1. Conventional Experiment Operation Guide

1. Growing overnight cultures

1. Prepare 5ml of broth in 15ml Falcon tubes (in the case of yeast, 3ml liquid culture)
2. Add 5μL of antibiotics
3. Inoculation [a. In case of a single colonyIn use of a sterile tip, picking a single colony dipping it underneath the surface of the broth and blend multiple times.] [b. In case of preserved bacteria culturePipette 10μL of culture into the broth, blend multiple times]
4. PS. Concentration of antibiotics 0.001 (i.e 1μL of antibiotics in 1ml of liquid culture)

2. Glycerol stock preparation

1. Sterile a 1.5ml Eppendorf tube with UV by placing it inside a clean bench for 2min
2. After labeling the Eppendorf tube, pipette 300μL glycerol into it
3. Make sure the label is 100 percent absolutely correct, then add 300μL of bacteria culture into the tube.
4. Gently invert the tube multiple times to mix thoroughly 5. Update strain list
5. PS. *Aseptic operation;
6. *Two copies of each bacteria is needed, placing each in 20˚C and -80˚C strain boxes;
7. *Label template (lid:coding number)(body: strain, plasmid, date)

3. Preparation of agarose gel

1. Place a weighing paper on an electronic balance, press ‘peeling’ to zero the balance
2. Decide concentration of agarose according to DNA length (usually 1.5%) and weigh the corresponded mass of agarose into the conical flask

3. Pour respective volume of TAE into the conical flask

4. Boil the solution with microwave

5. Boiling for 10s, then swing it for multiple seconds

6. Continue heating until boiled again for 5s. Swing for multiple seconds (repeat for 3 times)

7. After cooling, add SybrSafe®(1/10000 v/v solution of the mixture) to the solution 8. Set up the gel mould

8. Gently pour the gel inside the mould, making sure the liquid is evenly spread

Size	Agarose	TAE	SYBR
Large	1.5 g	100 mL	3 µL
Medium	0.7 g	50 mL	2 µL
Small	0.45 g	30 mL	1 µL

9. PS. *Fold the weighing paper to facilitate the process of adding agarose;

10. *Cooled until the flask could grabbed by bare hand—High temperature could inactivate DNA marker;

11. *Do not forget to put gel tray

4. Gel electrophoresis

1. Emerge the gel in the TAE buffer, making sure the liquid covers all the holes

2. Make sure the holes are placed toward the cathode

3. Pipette 20% volume of 6x Loading buffer into the samples, blend with vortex 4. Always put a marker in the leftmost lane

4. Add sample that is well mixed with loading buffer into the gel, record the order on the lab note

5. Electrophoresis should last for 30min, 150v. At 15min, place it on a blue light gel imager and check if bands appear. If so, stop electrophoresis immediately

6. After electrophoresis, obtain an image in the gel imaging system. Click UV Light and Start adjusting exposure, focus, and defocus. When a clear image of gel is obtained, save it at the file: iGEM

7. 29. [Naming of gel imageDate-Enzyme-Marker-Sample1-Sample2...]

8. PS. *TAE buffer inside electrophoresis cell is recommended to change routinely. Should preserve for 3 days at most;

9. *Do not relinquish before the gun is totally out of the liquid. So samples are are not drown back into the gun;

10. *Keep the volume of sample appropriate and annihilate the presence of bubbles, both of which preventing the overflow of samples

5. Gel extraction

1. Electrophoresis 30min at 150V

2. Obtain image using Gel imaging system

3. Prepare a 1.5ml tube already named

4. Placed the excised gel fragment in a 1.5ml Eppendorf tube

5. Compress the gel on the dip of the tube using a microcentrifuge. Estimate gel volume

6. Pipette equal volume of Buffer PN into the tube, mix with vortex, and place the tube inside a 50˚C metal bath until the gel fragment is fully dissolved

7. Balance a CA2 spin column with 500μL Buffer BL during the time of gel dissolving 8. Centrifuge the spin column for 1min at 12000rpm, discard the flow-through

8. Apply the dissolved gel solution into the CA2 spin column, letting stand for 2min and centrifuging at 12000rpm for 1min

9. Collect all the flow-through back to the CA2 spin column, centrifuge at 12000rpm for 1min

10. Discard the flow-through and pipette 600μL of Buffer PW to wash the CA2 column. Let stand for 2min then centrifuge at 12000rpm for 1min, discard the flow- through

11. Pipette again 600μL Buffer PW, let stand for 2min, 12000rpm for 1min, and discard the flow-through

12. Put back the CA2 spin column, centrifuge 12000rpm for 3min without adding anything to get rid of the buffer.

13. Apply the CA2 spin column to a new 1.5ml tube (already labeled with code on the lid, plasmid name on the body) and put inside a metal bath with lid open at 50˚C for 5min

14. Pipette 30μL of buffer EB to the silicon film of the CA2 spin column, let stand in a 50 ˚C metal bath with lid closed

15. Obtain DNA concentration with a microplate reader, note it on the body of the tube(under the name of plasmid) and date of extraction underneath that. Update into

the strain list

16. 48. PS. *Gel must be excised right after electrophoresis, or else the bands should disperse

17. *When gel volume could not be estimated, add 300μL of buffer PN

18. *The maximum volume of CA2 spin column is 800μL, thus when exceed of this value, repeat multiple times

19. *DNA fragment for sequencing could dissolve in ddH2O, whereas DNA for preservation should be dissolved in EB

20.

