The iGEMer's Guide to the Galaxy

Getting Started

Media

Most of the time you'll autoclave the media after putting it together, although certain chemicals (vitamins, antibiotics) need to be added after to prevent degradation.

LB

- Use for E. coli and B. subtilis
- Mix 20g/L of LB Broth powder into the desired volume of deionized water (which you can get in room 346, we'll show you how the filter system works)
- We highly recommend that you mix liquid media in small aliquots (100-200mL) so the whole batch doesn't get ruined when one gets contaminated (LB is a very rich medium, so this happens a lot)

BG11

- Use for Anabaena
- We have 50x stocks of BG11 in the fridge that are or can be aliquoted into 20mL samples
- Add 20mL of 50x stock to 980mL sterile water (autoclaved beforehand, and allowed to cool)
- This media doesn't have any solid carbon source, so it's pretty safe to make larger quantities

Antibiotic Stocks

We usually make our liquid stocks of antibiotics at 1000X, so that you add 1µl/mL to whatever media you are using. While the desired working concentration might change based on the plasmid, here are the stock concentrations we usually use for common antibiotics:

- Chloramphenicol: 34mg/mL (100% EtOH)
- Ampicillin: 100mg/mL (50% EtOH)
- Kanamycin: 20mg/mL (H20 only)
- Neomycin: 50mg/mL (H20 only)
- Tetracycline: 15mg/mL (50% EtOH)

You'll want to make these by filter sterilizing; you cannot autoclave antibiotics. We typically store them in 1mL aliquots in a -20C or -30C freezer. Those stocks made with ethanol will not freeze. Those in water only will require thaw time. Once you take an aliquot, it becomes yours.

Plates

Basics

- Using any typical media recipe, add 1.5% agar (15g/L) to the mixture before autoclaving

- After removing from autoclave, let cool until it is cool enough to hold for several seconds comfortably
    - Otherwise the media will be too hot and break down the antibiotic
    - Note: Chlor is a bit more heat-tolerant than other antibiotics
- Add the appropriate amount of antibiotic
- Pour enough media into each petri dish to just cover the bottom
    - *E. coli* grows on the surface, so the agar layer shouldn't be thick
    - Since the dishes come in sleeves of 25, it is usually good to make 500mL of the medium

Standard Molecular Workflow

Note: PCR is its own huge beast, so it's been given its own section following this one

Liquid Culture

- Inoculation:
    - Pipette tips work great for swiping or stabbing a colony
- Media:
    - LB works great for both E. coli and B. subtilis
- Temperature:
    - 37°C works fine for both E. coli and B. subtilis
- Shaker speed
    - 250 RPM for optimal growth, 200 OK
- Antibiotics:
    - If it is appropriate to select for the strain using antibiotics, add 1µl per mL of 1000X stock solution
- For best results (but certainly not necessary):
    - Pre-culture for ~6hrs in 20-25% final culture volume
    - Incubate in container with capacity >200% culture volume, overnight

Cryostocking

Any time you generate a new strain (i.e. transform a new combination of DNA parts) you should generate miniprep (for DNA) and a cryostock (for frozen cells).

It's pretty simple:

- In a cryostock tube (1.8mL tube with screw top), mix a dense liquid culture of the strain with glycerol to the proper percentage (I think there's some flexibility but Jesse usually goes for 20% glycerol for both E. coli and B. subtilis)
    - So this might look something like: 500µl liquid culture + 500µl 40% glycerol solution

Sterile technique is super important when making cryostocks

Miniprep (Qiagen, modified slightly)

1. Spin falcon tubes @7600rpm, @4C, for 5min to pellet
2. Discard supernatant by decanting
3. Reconstitute pellet in 250µl cold Buffer P1 and transfer to microcentrifuge tube
4. Add 350µl Buffer P2, invert 4-6 times to mix thoroughly
    a. Let stand for less than 5 minutes
        The timing for the next few steps is important. Don't delay.
5. Add 350µl Buffer N3, immediately invert 4-6 time to mix **thoroughly**
    a. This step will form a white precipitate
6. **Immediately** spin @max speed for 10min @room temperature
7. Pipette supernatant into spin column while avoiding the precipitate
8. Centrifuge 60sec, discard flow-through
9. Add 750µl Buffer PE to column, let sit for 60sec, spin 60sec
10. Discard supernatant and spin another 60s to dry
11. Transfer to clean microfuge tube and let sit 60s
12. Add 30 or 50µl qH2O and let sit 60s, spin 60s
    a. 30 results in higher concentration but lower total yield
13. Pipette and reapply flow through, sit 60s, spin 60s
14. Nanodrop

