium or maximum rotational speed.

Day2--Soft Agar Plate Preparation

Weigh LB broth with accuracy to 3 decimal places. Prepare a master 2% LB of desired volume in a Pyrex bottle.

Weigh BactoAgar with accuracy to 3 decimal places. If BactoTMAgar is used, add 0.3g per 100ml of LB. If Difco Agar is used, add 0.45g per 100ml of LB.

Add to a Pyrex bottle (autoclavable) the agar and the appropriate LB solution to dissolve the agar.

Autoclave the LB agar, then place the LB agar into a 55oC before it solidifies.

Leave the LB agar to cool to 55oC for at least half an hour.

Add glucose, IPTG (v/v) 1:1000, antibiotics (v/v) 1:1000 (if applicable) into LB agar. Mix well.
Aspirate 10cm3 of LB agar for each Petri dish, shake the plate to flatter the solution across the whole plate
Wait for at least one and a half hour before adding cells to the plate.
preculture for pattern development on Soft Agar Plate
Add 2ml LB with appropriate antibiotics to a Falcon 10ml preculture tube.
Add 40ul of the inoculation sample (dilution rate 1:50) to the tube.
Place tubes in 37oC shaking incubator until OD600 reaches 0.8 - 1.0.
Add 2ul of the cell culture to the centre of a soft agar plate.
Gently place the plates in 37oC and incubate overnight (12 – 19 hrs).
<Note>
Use fresh plate, do not use plates prepared the day before since water evaporates out from the plate and will cause the cells to swarm on the surface.
Glucose & Arabinose can be stored in room temperature but best is to store it in 4oC.
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PROTOCOL-2 Picture capture – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Preparation:
Light source: lamp
Frame: card-board box (e.g.with size37cm*28cm*22cm)
Background curtain: light-absorb cloth
Picture capture device: web camera
Computer: desktop all laptop
Location: warm room (37 ℃)
Device setting: (Fig.1.)
The lamp should be fixed on the wall with its light shooting directed to the wall. The reflected light will then enter the dark box with the light-absorb cloth wrapped inside. Drill hole(s) on the top of the box whose size is fit for the camera lens. A box of our size can hold up to 4 web cameras to take picture simultaneously.
The light-absorb cloth is simply stapled to the inside wall of the card-board box. It will largely reduce the light refraction inside the dark box as well as give a high contrast background to the bacteria pattern which is white in observation.
(If the light intensity is still too high, a piece of normal A4 paper or paper tissue can be used as a mask on the open side of the box.)