21. *Remember not to choose individual blanking when using micro plate reader

22. *Only apply sample when CV is less than 10%. If CV is higher than 10% please wash the plate and repeat blanking

23. *Do not touch the bottom of the plate when washing

6. Preparation of competent cells

1. Spread the bacteria needed for competent cells on plates and grow overnight

2. Pick a single colony at 5ml LB (no antibiotics)

3. Growing overnight at 37˚C 200rpm from 16h~18h (most recommended at 16h and do not exceed 18h)

4. Inoculate cells in needed broth with volume ratio 1:100(e.g pipette 500μL of bacterium solution inside 50ml of LB broth, cultivate at 37˚C 200rpm)OD value could reach

0.3~0.5 after 2h~2.5h

5. 4˚C 2000g centrifuge for 5min to collect bacteria

6. Resuspend the bacteria with 6ml of LB broth already cooled

7. Add competent cell preparation solution

8. Gently blend and distribute the solution

9. Add pure alcohol into a large ice box, pour appropriate amount of dry ice, and place a tube-holder inside (making sure the 1.5ml tubes would stay underneath the ice level when inserted)

10. Insert the competent cells inside the tube-holder

11. Mark the name of bacteria on a transparent bag and put the competent cells inside, put the bag inside -80˚C refrigerator (last for half a year)

7. Transformation of E.coli

1. Blend plasmid with 100μL of competent cells, ice bath over 30 min (Allow thorough contact of DNA and cells, enabling DNA to enter the cell wall)
2. Heat shock at 42˚C for 90s (Cells swell at high temperature, allow thorough contact of the cell membrane and cell wall, enabling DNA to pass through the cell membrane into the cytoplasm)

3. Ice bath for 5min (Cell shrinks at low temperature, dragging DNA on the wall into the cell)

4. Pipette 200μL of all nutrient broth into the tube(operate inside the bench), shake the bacteria in shaker at 37˚C for 1h30min

5. Spread the bacteria on plates. Observe after 16h~20h

6. PS. *E.coli competent is extremely vulnerable, do not shake them hardly, not to mention vortex

7. *Recovery should be done with all- nutrient LB broth. Plates with antibiotics should come after recovery

8. Transformation of yeast and reagents needed

1. Spread the yeast on YPD plates, incubate 2 days at 30˚C

2. Pick a single colony and inoculate it into YPD broth, and shake for 1 day at 30˚C 200rpm

3. Dilute yeast at 1:1000 into 30ml YPD until the OD value lies at 0.6~1.0. Collect yeast after 3000rpm for 5min

4. Resuspend using 30ml of ddH2O and discard the supernatant

5. Add 1.5ml of suspensions (TELiAcH2O=1:1:8) centrifuge at 3000rpm for 5min, discard the supernatant

6. Resuspend yeast with 1.5ml of suspensions, distribute to Eppendorf tubes for 50μL each

7. Add 5μL of plasmid, 10μL of Salmon sperm DNA, 300μL of transformation reagent (TELiAcH2O=1:1:8) into 50μL of bacteria, blend gently

8. Hot bath at 30˚C for 30min, blend each 15min

9. Hot bath at 45˚C for 15min

10. Cold bath for 2min

11. Discard supernatant after 50000rpm for 3min then rests-end with 500μL of ddH2O

12. Pipette 100μL of bacterium solution on a SC-U glucose plate, spread. Incubate for 3~4 days at 30˚C

13. PS. *Salmon sperm DNA 10mg/mL

9. PCR

[Random PCR]

1. Set up a PCR system
2. 87. Use microcentrifuge to annihilate bubbles in PCR tube
3. Blend with Vortex
4. Place in PCR machine and set the program as needed (see Program Setting for further details)
5. 30min before PCR ends, make agarose gel for DNA recycling (see Electrophoresis for further details)
6. Add 6x Loading Buffer into each system and blend using Vortex (If using rTaq mix then skip this step for the mix contains loading buffer)
7. The first lane of the left-hand side should always be the Marker (choosing of marker depends on the length of desired DNA fraction)
8. After electrophoresis, obtain an image using gel imaging machine
9. Recycle

Amplification system	50 μL
2x Q5 mix	25 μL
Primer F	2.5 μL
Primer R	2.5 μL
Template DNA	1 μL
ddH2O	19 μL

10. PS. *Identification system 30μL Recycle system: 50μL

11. *Program Setting [1] Choose the program named after the enzyme, click edit; [2] Annealing temperature (Ta) Ta=Tm-5˚C (When using different primers, set the value the one with the lowest Tm temperature); [3] Elongation timeDecided by DNA length and Enzyme efficiency

12. 97. * %Enzyme efficiency: %Q5, 2k/min; %rTaq, 1k/min

13. Set up a big PCR system without primers

14. Pick a single colony with gun tip and emerge it underneath the surface of the system. Blend multiple times

15. Pipette 1μL of PCR system on the blocks already drown on the replicate plate

16. Distribute the big system into 28μL per PCR tube

17. Add corresponded primers (Primer F & Primer R), 1μL each to the tubes

18. Centrifuge shortly with the microcentrifuge

19. Vortex blending

20. Place in the PCR machine and set the program as needed (see Program Setting for further details

21. 30min before PCR ends, make agarose gel for DNA recycle (see Electrophoresis for further details)

22. *Add 6x Loading Buffer into each system and blend with Vortex (If using rTaq mix then skip this step for the mix contains loading buffer)

23. The first lane of the left-hand side should always be the Marker (choosing of marker depends on the length of desired DNA fraction)

24. After electrophoresis, obtain an image using gel imaging machine

Colony PCR	**30 μL**
2x rTaq mix	15 μL
Primer F	1 μL
Primer R	1 μL
ddH2O	13 μL

25. PS. *Big system is needed because one colony coμLd have multiple reactions

26. *Gel image shoμLd be saved in file: iGEM

27. *Naming of gel follows: Date- Enzyme-Marker- Sample(note the order of) e.g 20180810-Q5-200bp- pUC20ptacGFP1~3-pSC101SP1 1~3