Digestion:

20 µl Recipe for any combination of the EcoRI, XbaI, SpeI, PstI

- 500-1000 ng DNA (as close to 1 µg as possible)
- 0.2µl Enzyme 1
- 0.2µl Enzyme 2
- 2µl appropriate buffer (see NEB enzyme doubledigest finder; for any
- combination of the biobrick enzymes, buffer 2 or buffer 3 will be great)
- 0.2µl BSA (if necessary, the newer buffers like CutSmart already have it)
- Top up with qH20

Mix reagents, adding enzymes last
Incubate at 37°C for 1-2 hrs (<30 min for HF)
Heat kill at 80°C for 20 minutes if proceeding to ligation

Verification

Gel Casting

0.75% agarose

- Use if DNA > 1000bp
- 40mL 1x TAE
- 0.3 g agarose
- 1 aliquot (~5µl) gel red

Add dry agarose to clean bottle (small enough to fit in microwave)
Add 40mL 1x TAE buffer
Microwave with cap on but loose, swish periodically, until solution is clear and smooth

- Agarose is very easy to overheat. Check it after 30 seconds.

Pipette in gel red, directly into solution (heat stable so don't worry about the temperature)
Pour into gel tray, making sure that tray is oriented and tightly inserted such that leaks will not occur, and that the gel is level

- It helps to pre-wet the rubber seals

Gel Loading & Running

- Lane 1 should be ladder; use 1kb ladder or 100bp ladder depending on the size of your DNA samples
- Digests can require more (~1.5x) than the usual amount of loading dye

Gel Imaging (using Typhoon scanner)

- Always scan a gel immediately after running
- Make sure the scanner area is clean; wipe ONLY with 70% ethanol (or DI) and kimtech wipes
- Gel should be placed on scanner face-up. That is, the wells should be oriented up, the same way the gel is oriented in the gel box

Gel Extraction & Cleanup

- Make sure to place gel on transilluminator face down (wells toward the glass)
- Remove as much excess gel matrix as possible without overexposing DNA to UV
- For cleanup, follow protocol for using the Wizard PCR Cleanup Kit, found below in PCR section

Ligation (adapted from openwetware ligation protocol):

10 µl Recipe

- 30-50 ng vector DNA (closer to 50 is better)
- Equation for calculating ligation ratios
    - A calculator to make life easy
    - $$\frac{[vector](V_{vector})(ratio\ of\ 3\ or\ 5)}{(bp_{vector}{:}bp_{insert})}(1/[insert]) = V_{insert}$$
    - where [vector] is concentration of vector (ng/mL)
    - (V_{vector}) is volume of vector (µL)
    - ratio of 3 or 5 relates to the 3:1 or 5:1 ratio of insert to vector
    - bp_{vector}:bp_{insert} is the ratio of vector to insert base pairs
    - [insert] is the concentration of insert (ng/µL)
    - V_{insert} is the volume of insert to add (µL)
- 1µl (10%) 10X T4 DNA ligase buffer
- 0.5µl (.5%) T4 DNA ligase
- Top up w/ qH_20 up to 10uL

Procedure

- Usually heat inactivation of digests is sufficient; difficult ligations might require a proper

cleanup
- As often as possible, use isolated inserts and vectors to avoid unwanted ligations
- If the reaction needs to be greater than 10µl, adjust amount of 10X ligase buffer and T4 DNA ligase so that they remain at 1% and .5% by volume, respectively
- For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
- For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours*(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.

Chemically Competent Transformation (protocol from Kosuke)

Materials
- 1 aliquot of competent cells
- 2-4µl ligation mixture
- 500µl SOC media

Procedure
1. Thaw cells at 4°C for 5 minutes
2. Gently mix in ligation product
3. Incubate at 4°C for 20 min
    a. Meanwhile, warm SOC media to 37C
4. Heat shock at 42°C for 30 sec
    a. 45 sec for NEB5-alpha cells
5. Return to 4°C for 1 min
6. Add 500µl pre-heated SOC
7. Incubate at 37°C for 1hr with shaking
    a. Meanwhile, pre-heat plates to 37°C
8. Plate, one plate w/ 100µl, one plate w/ 150µl

Electrocompetent Cells

Alex is a fan of electroporation because it's faster and more efficient than the chemical protocol. The only downside is that it's incompatible with the NEB Instant Ligase mixes.
We got good results from these protocols.