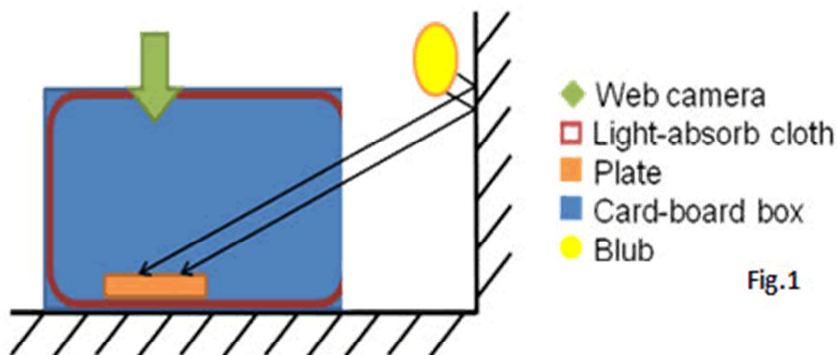

Fig.1

Picture capturing:
Software used for monitoring and capturing pictures may be differed from various web cameras made from different companies. Since the web camera we purchased is a product of Vimicro, we basically used their software Vimcam (which can be found here) for the analysis.
Since we may want to take picture for the same plate repetitively in a fixed time interval and a long time duration, we used a automatic approach with the help of a simple Java program. (You can find the Java code and a simple description here) The Java program is will simulate a robot who will click and release the left mouse button for a given time interval and this program will stop after a certain time duration.
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PROTOCOL-3 Brightness cell concentration relationship Measurement – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Day 1 –Streak the strand of cells to be tested on agarose plate to form single colony
Day 2 – Start seed culture
Morning: inoculate a single colony of cells (or from a patch amplified from a single colony) into X ml LB. Incubate on shaker at 250 rpm at 37oC
Day 2 – Start pre-culture before leaving
2) Afternoon or evening: centrifuge the LB seed culture at RT; drain the medium as clean as possible; resuspend the cells with the culture medium using conical flusk with as large volume as possible, I liter or 500 ml flusk is recommeded (it's better to centrifuge and resuspend for another time); measure OD; inoculate the seed cells into 1/4 the flusk volume's (exp, 250ml for 1 liter flusk) minimal medium (2% LB medium)
Day 3 – Start experimental culture after arriving
Morning: Prepare 200 ml LB-Agar with 2% LB and 0.8% Agar and autoclave, put on 50oC water bath to keep it in liquid form.
Centrifuge the pre-culture at RT; drain the medium as clean as possible; resuspend the cells into 100 ml culture medium (2% LB medium), shake it on votex to make sure cells are separated thoroughly and unified throughout the medium, measure OD(if OD is much higher than 1, then dilute with power of 2 and measure).
Prepare several clean 20 ml tubes. Pull 10 ml bacteria LB-Agar mixture with cell concentration gradient onto plates
Add different volume of bacteria into 20 ml tubes. (exp: 1ml, 2ml, 3ml, 4ml, and 5ml)
Add 2% LB medium to the 20 ml tube to fill the solution to 5 ml
Add 5 ml of LB-Agar and pull plate sequentially.

Tube	1	2	3	4	5	6
Bacteria(ml)	0	1	2	3	4	5
Cell(ml)	5	4	3	2	1	0
LB-Agar(ml)	5	5	5	5	5	5

After the mixture, the Agar Concentration will become 0.4%, which equals to the agar concentration where cells can swim into the gel, and contain different concentration of cells.
Fix a plate cover below the camera, then place the plates upside down on the cover in order to keep position fixed, and take photos one by one.
Measure the average brightness of each plate.
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PROTOCOL-4 Migration rate measurement – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Day 1 –Streak the strand of cells to be tested on agarose plate to form single colony
Day 2 – Start seed culture

Morning: inoculate a single colony of cells (or from a patch amplified from a single colony) into X ml LB. Incubate on shaker at 250 rpm at 37oC
Prepare 100 ml LB-Agar with 2% LB and 0.4% Agar and autoclave, put on 50oC water bath to cool down, and pull plate.
Day 2 – Start experimental culture
Afternoon or evening: centrifuge the pre-culture ar RT; drain the medium as clean as possible; resuspend the cells with the culture medium (it's better to centrifuge and resuspend for another time); measure OD; When it is below 1.000 inoculate the pre-culture cells into 5 ml minimal medium
When OD reaches 1.000, use pipette to drop 2 µl bacteria solution onto the centre of the prepared plate. Rotate the plate upside down and fix it below the camera, use automatic Photo taking to capture Cell migration status over-night.
Day 3 – Collect data
Afternoon: 24 hours after photo taking, retrieve the image data and measure the brightness of whole plate along time course to analyze the migration rate.
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PROTOCOL-5 Harvest sample from agar plate – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Culture of the E.coli on the 35mm petradish
Measure the OD600 of the cells
Autocleave the LB–agar and let it cool down to 42'C in water bath
Mix the cells with the agar to a final concentration of OD600 0.01
Pipet 1ml mixture into each 35mm petradish
Let grow at 37'C
Harvest the agar from the petradish
Remove the agar from the plate completely with the spoon made of soft plastic(refer to the photo attached)
Wash the plate with 0.5ml PBS?
The agar and the washing PBS is to be collected into the 1.5mL epindoff tubes?
<Note>
Cool the agar to 42'C before mix it with cell otherwise the cell will be killed.
Cool the plated agar to the room temperature before put into warm room; otherwise the solidification of the agar will be prolonged.
While culturing, the plates should not be piled up vertically, otherwise the temperature will be lower for the plates in the middle, which will result in slow growth.
All agars should be transferred to the tubes for the accuracy of later measurement.