2. Grow Pichia for Spheroplasting

1. Background

Spheroplasting: The cell wall of yeast prevents uptake of DNA. To enable yeast to take up DNA, it is necessary to partially remove the cell wall. Zymolyase is a β-glucanase that hydrolyzes the glucose polymers with α1,3 linkages in the cell wall. Addition of Zymolyase partially digests the cell wall. It is critical not to overdigest the cell wall as doing so will cause the cells to die. Zymolyase digestion is monitored by the sensitivity of the cells to SDS. Aliquots of cells are added to SDS, lysing the spheroplasts. This causes a clearing of the solution that is monitored by the absorbance (light-scattering) at 800 nm. It has been empirically determined that when 70% spheroplasting has been achieved, digestion is optimal. Cells are then washed with an isotonic solution to remove the enzyme and incubated with DNA. The cells are resuspended in sorbitol to facilitate cell wall regeneration and plated.

2. Material

1. Prepare the following media several days in advance and store at 4°C :

   YPD (Yeast extract Peptone Dextrose) medium, 1 liter
   YPD plates, 1 liter
   RDB (Regeneration Dextrose Base) plates, 1 liter

RDHB (Regeneration Dextrose Histidine Base) plates, 1 liter

2. Prepare the following solution on the day of transformation and maintain at 45°C:

   5% SDS solution in water

   RD (Regeneration Dextrose), molten agarose, 100 mL

3. Solutions

Spheroplasting and Transformation Reagents Provided:

- 1 M Sorbitol
- SE: 1 M sorbitol, 25 mM EDTA, pH 8.0
- DTT: 1 M DTT in water
- SCE: 1 M sorbitol, 1 mM EDTA and 10 mM sodium citrate buffer, pH 5.8 CaS: 1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM CaCl2
- Zymolyase: 3 mg/mL in water
- 40%PEG: 40% (w/v) PEG 3350 (Reagent grade) in water
- CaT: 20 mM Tris, pH 7.5 and 20 mM CaCl2
- SOS: 1 M sorbitol, 0.3X YPD, 10 mM CaCl2
- Prepare fresh for each transformation:
- SED: 19 mL of SE and 1 mL of 1 M DTT (see page 37)
- PEG/CaT: 1:1 mixture of 40% PEG and CaT (see page 39)

4. Procedure

1. Streak GS115 or KM71 onto a YPD plate such that isolated, single colonies will grow. Incubate the plate at 28–30°C for 2 days.

2. Inoculate 10 mL of YPD in a 50 mL conical tube or 100 mL shake flask with a single colony of GS115 or KM71 from the YPD plate and grow overnight at 28–30°C with vigorous shaking (250–300 rpm). You may store this culture at 4°C for several days.

3. Place 200 mL of YPD in each of three 500 mL culture flasks. Inoculate the flasks with 5, 10, and 20 µL of cells from the culture made in Step 2 and incubate them overnight with vigorous shaking (250–300 rpm) at 28–30°C.

4. The next morning, bring the transformation solutions (SE, SCE, Sterile Water, SOS, PEG, CaS, CaT, 1 M sorbitol) provided in the kit, the RDB plates (for plating transformants), and the RDHB plates (for viability control) to room temperature.

5. Check the OD600 of each of the three culture flasks. Harvest the cells from the culture that has an OD600 between 0.2 and 0.3. Centrifuge the cells at room temperature for 5–10 minutes at 1,500 × g. Decant the supernatant and discard the other cultures. Proceed to Prepare Spheroplasts.

6. Note: If the cultures are all over 0.3, choose one of the cultures and dilute (1:4) with fresh medium and incubate at 28–30°C until the OD600 is between 0.2 and 0.3

7. (2–4 hours). Harvest the cells and proceed as in Step 5, above.

3. pAO815-In Vitro Multimerization

1. Digesting Recombinant pAO815

1. Set up two separate digestions of recombinant pAO815 containing one copy of your gene:

2. Double digest 1–2 ug recombinant pAO815 with 10 units each of *Bgl* II and *Bam*H I. Use a 20 uL reaction volume and digest for 1–2 hours at 37°C to release your expression cassette. Proceed to *Producing Expression Cassettes* for Multimerization, Step 1.

3. Digest 2 ug recombinant pAO815 with 10 units of *Bam*H I only. Use a 20 uL reaction volume and digest for 1–2 hours at 37°C to linearize recombinant pAO815. Proceed to Dephosphorylating the Vector, Step 1.

2. Producing Expression Cassettes for

Dephosphorylating Rxn		
	A	B
1	BamH I Digested recombinant pAO815	17 ul
2	10x CIP Buffer	2 ul
3	CIP	1 ul
4	Total Volume	20 ul

Multimerization

1. Uses Gel Purification Kit, Follow the instruction on it. Store the purified DNA in 15 uL of sterile water and on ice. (Store at -20 if long term storage(overnight).

3. Dephosphorylating the Vector

1. Take your digest from Digesting Recombinant pAO815, Step 2 and phenol extract, then ethanol precipitate the DNA. Resuspend in 17 mL of sterile water.