Preparing Electrocompetent Cells

1. Prepare a 10ml pre-culture on LB medium. For best results, avoid using overnight preculture.
2. Dilute pre-culture as follows: 4 ml in 200-ml of fresh LB pre-warmed at 37°C.
3. Grow the cells at 37°C.
4. When OD (600) = 0.6 is reached, chill the culture on ice as quickly as possible.
5. Centrifuge in disposable tubes (50ml disposable type) for 5 minutes at 3000 rpm.
6. Resuspend the pellets in 25ml freshly prepared water[1] (MilliQ®quality) at ice temp.
7. Repeat steps 5 & 6 twice more.
8. Resuspend the pooled pellets in 400µl (cell concentration should be 1 x 10^{10} cells x ml^{-1}) freshly prepared water (MilliQ® quality) at ice temp.
9. Check the final volume and add 10% of glycerol (molecular biology grade).

10. Use immediately or aliquot the electrocompetent cells to 100µl in 10% glycerol and freeze at - 70°.

Transforming Electrocompetent Cells

1. Defrost an aliquot of electrocompetent cells
2. Load an Eppendorf tube chilled on ice with 40µl of cell suspension
3. Add 1 to 5µl of ligation mix (DNA)
4. Mix well and keep on ice for >1 minute
5. Select 1800 Volt as the output voltage (for 1mm cuvettes, for 2mm use 2500)
6. Load an electroporation cuvette chilled on ice with the cell suspension
7. Avoid putting your finger on the aluminium electrodes or it will dramatically increase the temperature of the sample and increase the risk of arcing
8. Trigger the pulse immediately
9. As soon as possible (less than 30 seconds) resuspend the cells in the cuvette with 1ml SOC medium (the quality of the SOC is important)
10. Transfer the cells in an appropriate vessel and incubate at 37°C for 1 hour (30 minutes is usually enough)
    a. 250 rpm shaker is best
11. Plate the cells on the selective medium. 100uL and 300uL are good starting amounts.
12. Incubate overnight and look for transformant colonies in the morning

PAGE Gel Preparation, Running, and Scanning (proteins only)

Setting up and Running the Gel

1. Use NuPAGE (NOT Bolt) gels,

2. Keep the gel in its case and rinse off with DW water

3. Carefully remove comb from the case by pulling out from both sides, be gentle! Also remove white tape on bottom for current circulation when gel is running

4. Keep gel in its case. Load gel into running box (upright). Make sure gel is secure and the segment for loading the wells is on the side opposite you.

5. Add SDS running buffer (not MOPS!) so wells overflow into front of the box

6. Before loading must wash out each gel well by pipetting gently up and down

7. Prepare loading samples (also refer to gel kit instructions)

    a) 7.5ul of product + 2.5ul SeeBlue loading dye in each well

    b) 6ul SeeBlue NuPAGE ladder (purple top, keep refrigerated)

8. BEFORE LOADING SAMPLES HEAT THEM for 10 minutes at 70 C

9. Load gel with ladder and dyed samples

NOTE: When you load a PAGE gel push pipette against the front of the box. The gel has 12 wells, if you do not need to use all 12, then avoid using the very first and the very last well; as the gel runs the current pulls unevenly from the sides (creates “smiling effect” that can make interpreting the scan more difficult)

10. Run gel for 35 minutes at 150-200V

Fixing and Staining the Gel

1. After running, the gel needs to be fixed, stained overnight, and then washed before it can be scanned on the Typhoon scanner. Prepare fixing solution for the gel (ideally you should do this while the gel is running)
    Fix solution recipe:
    50% methanol, 7% acetic acid, fill with milliQ water to 200ul
2. Remove the gel from the running case and place it in a clean container with fixing solution
3. Put gel in 100ul of fix solution and shake in RT at 80rpm for 30 minutes
4. Repeat step 3 with remaining 100ul fix solution
5. Remove all fix solution from container with the gel
6. Soak gel in 60ml SYPRO Ruby gel stain, shake overnight at 80rpm in RT.