PROTOCOL-6 β-galactosidase assay – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

1. Sample treatment
a) Harvest the culture by centrifuge (13krpm*1.5min) followed by washing with 1ml PBS twice
b) Resuspend the pellet with PBS and protease inhibitor cocktail.
c) Sonication for (5.5seconds+1second pulse)*10 minutes
d) Centrifuge (13krpm * 1min)
2. ONPG enzymatic reaction
a) Aliquot 200 microliter of treated sample into the 96 wells plate
b) Add 40 microliter of ONPG(4mg/ml in ddw) into each well
c)Read the OD420 every 10minutes for 5 minutes at 37'C on the spectrometer
3. Trouble shooting

a) Raw reading beyond most accurate region of o.2~0.4
b) Loss of activity due to high temperature or malfunction of the protease inhibitor
c) Incomplete cell lysis because too short sonication time or incomplete resuspension of the pellet
d) The temperature for the enzymatic reaction should be 37'C, so the spectrometer should be prewarmed.
< Note>
Must keep the sample at low temperature to avoid the loss of enzymatic activity.
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PROTOCOL-7 Western blot – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Buffer preparation
Rususpension buffer: 1ml stock + 0.1ml protease inhibitor (10x)
Loading buffer: 1ml stock + 0.2 ml DTT + Phenol Blue
Sample treatment
centrifuge the culture into pellet (13k rpm 10min)
Resuspend the pellet with 5xVolume(pellet) Resuspension buffer
Add 5xVolume(pellet) loading buffer
Boil the resuspend for 10 minutes at 100'C
Centrifuge for 1 min at 13krpm
SDS-PAGE
15% separation gel
5% stacking gel
Load the sample and run under the constant voltage of 100V
Western blotting
Transfer from gel to membrane
Assemble "sandwich" Transblot.
Prewet the sponges, filter papers (slightly bigger than gel) in 1x Blotting buffer.
Transfer for 1 hr at 1 amp at 4°C on a stir plate. Bigger proteins might take longer to transfer. For the Mini-Transblot, it's 100 V for 1 hr with the cold pack and prechilled buffer. When finished, immerse membrane in Blocking buffer and block overnight.
Hybridization with antibodies
Incubate with primary antibody diluted in Blocking buffer for 60 min at room temp.
Wash 3 x 10 min with 0.05% Tween 20 in PBS.
Incubate with secondary antibody diluted in PBS for 45 min at room temp.
Wash 3 x 10 min with 0.05% Tween 20 in PBS.
Detect with SUPER SIGNAL WEST PICO Kit (1ml luminol solution + 1ml stable peroxide solution
Result analysis
The size of the tagged protein can be determined by the marker (protein ladder)
The amount of the protein can be estimated by the brightness of the band, or accurately analysed by the software.
Trouble shooting
Unspecific binding : 1st antibody-membrane and irrelevant protein to 1st antibody
Film over-exposure or lack of exposure
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PROTOCOL-8 Recombineering – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

The procedure used was similar to that described by Watt et al [1]. About 500 µl from overnight cultures [5 ml L medium (containing antibiotic where applicable) inoculated from single colonies, grown at 32°C for 18 h] was expanded into 50 ml of L medium in a 250 ml Erlenmeyer flask, and incubated at 32°C for