2. Set up the dephosphorylation reaction in a microcentrifuge tube as follows: (*expand the system to get high concentration products (5x))

3. Incubate at 37°C for 15 minutes.

4. Uses DNA Clean Up kit to remove remaining CIP and CIP Buffer.

5. Resuspend the DNA pellet in 8ul of ddH2O

6. Save On Ice/-20

4. Ligating and Digesting the Expression Cassette

1. Set up a 20 ul ligation rxn as follows

Ligation Rxn

	A	B
1	Bgl II-BamH I digested expression cassette	15 ul
2	Sterile Water	2 ul
3	10x Ligation Buffer (w/ ATP)	2 ul
4	T4 DNA Ligase (2.5 units / ul	1 ul
5	Total Vol.	20 ul

2. Incubate at 16 Celsius for 2.5 Hrs

3. Heat inactivate the ligase by incubating at 65 Celsius for 20 min.

4. Add the following reagents for the restriction enzyme digestion. BamH I and Bgl II may be used with the same restriction buffer.

Digestion Rxn.

	A	B
1	Sterile Water	23 ul
2	10x Fast digest green buffer	5 ul
3	Bgl II (10 units / uL)	1 ul
4	BamH I (same above)	1 ul
5	Total Volume	30 ul

5. Incubate the reaction at 37 Celsius for 2 Hrs.

6. Gel run

7. Extraction

5. Ligating Multimers into Linearized Vector

* Incubate overnight at 16 Celsius

6. Transform into E. Coli

1. Transform competent *E. coli* by your method of choice.

1. Set up the following ligation reactions:

Dephosphorylated vector (page 17, Step 10)	4 µL
Expression cassette multimers (Step 10, above)	4 µL
10X Ligation Buffer	1 µL
T4 DNA Ligase (2.5 units/µL)	1 µL
Total volume	10 µL

For the vector only control:

Dephosphorylated vector	4 µL
Sterile water	4 µL
10X Ligation Buffer	1 µL
T4 DNA Ligase (2.5 units/µL)	1 µL
Total volume	10 µL

2. After adding medium to the transformed cells and allowing them to recover, plate 10 L and 100 L of each transformation mix onto LB plates with 50– 100 g/mL ampicillin. Save the remainder of your transformation mix at 4°C.

3. Incubate overnight at 37°C. If you do not get transformants or very few transformants, plate out the remainder of the transformation mix onto LB- ampicillin plates.

7. Analyzing Transformants

1. Pick 20 transformants and inoculate 2 mL LB containing 50–100 g/mL ampicillin. Grow overnight at 37°C.

2. Isolate plasmid DNA and digest with *Bgl* II and *Bam*H I to release any multimers from pAO815.
   (Be sure to include *Bgl* II-*Bam*H I digested pAO815 as a control. It is possible to get vector rearrangements and deletions with large vectors in *E. coli*. Including *Bgl* II-*Bam*H I digested pAO815 will allow you to detect these rearrangements-deletions in the vector backbone.)

3. Analyze your digests on a 1% agarose gel. You should see bands corresponding to 1 copy, 2 copies, 3 copies, etc. of your expression cassette along with the vector backbone.
   (The number of copies you obtain may depend on how well a large vector is tolerated by the host strain.)

4. Once you have identified plasmids with multiple copies of your expression cassette, be sure to purify by streaking for single colonies and confirming your construct.

5. Prepare frozen glycerol stocks of *E. coli* containing each of your multimeric constructs.

6. Prepare 5–10 ug of each plasmid for transformation into *Pichia*.

4. Plasmid Miniprep (Monarch)

1. Preparation

1. Add 4 volumes of ethanol(≥95%)to one volume of Plasmid Wash Buffer 2.
2. All centrifugation steps should be carried out at 16,000 x g(~13,000 RPM)

3. If precipitate has formed in Lysis Buffer (B2), incubate at 30-37°C, inverting periodically to dissolve.

4. Store Plasmid Neutralization Buffer (B3) at 4°C after opening, as it contains RNase A.

2. Steps

1. **Pellet 1-5 ml (not to exceed 15 OD units) bacterial culture by centrifugation for 30 seconds. Discard supernatant.** 1.5 ml of culture is sufficient for most applictions. Ensure cultures are not overgrown(12-16 hours is ideal).

2. **Resuspend pellet in 200 µl Plasmid REsuspension Buffer (B1).** Vortex or pipet to ensure cells are completely resuspended. There should be no bisible clumps.

3. **Add 200 µl Plasmid Lysis Buffer (B2), gently invert tube 5-6 times, and incubate at room temperature for 1 minute.** Color should change to dark pink, and solution will become transparent and viscous. Do not vortex.

4. **Add 400 µl of Plasmid Neutralization Buffer (B3), gently invert tube until neutralized, and incubate at room temperature for 2 minutes.** Sample is neutralized when color is uniformly yellow and precipitate forms. Do not vortex.

5. **Centrifuge lysate for 2-5 minutes.** For best results, and especially for culture volumes > 1ml, we recommend a 5 minute spin to ensure efficient RNA removal by RNase A. Pellet should be compact; spin longer if needed.

6. **Carefully transfer supernatant to the spin column and centrifuge for 1 minute. Discard flow-trough.**

7. **Re-insert column in the collection tube and add 200 µl of Plasmid Wash Bufffer 1. Centrifuge for 1 minute.**
   Discarding the flow-through is optional.

8. **Add 400 µl of Plasmid Wash Buffer 2 and centrifuge for 1 minute.**

9. **Transfer column to a clean 1.5ml microfuge tube.** Use care to ensure that the tip of the column does not come into contact with the flow-through. If there is any doubt, re-spin the column for 1 minute.

10. **Add ≥ 30 µl DNA Elution Buffer to the center of the matrix. Wait for 1 minute, then spin for 1 minute to elute DNA.** Nuclease-free water (pH 7-8.5) can also be used to elute the DNA. yield may slightly increase if a larger volume of DNA Elution Buffer is used, but the DNA will be less concentrated. For larger size DNA, (≥ 10 kb), heating the elution buffer to 50°C prior to use can improve yield.