Washing and Scanning the Gel

1. Remove PAGE gel from overnight staining and put into new, clean container
2. Wash gel in 100ul of wash solution for 30 minutes in 70-80rpm for 30 minutes
    Wash solution recipe:
    10% methanol, 7% acetic acid, fill with milliQ water until 100ul
3. Remove gel from wash and rinse twice with DI water for 5 minutes to remove all wash to prevent damage to scanner

Scanning a Protein PAGE gel

1. Same as DNA gel, specify size of the gel for the scanner. When loading, make sure to be gentle with the gel (it's fragile!) and carefully separate combs when on the scanner so you can tell which well is which

PCR (Polymerase Chain Reaction)

Templates

1. Amplifying from a plasmid or isolated sample of DNA

You have a tube of linear or plasmid DNA like that from the registry directly and don't want to wait for the the transformation and miniprep. (note: you should go through the time-intensive transformation in parallel regardless).

In this case, you need first to know the concentration of your sample. If you don't know it or it was not provided, you can learn the concentration for your sample by using the nanodrop machine located in room 347. It depends on the size of your template, but as a general rule, you need on the order of 25-50 ng template minimum for a successful PCR, so adjust the volume of your template in your PCR accordingly.

2. Colony PCR

You can also amplify plasmid or genomic DNA straight from live cultures of organisms containing your desired sequence. You will usually have cultures in one of two forms: either in liquid culture, or spread on an agar plate. If you are amplifying from liquid culture, grow it up as much as you can and add 1µL of the culture to the PCR mix. If you're amplifying from the plate, there is no need to add a volume; instead, simply take a pipette with a pipette tip from the green box, gently touch the pipette tip to the desired colony on the plate (try to take as little from the plate as possible; agar can screw up PCRs), and then insert your pipette tip into the PCR mixture and pipette up and down to mix.

Polymerases and Master Mixes

GoTaq Green

http://www.promega.com/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-m712-protocol/

Q5 Polymerase

Q5 is a fast, high-fidelity polymerase that even beats Phusion. Unlike Taq, Q5 produces blunt-end amplicons. It's also very expensive so treat it carefully.
25 µL recipe:

- 5 µL 5x Q5 buffer
- 0.5 µL 10mM dNTPS
- 1.25 µL forward primer (10µM dilution)
- 1.25 µL reverse primer (10µM dilution)
- Template DNA (a couple nanograms worth)
- qH2O to 24.75 µL
- 0.25 µL Q5 enzyme (add last)

The 50uL recipe (when you needs lots of product) is simply double.

Thermocycler Conditions

Taq polymerase (GoTaq Green)

1. Initial Denature: 95°C 2 min
    a. The official Platinum Blue protocol calls for
    b. 94°C for 3 min, although I have never done it that way. Either will work, I am sure.
2. Denature: 94°C 15-30 secs
    a. Use a shorter time if the amplicon is a relatively short segment of DNA, and a longer time if it is a relatively long piece of DNA.
3. Annealing X°C 15-30 secs
    a. This is the most crucial step of the thermocycle! Your annealing temperature will be determined by the melting temperature of your primers. As a general rule, your annealing temperature should be about 5° lower than the lowest melting temperature of your primer pair. Additionally, if you are trying to add tails to you amplicon (e.g. you are trying to add restriction sites to the ends of your DNA template), you may need to drop the annealing temperature down even more. I

have had primers with melting temperatures above 65° that needed to be annealed at 42°.
    b. Additionally, if a primer may be difficult to anneal to the template, you can increase the annealing time for better results.
4. Extension 72° X seconds
    a. Taq extension runs at 1kb per minute. Therefore, allow the extension step enough time to fully copy your entire amplicon.
5. Repeat steps 2-4 32X
6. Final Extension 72°C 5 min
7. Hold 4°C forever

Q5

1. Initial Denature at 98°C for 30 sec
2. Denature at 98°C for 10 sec
3. 3. Annealing at X°C for 15-30 sec
    a. Use the NEB calculator: https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator
4. Extension at 72°C for X seconds
    a. Q5 is much faster than Taq, and requires 20-30 sec per kb.
5. Go to step two 25-35X
6. Final extension at 72°C for 2 min
7. Hold 10°C forever (zero minutes=forever)

The standard protocols for various polymerases can be found at these addresses:
GoTaq:

- http://www.promega.com/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-m712-protocol/

Q5:

- https://www.neb.com/protocols/2012/09/27/pcr-using-q5-high-fidelity-dna-polymerase-m0491

PCR Cleanup (using Wizard SV Gel and PCR Purification System)

Sample Prep

Gel Extraction:

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
    a. Trim the slice of parts that don't contain DNA
2. Weigh gel slice (by weighing the tube containing the slice and subtracting the mass of the empty tube)
3. Add 10µl Membrane Binding Solution per 10 mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved (usually 10-15 minutes)

PCR Amplifications:

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at max speed for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube. If you are worried about the final concentration of your purified product, you can repeat this step to maximize the amount of DNA bound to the filter.