2 h (until OD600 of ca. 0.4–0.6). Flasks were transferred to a shaking water bath at 42°C and incubated for 14–15 min, before cooling to 0°C as rapidly as possible in iced water. After 15–20 min, cells were harvested by centrifugation at 0°C (4000 g, 9 min). Cell pellets were carefully washed three times with sterilized ice-cold water (2 × 50 ml, then 1 × 1.5 ml) then re-suspended in 100–200 μl of ice-cold water. Competent cells (50 μl) were transformed with 50–200 ng of (gel purified) linear dsDNA targeting cassette using a BioRad electroporator (1.8 kV, 25 mF, 200 W). The L medium (1 ml) was added to the transformed cell mixture, which was incubated at 32°C, for 2–2? h. Cells were collected by centrifugation, ca. 900 μl of supernatant media was discarded, and then the resuspended cells were plated onto LB agar containing the appropriate antibiotic to select for resistant colonies.
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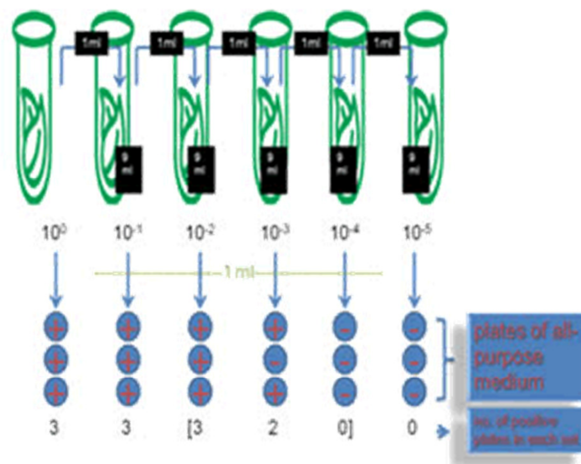
PROTOCOL-9 Most Probable Number method (MPN) – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Preparation:
A original undiluted cell culture sample (> 1ml)
Distilled water
Vortex**
OD600 reader
Fresh medium (liquid or solid)



Steps:
Measure and record the OD600 value of the original undiluted sample.
Shake the sample thoroughly and draw 100μl into a new tube which contains 900μl water. Mark the new tube with 1.
Repeat step 2 for several times according to the OD600 value which have been recorded. Every time dilute the previous sample 10 times and mark the new sample in an ascending order, i.e. 2, 3, 4… (Since it is commonly believed that there are around 109 cells in 1ml sample with OD600 measured 1.00, if this sample is used, loop for 9 or 10 times is needed)
Up to this step, a series of diluted samples should be obtained. Every time draw 100μl from the sample and drop it on a plate. For every sample, repeat this procedure for 5 separate plates.
Incubate the plates over night, at 37℃.
Record the outcome in the following format, in which 1 represents a bacteria growth is observed while 0 means not.

dilution / Plates	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
1	1	1	1	1	1	1	1	1	0
2	1	1	1	1	1	1	1	1	0
3	1	1	1	1	1	1	1	0	1
4	1	1	1	1	1	1	1	0	0
5	1	1	1	1	1	1	0	1	0
total	5	5	5	5	5	5	4	3	1

Check up the MPN table for the final result.
To see our result, please refer to the RESULT part of our wiki.
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PROTOCOL-10 Bradford assay – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

1. Bacteria culturing
A 50 mL culture of EColi. was grown in 2% LB media overnight (16 hours) in 37'C shaker. A fresh 50 mL LB culture was inoculated with 0.2 mL of these cells and grown for 5 hours to an OD600 between 0.100 and 3.000. The cells were collected with centrifugation (Beckman, 13,000rpm, 2min, rt'C), washed twice with chilled 200 λ PBS Buffer, and re-suspended in 1mL of chilled PBS Buffer.

2. Protein quantization
Sonication Settings:
1,000 µl aliquots of re-suspended bacteria were transferred to 1.4 mL polyethylene tubes tubes were subjugated to sonication times of (10"+5")*10cycles seconds at 40% power (≈4.8 watts/pin). The samples were then centrifuged at 13,000 rpm for 2 minutes to pellet debris. The supernatant containing solubilized proteins was collected for analysis.
Or B-Per protein extraction kit(recommended):
1000 µL aliquots of re-suspended bacteria were centrifuged at 13k rpm for 10 min. to pellet cells. The cells were resuspended in 200 µL of B-Per Reagent (Pierce, Rockford, IL) and vortexed for 1 minute corresponding to the Pierce protocol. 800 µl of PBS buffer was added to the solution and the solution centrifuged 10min 13k rpm to pellet debris and the supernatant collected for analysis
Protein Assay:
The amount of protein released after each sonication time was qualitatively determined by use of Bradford Reagent. An aliquot of 10 µl of each sample was mixed with 200 µl of 1:5Diluted Bradford Reagent and the absorption at 595 nm recorded (CARY300 Bio UV-Visible spectrometer) after 10~30 minutes of mixing time.
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Casting agarose gels – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Assemble casting trays, gel box and gel comb, choose the comb of appropriate width, size, and number into niche at end of the tray.