5. Prepare spheroplasts

1. Preparation

Cell pellet from Step5, "Grow *Pichia* for sheroplasting"

2. Before starting

1. You should have a cell pellet from Step 5, "Grow *Pichia* for spheroplasting".
   · Prepare 100 mL of molten RD agarose and keep at 45°C (see Appendix, page 68)
   · Thaw one tube of 1 M DTT (provided in the kit)
   · Prepare fresh SED for one batch of spheroplasts as follows:

   Using sterile technique, transfer 19 mL of SE (provided) to an appropriate sterile container (e.g., 50 mL conical tube). Add 1 mL of 1 M DTT and mix well. For best results this solution of SED should be made and used immediately.

2. **Note**: The quality and freshness of DTT is critical for a successful spheroplast preparation. The 1 M DTT provided is analytical reagent grade and must be stored at –20°C.

3. Wash the cells

1. Wash the cells from Step 5, "Grow Pichia for spheroplasting", by resuspending the pellet in 20 mL of sterile water (provided). Resuspend the pellet by swirling the tube. Transfer to a sterile, 50 mL conical tube.

2. Pellet the cells by centrifugation at 1,500 × g for 5 minutes at room temperature. Decant and discard the supernatant. The cell pellet will be used to prepare spheroplasts.

3. Wash the cell pellet once by resuspending in 20 mL of fresh SED, prepared above and centrifuge at 1,500 × g for 5 minutes at room temperature.

4. Wash the cells once with 20 mL of 1 M sorbitol and centrifuge as described in Step 2.

5. Resuspend the cells by swirling in 20 mL of SCE buffer and divide the suspension into two 50 mL conical tubes (~10 mL each).

6. Remove one tube of Zymolyase from –20°C and place it on ice. Mix well by flicking the tube several times. Zymolyase is provided as a slurry and does not go into solution. It is important to mix the slurry thoroughly before each use to ensure addition of a consistent amount of Zymolyase.

4. Add Zymoplyase

1. Use one tube of cells prepared above to determine the optimal time of digestion with Zymolyase to make spheroplasts. After you determine the optimal, use the other tube of to make spheroplasts.

2. Zymolyase digests the cell wall and makes the cells extremely fragile. **Handle the sample gently.** The moment after adding Zymolyase, the cell wall begins to get digested.

3. · Prepare at least 20 mL of a 5% SDS solution (not provided) for use below.
   · Set your UV-Vis spectrophotometer to 800 nm and blank with 800 µL 5% SDS and 200 µL SCE.
   · Set up 17 sterile microcentrifuge tubes and label them 0, 2, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50. Add 800 µL of 5% SDS to each tube.

4. From one tube of cells (Step 5, "Wash the cells"), withdraw 200 µL cells and add to the tube marked "0". This is your zero time point. Set the tube aside on ice.

5. Add 7.5 µL of Zymolyase to the same tube of cells, mix it gently by inversion, and incubate the cells at 30°C. **Do not shake the sample.** This sample will be used to establish the incubation time for optimal spheroplasting as described below. Keep the second tube of cells at room temperature for use in Step 6 below. Keep the remainder of the Zymolyase on ice.

6. Monitor the formation of the spheroplasts as follows: At time 2 minutes, withdraw 200 µL of cells (from the suspension in Step 2) and add to the tube marked "2". Repeat at time t= 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, and 50 minutes after adding Zymolyase. Read the OD800 for all samples.

7. Determine the percent of spheroplasting for each time point using the equation: % Spheroplasting = 100 – [(OD800 at time t/OD800 at time 0) × 100]
   For example:
   At time t = 0, the OD800 = 0.256; At time t = 15, the OD800 = 0.032 Calculation:
   % spheroplasting = 100 – [(0.032/0.256) × 100]
   = 100 – [(0.125) × 100] = 100 – 12.5= 87.5%

8. Determine the time of incubation that results in approximately 70% spheroplasting. This time of incubation is variable due to differences in lots of Zymolyase. In Life Technologies labs, it takes approximately 15–40 minutes of Zymolyase treatment to achieve optimal spheroplasting.

9. Note: It is important to establish the minimum time required for the desired amount of spheroplasting. Prolonged incubation with Zymolyase is deleterious to spheroplasts and will result in lower transformation efficiency.

10. Add 7.5 µL Zymolyase to the remaining tube of cells as described in Step 1 above. Incubate the tube at 30°C for the time that was established in Step 5 to obtain the optimal level (70%) of spheroplasting.

11. Harvest the spheroplasts by centrifugation at 750 × g for 10 minutes at room temperature. Decant and discard the supernatant.

12. Wash the spheroplasts once with 10 mL of 1 M sorbitol (**gently** disperse the pellet by tapping the tube, **do not vortex**). Collect the spheroplasts by centrifugation at 750 × g for 10 minutes at room temperature.

13. Wash the spheroplasts once with 10 mL of CaS and centrifuge as in Step 7. **Gently** resuspend the spheroplasts in 0.6 mL of CaS. The spheroplasts must be used immediately (up to 30 minutes) for transformation (Transform *Pichia*). They cannot be stored for much longer. This preparation yields enough spheroplasts for six transformations

6. Preparing Electrocompetent G. xylinus Cells

1. Introduction

This is the protocol for preparing electrocompetent G.xylinus cells. This protocol was developed by 2014 iGEM Team Imperial UK.