agarose solution(1% gel)：0.4g agarose + 40ml 1X TAE for small gels or 1g agarose + 100ml 1X TAE for large gels.
add agarose solution in a 500ml beaker.
Microwave bottle for 5min
Remove from microwave and let cool
Once gel is cooled so that it can be touched comfortably with your gloved hand, add 4µL(for small gel) or 10 µL(for large gels) EB dye.
Pour gel into casting trays.
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Running agarose gels – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Place the gel in gel box.
Add enough 1X TAE to fill the reservoirs at both ends of the gel box and cover the surface of the gel--the gel should be immersed.
Mix6X loading dye with samples: Typically, use 1 µL loading dye per 5 µL of sample.
Load samples left to right.
Place gel box cover on gel box such that your samples will run towards the positive, red electrode. Make sure that the cables from the cover are connected to the power supply correctly.
Turn on the power supply and run your gel at ~120 V for 30 mins.
Verify that bubbles are rising from the electrodes once you start your gel to ensure your gel is running properly.
Marker standard
We use 1kb plus DNA ladder.
Quick gel purification – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol
according to QIAprep kit protocol
Agarose gel electrophoresis, cut the DNA band as thin as possible.Then put it into a clear EP tube.
weighing,if the weight is 100mg,the volume can be considered as 100uL,add QG beffer,put into 55℃ water bath until complently merge, it takes about 10 minutes
cool the tube to room temperature
Add the solution into the collection tube, standing for 1 minute,13,000rpm for 1 min, discard the flow-through
Add 650 µl WB,13,000rpm for 1 min, discard the flow-through
Again
13,000rpm for 2 min, discard the flow-through
Put the collection tube into a clean EP tube, Add 30 µl ddH2O in the center of the collection tube, standing for 5min
13,000rpm for 2 min.
store the DNA solution at -20℃
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PCR product purification – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol
according to QIAprep kit protocol
Take 100uL PCR product solution, add 5 times volume Binding Buffer,mix, add into spin column, standing for 2 min, 13000rpm for 1 min,discard the flow-through.
Add 650 mL buffer WB ,13000 rpm for 1min,discard the supernatant
Again
13000rpm for 2 min to wipe out the remaining WB
Put the collection tube into a clean EP tube, Add 50uL 60 ℃ddH2O in the center of the collection tube, standing for 15min

13000rpm for 12min.
store the DNA solution at -20℃
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Preparation of Competent Cells for electro transformation – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol
Materials
Media
LB(Both liquid media and media containing agar. Add certain antibiotic if it is necessary.);
Buffers and Solutions
10% Glycerin.
Special Equipments
EP tubes(1.5mL), micropipette tips, centrifugation bottles(polypropylene tubes, 50mL), graduated flask(250mL*2, 5mL*1), plates and test tubes.
Protocol
Sterilization
Including all materials in part2.
Caution (Remind): use some special marks to distinguish the sterilized materials from the unsterilized ones.
Preparation after sterilization
Chill the 10%Glycerin to 4 centigrade degree
Decant LB containing agar into the plates.(If antibiotics are necessary, be sure that they are added when the media temperature is below 60 centigrade degree.)
Streak the prepared strains onto the agar plates. Then incubate it at 37℃ for 10-16 hours.
overnight preculture
take? 0.5mL overnight culture to 50mL LB bottle.
37℃ shaking 100~120 min to O.D. 600=0.45~0.6
on ice for 30min
4000rpm 7min at 4℃
Add origin volume 10% glycerol,suspend softly.
4000rpm7 min at 4℃
Add origin volume 10% glycerol,suspend softly.
Add origin volume 1/10 10% glycerol, suspend softly.
4000rpm7 min at 4℃
Add origin volume 1/100 10% glycerol, suspend softly, store at -80℃.
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Electro tranformation
Hold competent cells (from -80℃ refrigerator) on ice.
Gently mix ligation product (1-5 μL) with cells.
transfer the cell/DNA mix into an electroporation cuvette
Note: the gene pulser should already be set properly
- time constant = 4.5 - 5.0 ms
- resistance = 200 W
- capacitance = 25 mFD
for 0.1 cm gap cuvettes, set the volts to 1.7 kV
pulse the cells once; the voltage display blinks, and the gene pulser beeps
quickly transfer 37 ℃ SOC to cuvette, mix by gently pipetting up and down, and transfer SOC/cells back to culture tube
Bath in 37℃ for 60~90 min.?
Separate cells on petri-dishes, and cultivate them in 37℃ for 12 hour.

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Ligation – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol
We use new England Biolabs T4 DNA ligase
Choose reaction volume: 5-10 μL
Mix proper proportion (usually 3~5:1) of DNA fragment and vector.
Add 10×(meaning 1/10 of final volume) ligase buffer.
Add 0.5 μL ligase per 10 μL final volume.
Bath in 16℃ water for 12 hour,
Begin transformation.
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DNA extraction
Use QIAGEN kit
Polymerase Chain Reaction – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Choose enzymes
1.We use rTaq in testing but not in cloning genes.
2.Ex Taq and LA Taq are modified Taq by Takara, they have 3'->5'exonuclease activity and a relatively high fidelity.
3.Generally speaking, genes shorter than 1000bp can be cloned by Ex Taq. Genes shorter than 1.5kb can be cloned by LA Taq.
4.Taq can add A at the end of each fragment, so their PCR product can direcly link to T plasmid.
PCR system
Normal Taq polymerase reaction component has a template of 1ng~1ug, dNTP 200uM each， primer 50pmol each， polymerase 1U， buffer and water。Template can be plasmid， colony and genomic DNA.
Taq series polymerase reaction components
Colony PCR
Template:Bacterial Colony
10×PCR rTaqbuffer 2uL
10×dNTP 1.6uL
Primer-F 0.1uL
Primer-R 0.1uL
rTaq 0.2uL
ddH2O 16uL
20uL system
Cloning
Template 1uL (above 10ngDNA)
10×PCR buffer（Ex/LA） 5uL
10×dNTP 4uL
Primer-F 1uL
Primer-R 1uL
Ex/LA Taq 0.25uL
ddH2O 37.75uL
50uL system
reaction condition

1. 95℃ 5min Taq enzyme activation by heat
2. 95℃ 30s DNA denaturing
3. Tm-5~10℃ 30s Tm is annealing temperature, with a range of 45～60℃
4. 72℃ ETs ET is elongation time, 1kb/min
28cycles
5. 72℃ 10min add A at the end of each fragment
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Plasmid digestion – 2008 – iHKU
http://2008.igem.org/Team:iHKU/protocol

Digestion temperature
Most endonuclease has the optimal activity temperature of 37℃.
enzymatic system
Includs plasmid/PCR product, enzyme, buffer and water, sometimes with BSAin it. The system for enzymatic test should be no more than 20uL. The system for recruiting vectors and fragments should be 50-100uL.
DNA concentration in digestion system
The vector concentration should be no more than 1ug, PCR fragment concentration should be no less than 1ug.
Star activity
Some endonuclease will have a less specific substrate selection under certain conditions. It will cut different sequences from original recognitions. This is called star activity.
In order to reduce the star activity, glycero concentration should not be too high and DNA concentration should not be too low. In the storage of enzyme there is glycerol. So the maximal amount of enzyme added in a system should be 10%, especially for enzymes with star activities.
The influence of Methylation
Some endonuclease activity will be affected by methylation of DNA.
There is a methylation form we can refer to.
Terminal digestion of PCR product
The terminal digestion always has a low efficiency. Some additional base pairs are always added at the end of PCR product during primer design in order to improve the digestion efficiency.
There is a form about PCR terminal digestion.
Inactivation
After the digestion, endonuclease must be inactivated before applied to ligation system. Different enzymes have different inactivation condition.
There is a form about inactivation conditions of all usual endonuclease.
Digestion Timing
1 hour enables a test digestion. If digestion is used for cutting vectors and PCR fragments, in order to digest thoroughly, about 16 hours is a relatively good choice. However, some endonuclease used in the digestion system inactivates quickly. So we need add new enzymes at intervals during digestion.
Refer to the form of different endonuclease about their remaining activity after different periods of time.