2. Material

1. Cells , Reagents and Materials

1mM HEPES (ph7.0) 80mL

HS+cellulase media

15% glycerol

50 mL tubes

Ice bucket and ice

2. Instruments

Temperature controlled centrifuge

Shaker at 30 °C, 180rpm

3. Preparation steps

1. f the goal of transformation is to produce cellulose-producing transformed G.xylinus, use regular HS media for culturing. If cellulose production after transformation is not primary and speed is required, HS-cellulase medium can be used. Usage of HS-cellulase medium during growth results in the formation of a higher number of cellulose negative mutants, but results in much higher transformation efficiencies, and requires less time. Even with HS- cellulose medium, cellulose producing colonies can be identified on the plate after transformation, as cellulose- producing colonies differ in morphology from cellulose non-producing colonies (see section *Gluconacetobacter* for images of colony morphology).

2. Inoculate 5ml of HS or HS+cellulase medium with *Gluconacetobacter*.

3. Incubate at 30°C, 180 rpm shaking overnight.

4. Next day, pour 30mL of HS or 15ml of HS+cellulase medium into each of four 50mL tubes. If using regular HS medium, vortex the tubes for 3 minutes to release cells from the cellulose pellicle

5. To each tube, add 1mL of overnight culture and incubate with shaking at 180rpm, 30C.

6. Incubate overnight or until OD600 of around 0.4-0.7 is reached. When using HS-cellulase media, OD600 can reach up to 0.6 -0.7, however when using regular HS medium, OD600 measurement is disturbed by the cellulose pellicle, and can reach up to 0.2. Vortex tubes for 3 minutes before taking the measurements.

7. If using regular HS medium, add 62ul (0.2% v/v) Celluclast cellulase to each tube and incubate at 30C, 180rpm shaking for 2 hours, or until the cellulose pellicle is degraded to completely release the cells. This does not result in higher formation of cel- mutants, as short incubation time and nutrient depletion does not allow for proliferation of cel- mutants.

8. Before continuing, set up the necessary materials:
   Pre-cool centrifuge to 4°C
   Prepare ice bucket

Chill 1mM HEPES buffer and 15% glycerol buffer on ice

9. Once the cultures reach desired OD600, take them out of incubation and put them on ice for 10 minutes (the tube should feel cool).

10. From here on, keep cells always cool, at or below 4°C

11. After cooling, spin the tubes in a refrigerated (4°C) centrifuge for 12min at 4100rpm.

12. Pour off supernatant carefully, taking care not to pour off the pellet. *G. xylinus* does not pellet as easily as E.coli, most likely due to the buffering effects of cellulose. If the pellet is not attached to the wall after centrifugation, smear the pellet onto the wall of the tube and centrifuge again using longer centrifugation times. Re-suspend bacteria in 10mL HEPES: re-suspend first using 1ml HEPES and a P1000 pipette, then add 9ml of HEPES using a stripette; it is much easier to re-suspend the pellet fully using a P1000. Do not use a vortexer.

13. Pool the samples into a single 50ml tube.

14. Centrifuge again for 14 minutes at 4100 rpm and 4 °C temp. Use a balancer tube with 40ml water.

15. Pour off supernatant again, re-suspend pellet in 10mL ice-cold HEPES on ice as before.

16. Centrifuge again for 14 minutes at 4100 rpm, 4 °C.

17. Pour off supernatant and re-suspend pellet in 4mL ice cold 15% glycerol solution.

18. Pipette 50ul aliquots into tubes. Label tubes properly.

19. Store samples on ice for immediate use or freeze 50ul aliquots in-80°C. According to some reports, the efficiency of electrocompetent cells reduces after each freezing, so immediate use may result in highest efficiencies.

7. Quantification of cellulose production

1. Introduction

This is the protocol for preparing electrocompetent G.xylinus cells. This protocol was developed by 2014 iGEM Team Imperial UK.

2. Material

1. Reagents & Instruments

G.xylinus culture

0.1M NaOH

Distilled water

Baking paper

Drying Oven

Digital Scale

3. Experiment steps

1. Add 50ml of HS medium (or other medium of choice) to 250ml conical flask

2. Grow G.xylinus in HS medium for 7 days standing, at 30C. Don’t seal the flasks hermetically in order to allow diffusion of oxygen (seal using foam buns)

3. After 7 days of growth, wash the cellulose twice with distilled water

4. Add 50ml of 0.1M NaOH to cellulose, incubate at 65C for 4 hours

5. Wash the cellulose twice using distilled water

6. Place the formed cellulose pellicle on baking paper and dry the pellicle at 65 degrees for 4 hours-overnight. Before drying, cut out and measure the weight of a piece of baking paper, and dry the pellicle together with the paper. This is because the pellicle will invariably stick to the surface, and removal of it results in loss of cellulose.

7. Place the pellicle+paper into a vacuum desiccator for 2 hours

8. Weigh the pellicle+paper using a high-sensitivity scale. Subtract the weight of the paper to determine the weight of cellulose.

4. Important Notice

CRITICAL

1. Sodium hydroxide used in this experiment is **HIGHLY CORROSIVE**. All personal operating this experiment MUST wear proper protection.

8.Sequential Double Digest

1. Introduction

1. This is the Sequential Double Digest Protocol with Standard Restriction Enzymes. If there is no buffer in which the two enzymes exhibit > 50% activity, this sequential digest can be performed.
2. More information from NEB can be found here.
3. Double Digests can be designed using NEB's Double Digest Finder.
4. NEBcloner will help guide your reaction buffer selection when setting up double digests.

5. See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

2. Material

1. DNA **1 µg**
2. NEBuffer (1X)
3. NEB Restriction Enzymes
4. Deionized Water

3. Sequential Double Digest

1. Set up the following reaction using the restriction endonuclease that has the lowest salt concentration in its
2. Recommended buffer (total reaction volume **50 µl**). 2. Set up the following digest reaction on ice.