Transformation – 2008 - Chiba
Day 1 morning
100 ml SOB medium in 1L or 500 mL flask and sterilize
E.coli culture grown in 2 mL of fresh LB medium.
Day 1 night

Inoculate preculture (100 µL-1 mL) to sterile SOB medium. Shake culture vigoruoussly at 20-25 ℃ until OD is 0.4-0.6.
Day 2
Transfer the culture to ice 10min.
Prepare Wash buffer and Competent buffer by adding 3 mL Dilution buffer to 3 mL of Wash buffer(x2) and to 2.5 mL of Competent buffer(2x), respectively.(on ice)
Pellet the cells by centrifugarion at 2500rpm for 6 min.
Remove supernatant and genlly resupend the cells in 6 mL ice-cold Wash buffer(1x).
Pellet the cells by centrifugarion at 2500rpm for 6 min.
Completely remove the supernatant and gently resuspend the cells in 6 mL ice-cold Competent buffer(1x).
Aliquot (on ice) 100µL of cell suspension into sterile 1.5 mL microtube and store in deep freezer.

Agarose gel electrophoresis – 2008 - Chiba
Agalose Gel casting
Measure out the appropriate mass of agarose into glass bottle with the appropriate volume of TAE buffer
Microwave until the agarose is fully melted
Pour the agarose solution into the gelbox and let it cool for about 30 minutes, until the gel is solid
Remove comb

Running agalose gel
Load 5 µL prepared 1kbp ladder
Mix DNA solution with loading dye(6x) and water
Load it into agalose gel
Run the gel at ~100 volts for 35 mins.
Visualizing agarose gels
Remove gel from gel box
Soak the gel in ethidium bromide solution
Let it 30 min.
Place the gel in Trans-Illuminator and turn on UV light after make sure the door closing.
Print the picture.
Remove gel and throw in trash
Wipe down Trans-Illuminator if necessary.

PCR – 2008 - Chiba
Resuspend primer in Nuculease free water to 100 µM
PCR mix
DNA template 1µL
Fwd primer 10µL (final con. 10 pM)
Rev primer 10µL
10x thermo pol buffer 10µL
dNTP mix 10µL
DNA pol. 1µL
dH20 58µL

100µL
PCR cycle
Start: 94 °C for 5 min. (melt)
cycle: melt: 1 min.
anneal : 30 sec.
cycle end: extension:72 °C for 3.5 min.

25 cycles
72 °C for 10 min
store: keep at 6 °C forever

Gel extract – 2008 - Chiba
Run the Digested DNA solution
Cut the agarose target band
The chip of the gel into 2mL ADB buffer
Let it in 37 degree 30 min to solve the agarose gel.
Purify the DNA with Zymo DNA Cleam&Concentrator Kit

DNA Cleam&Concentrator Kit – 2008 - Chiba
Add 2 volume of DNA binding buffer to each volume of DNA sample, Use voltex to mix.
Load mixture silica column and place column into a 2 ml collection tube
Centrifuge at full speed for 30 sec. Discard the flow-through.
Add 200μL of wash buffer and spin 30 sec.
Place siliva volumn into a new 1.5 ml tube. Add water directly to the column matrix and spin to elute the DNA.

Dephosphorylation – 2008 - Chiba
SAP: Alkaline Phosphatase (Shrimp)
Mix
DNA fragment 1~10 pmol
Shrimp Alkaline Phosphatase (1~5 μ l) 1~5 U
10X SAP Buffer 5 μ l
Sterilized distilled water up to 50 μ l
Incubate at 37℃ for 15~30 min.
Incubate at 65℃ for 15 min. (for inactivation by heat treatment)
Purify the DNA with Zymo DNA Cleam&Concentrator Kit

Time Delay Test - 2008 - Chiba
Transformed sender and receiver into E coli strains.
Inoculated them independently in liquid media. Incubated at 37℃ 12h.
Inoculated again in Fresh liquid media upto about OD600=2 at 37℃
Washed sender and receiver.
Mixed them. (Sender:Receiver=1000μL:1000μL)
Incubated at 25°C, 30°C or 37°C.
Measured intensity of green fluorescence at regular time intervals.(Fluoroskan Ascent^R FL＆Fluoroskan Ascent^R Thermo ELECTRON CORPORATION)

Procedure for growth curve (Before Standardization) – 2008 – IIT Madras
http://2008.igem.org/Team:IIT_Madras/Project#Protocols
Inoculate the LB broth with a single colony and grow it as an overnight culture.
Add 50μg/ml spectinomycin for the flask containing the k12z1 strain and 100μg/ml of ampicillin for all the other flasks containing the constructs
We transfer X% inoculum such that the cells reach the OD of 0.1 in the M9 minimal media, transfer 1% of the overnight LB culture into the flasks containing the plasmids grown in M9 minimal media.
Take two sets of are samples (1ml each) from each flask for OD and CFP measurements.
Samples were taken hour.

To measure OD we centrifuge the sample for 10 min at 10000 rpm
Then resuspend them in saline and take the reading using a UV spectrometer @600nm
Saline was used as blank solution
For YFP measurements 1 ml of the sample was taken and measurement was done at a 434nm excitation and 470nm emission in fluorescence microscope
.
Growth curve (standardized) – 2008 – IIT Madras
http://2008.igem.org/Team:IIT_Madras/Project#Protocols
Inoculate the LB broth with a single colony and grow it for 6hours.
Add 100µg/ml spectinomycin for the flask containing the k12z1 strain and 50µg/ml of ampicillin for all the other flasks
Transfer 1% of the overnight LB culture into the flasks containing the plasmids grown on M9
Take two sets of are samples (1ml each) from each flask for OD and YFP measurements.
Samples should be taken hour(up to 8 hrs)
The OD was measured without centrifugation @600nm
M9 medium was used as blank solution
The YFP was measured at 514nm excitation and 527nm mission in fluorescence microscope.
Standardized protocol for stress – 2008 – IIT Madras
http://2008.igem.org/Team:IIT_Madras/Project#Protocols
Inoculate the LB broth with a single colony and grow it for 6hours.
Two flasks were assigned for each plasmid. One flask with IPTG (0.25mm) and the other without IPTG.
Transfer 1% of the overnight LB culture into the flasks containing the plasmids grown on M9
Allow it to grow for 4hrs
Then we stress it using the following conditions:
500mM NaCl in Minimal Media M9
100microM H2O2 in Minimal Media M9
Heat Shock by keeping in 42 degrees for 120 seconds
PH of 5.5 achieved by adding 4.150 ml of 3% HCl in 50 ml Minimal M9
Starvation stress: Pellet out cells in early exponential phase and re suspend them in M9 media without glucose. This is called severe CARBON - LESS stress
The first sample was taken after 30min of stressing.
Samples were taken every subsequent hour (up to 5hours)
The OD was measured at 600nm using UV spectrometer.
The YFP was measured at 514nm excitation and 527nm emission in fluorescence microscope.
Preparation of M9 – 2008 – IIT Madras
http://2008.igem.org/Team:IIT_Madras/Project#Protocols
Prepare 10x m9 stock solution
For 50 ml of M9 media
10x m9 -5 ml
20% glucose - 1ml
MgSO4 - 0.1 ml