Table1

	A	B	C
1		Volume (µl)	
2	**Buffer (10x)**	5	
3	**DNA ***	Input Volume for ng	
4	**Restriction Enzyme #1 ****	1	
5	**Deionized Water (µl)**	1	
6	**Total Volume (µl)**	#VALUE!	

3. *A 50 µl reaction volume is recommended for digestion of 1 µg of substrate.

** Restriction Enzyme, 10 units is sufficient, generally **1 µl** is used
***The enzyme should be the last component added to reaction

3. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

4. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

5. Incubate for 1 hour at the enzyme-specific appropriate temperature.

6. Can be decreased to 5-15 minutes by using a Time-SaverTM Qualified Restriction Enzyme
   See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

7. Adjust the salt concentration of the reaction (using a small volume of a concentrated salt solution) to approximate the reaction conditions of the second restriction endonuclease.
   Alternatively, a spin column can be used to isolate the DNA prior to the second reaction.

8. Add the second enzyme.
   ** Restriction Enzyme #2, 10 units is sufficient, generally **1 µl** is used

9. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

10. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

11. Incubate for 1 hour at the enzyme-specific appropriate temperature.
    Can be decreased to 5-15 minutes by using a Time-SaverTM Qualified Restriction Enzyme
    See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

9. Single-temperature Double Digest

1. Introduction

1. This is the Double Digest Protocol with Standard Restriction Enzymes, using a common reaction and same incubation temperature for both enzymes.

2. More information from NEB can be found here.
   Double Digests can be designed using NEB's Double Digest Finder.
   See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures. NEBcloner will help guide your reaction buffer selection when setting up double digests.

2. Material

1. DNA **1 µg**
2. NEBuffer (1X)
3. NEB Restriction Enzymes
4. Deionized Water

3. Single Temperature DD Reaction

1.

1. Set up the following reaction (total reaction volume 50 µl).

*Recommended maximum of 1 µg of substrate per 10 units of enzyme.

** Restriction Enzymes should be added to the mixture last.

2. Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

Table2

	A	B
1		**Reagent Volumes (µl)**
2	**Buffer (10x)**	5
3	**DNA ***	Input Volume for ng
4	**Restriction Enzyme #1 ****	1
5	**Restriction Enzyme #2 ****	1
6	**Deionized Water (µl)**	48
7	**Total Volume (µl)**	50

3. Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

4. Incubate for 1 hour at the enzyme-specific appropriate temperature.

5. Can be decreased to 5-15 minutes by using a Time-SaverTM Qualified Restriction Enzyme
   See the NEBuffer Activity/Performance Chart with Restriction Enzymes for the incubation temperatures.

10. Transformation of Pichia (spheroplast)

1. Procedure

1. For each transformation, dispense 100 µL of the spheroplast preparation from Step 9 (previous page) into a sterile 15 mL snap-top Falcon 2059 tube (or equivalent).

2. Add 10 µg of DNA and incubate the tube at room temperature for 10 minutes.

3. During the 10 minute incubation, make a fresh PEG/CaT solution. Since each transformation requires 1.0 mL of the PEG/CaT solution, calculate the amount you

need and prepare this volume by adding together equal volumes of 40% PEG and CaT (a 1:1 solution).

4. Add 1.0 mL of fresh PEG/CaT solution to the cells and DNA, mix gently, and incubate at room temperature for 10 minutes.

5. Centrifuge the tube at 750 × g for 10 minutes at room temperature and carefully aspirate the PEG/CaT solution. Invert the tube and tap it gently to drain the excess PEG/CaT solution.

6. Resuspend the pellet of transformed cells in 150 µL of SOS medium and incubate it at room temperature for 20 minutes.

7. Add 850 µL of 1 M sorbitol. Proceed to Plating, next page.

2. Plating

8. Mix together 100–300 µL of each spheroplast-DNA solution from Step 7, previous page, with 10 mL of molten RD agarose and pour on RDB plates. Allow the top agarose to harden.
   Note: There is enough of the spheroplast-DNA solution to plate duplicate and triplicate plates.

9. Invert plates and incubate at 28–30°C. Transformants should appear in 4–6 days.

10. For cell viability: Mix 100 µL of spheroplasts with 900 µL of 1 M sorbitol.

11. Mix 100 µL of this diluted sample with 10 mL of molten RDH and pour on a RDHB plate. Allow top agarose to harden.

12. Invert the plates and incubate at 28–30°C. Appearance of colonies after 4–6 days demonstrates that the spheroplasts can regenerate into dividing cells.

11. Transform Pichia

1. Before starting

1. Make sure your RDB plates are at room temperature and that you have molten RD top agarose available. Thaw your linearized DNA and keep on ice. You should have the following:

- · Your construct linearized with *Sal* I, *Stu* I, or *Sac* I to favor isolation of His+ Mut+ recombinants in GS115
- · Your construct linearized with *Sal* I, *Stu* I, or *Sac* I to favor isolation of His+ MutS recombinants in KM71
- · Your construct linearized with *Not* I, *Bgl* II, or equivalent to favor isolation of His+ MutS recombinants in GS115
- · Parent plasmid linearized with same restriction enzyme

2. Controls should include no DNA or linearized pBR322 DNA and plasmid only (no cells) to check for contamination.

2. Procedure

1. For each transformation, dispense 100 µL of the spheroplast preparation from Step 9 (Prepare shperoplasts) into a sterile 15 mL snap-top Falcon 2059 tube (or equivalent).

2. Add 10 µg of DNA and incubate the tube at room temperature for 10 minutes.

3. During the 10 minute incubation, make a fresh PEG/CaT solution. Since each transformation requires 1.0 mL of the PEG/CaT solution, calculate the amount you need and prepare this volume by adding together equal volumes of 40% PEG and CaT (a 1:1 solution).

4. Add 1.0 mL of fresh PEG/CaT solution to the cells and DNA, mix gently, and incubate at room temperature for 10 minutes.

5. Centrifuge the tube at 750 × g for 10 minutes at room temperature and carefully aspirate the PEG/CaT solution. Invert the tube and tap it gently to drain the excess PEG/CaT solution.

6. Resuspend the pellet of transformed cells in 150 µL of SOS medium and incubate it at room temperature for 20 minutes.

7. Add 850 µL of 1 M sorbitol. Proceed to Plating, followed.

3. Plating

1. *Pichia* spheroplasts need to be plated in top agarose or agar to protect them from lysis prior to selection.

2. Mix together 100–300 µL of each spheroplast-DNA solution from Step 7, previous page, with 10 mL of molten RD agarose and pour on RDB plates. Allow the top agarose to harden.
   Note: There is enough of the spheroplast-DNA solution to plate duplicate and triplicate plates.

3. Invert plates and incubate at 28–30°C. Transformants should appear in 4–6 days.

4. For cell viability: Mix 100 µL of spheroplasts with 900 µL of 1 M sorbitol.

5. Mix 100 µL of this diluted sample with 10 mL of molten RDH and pour on a RDHB plate. Allow top agarose to harden.

6. Invert the plates and incubate at 28–30°C. Appearance of colonies after 4–6 days demonstrates that the spheroplasts can regenerate into dividing cells.

4. Evaluate transformation experiment

1. After 4–6 days, His+ transformants on your sample plates will become apparent. Transformation efficiency using the spheroplast method is generally 103 to 104 His+ transformants/µg of DNA. There should be no colonies on the "No DNA", pBR322 plate, or the plasmid only (no cells) plate

5. Optimal method

1. Plating in top agarose can cause the transformants to be on top or be imbedded in the top agarose, making it difficult to pick and patch colonies as described in the next section. The following protocol allows you to collect the transformants and re-plate them directly onto plates without using top agarose.

2. Scrape the agarose containing the His+ transformants with a sterile spreader into a sterile, 50 mL, conical centrifuge tube and mix with 20 mL sterile deionized water. Vortex the suspension vigorously to separate the cells from the agarose.

3. Filter the suspension through 4 folds of sterile cheesecloth. Centrifuge the filtrate at 1,500 × g for 5 minutes at room temperature. Centrifugation pellets the cells on the bottom of the tube and any remaining agarose on top of the cells.

4. Remove the agarose pellet carefully from the top of the cells by gently shaking the tube to disperse only the agarose pellet into the water. Decant the supernatant with the agarose pellet.

5. Resuspend the cell pellet in 5 mL of sterile deionized water and sonicate for 10 seconds using a microtip and 20–30% power. Sonicate to get the cells into solution and not to lyse the cells.

6. Dilute cells by 104 and plate 50 µL and 100 µL onto MD plates. Incubate the plates overnight at 30°C. Proceed to Screen for Mut+ and MutS Transformants.

6. Chemically competent Pichia cells

1. The *Pichia* EasyCompTM Kit (see Accessory products) provides enough reagents to produce 6 preparations of competent cells; each preparation yields enough competent cells for 20 transformations. You may use these cells immediately or store them frozen for future use. Each 50 µL aliquot of competent *Pichia* cells with 3 µg linearized plasmid DNA yields approximately 50 colonies on selective medium.

7. Screen by functional assay

1. Some researchers have used a functional assay to directly test for high expressing *Pichia* recombinant clones without first screening for MutS or Mut+ phenotypes. After testing for high expression, be sure to also check the Mut phenotype. This will help you optimize expression of your recombinant clone.

12. GS115 Pichia Pastoris

1. Linearizied Plasmid's Preparation

1. Take 3ml plasmid culture that is cultured overnight, and use the plasmid extraction box to extract the plasmid. Then solute the plasmid into 50ul TE or water. Put all plasmid fragments into the 200ul system to retriction enzyme cutting linearization. Use phenol chloroform solution to remove protein and condense the fragments into a 20ul system. Repeat the preparation multiple times to unsrue over 50ug plasmid fragments as whole.

2. Transformation

1. Directly put it into the frozen pichia pastoris in order to have the maximum transform rate. (Put it into the frozon, not
   the of thawy, cells)

2. Put the pichia pastoris in to 37°C water quickly for 5min, mix the sample 1-2 times while in the water.

3. Take out the centrifuge tube, put 1.0ml Solution 1, and mix them completely and gently. (Do not mix them violently and do not use a vortex oscillator.)

4. Put it into 30°C water for 1h.

5. 2000g centrifuge for 10min under room temperature, remove the supernatant and re-suspend the precipitation in 0.8ml Solution 2.

6. 2000g centrifuge for 10min, remove the supernatant and re-susoend the sample in 0.2ml Solution 2.

7. Tile all transformation solution on selective culture plate, culture it for 3-4 days at 30°C ,and test afterward.

3. Attention

1. In step2, put DNA into the frozen cells, not the thawed cells. Make sure not put the DNA into the thawed cells.
2. 10. It's necessary to mix the sample in step3.
3. 11. In step4, make sure mixng the samples completely and gently. Do not mix the samples violently.
4. 12. Make sure all the mixation of cells are gentle.
5. 13. The usage range of yeast competent cell:
6. - X33 Competent Cell: Only suitable for plasmids that are selected by Zeocin, like pPICA/B/C and pPICαA/B/C. - GS115 Competent Cell: Only suitable for plasmids that are selected by histidine deficiency, like pPIC3.5K and pPIC9.0K.
   - KM71H Competent Cell: Only suitable for plasmids that are selected by Zeocin, like pPICA/B/C and pPICαA/B/C.