Washing

1. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at max speed for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
2. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at max speed for 5 minutes.
3. Empty the Collection Tube and re-centrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

1. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
2. Add 30-50 µL of Nuclease-Free Water to the **center** of the minicolumn. Incubate at room temperature for 1 minute. Centrifuge at max speed for 1 minute. By adding less water, like 30 µl, you will increase the concentration but decrease the total amount of product. On the flipside, if you want to maximize product, you can maximize elution volume so long as you don't care about concentration.
    a. Note: you can also increase yield by warming the elution water before hand. I usually warm it to 40°C with good results.
3. Discard Minicolumn and take sample to nanodrop (see 'Nanodrop', below)
4. Store DNA at –20°C.

The standard protocols for the SV Wizard Gel and PCR purification kit can be found here:
http://www.promega.com/resources/protocols/technical-bulletins/101/wizard-sv-gel-and-pcr-cleanup-system-protocol/

ELIM Biopharm: Primers & Sequencing

Primers

Designing Primers

- Choose a forward and reverse primer from a location in the gene or plasmid that is sure to include the portion desired for amplification or sequencing
    - For sequencing, it is desirable if possible to have primers that fall 50-150bp outside your desired region, to ensure that accurate reading occurs for the whole

gene (often the first and last ~100bp in the read are very inaccurate)
    - For PCR remember that the sequence portion corresponding to the primers themselves will be amplified also
- Primers should normally be between 15-30bp in length (around 20bp is ideal)
- Desired melting temperatures are generally between 55-65°C
    - As you will see, melting temperature is a function of length and GC content, so it is often difficult to design primers in regions much greater than 50% AT
- Forward and reverse primers should have the same melting temperature, or with a difference of no more than 3 degrees
- The annealing temperature used for a pair of primers should be set at 5 degrees below the lower melting point of the primer pair
- Using a tool like ApE or Geneious makes it easy to select certain sections of a sequence to check for primer features like melting point and GC content
- IDTs 'Oligo Analyzer' is a great tool to check for primer dimerization, hairpin structures, etc.
    - http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/
    - Use this tool or something like it as a final check to make sure your primers will not be likely to react with themselves or each other around the temperatures they will be active for gene interaction
- NCBI Primer Blast is another great tool. It can be used both to help design the primers and to ensure that the primers you choose will not amplify any genomic DNA in a colony based amplification
    - http://www.ncbi.nlm.nih.gov/tools/primer-blast/
    - Primer Blast isn't perfect. It will often miss off-target products, or predict ones that don't happen.

Special BioBrick considerations

Primer Dilution (stock preparation)

- Once you receive your primers, you need to dilute them; Kosuke does 1/10 dilutions,
- iGEM typically uses 1/20 (10μM) dilutions
- Typically we create 100-200μl working stocks; it will take a long time to use up that much primer
- Example:
    - 10μL primer stock
    - 190μL qH2O or TE buffer

Sequencing

Two main reasons:

- After a difficult pcr/gel extraction to ensure the product is correct
- After cloning/biobricking to ensure no errors were introduced during PCR

Ordering Sequencing

- You can put in the order using the same general procedure for ordering primers

- The important difference is either before or right after ordering, you need to actually prepare the DNA that will be picked up for sequencing (see Premix Specifications below)

Premix Specifications (plasmid DNA)

Prepare the DNA as specified by ELIM. For plasmids, this looks like:

- 500ng DNA
- 0.8µl primer (one primer per sequencing reaction)
- Top up w/ qH20 to 15µL

For sequencing other DNA (e.g. PCR product) see the ELIM website for specifications: http://www.elimbio.com/Sample_Preparation.htm

Checking the Data

- Usually sequencing data will be available the morning after you put in the order, sometimes early, sometimes closer to lunchtime
- The results can be accessed again through the ELIM site; after signing in, there is an option to "retrieve/download sequencing data"
- Tools like ApE or Geneious will be needed to properly read the files
- Each sequence read will come with a '.ab1' file that visualizes the data, and a '.seq' file that actually gives you the sequence they read
    - Check the ab1 file first; you're hoping for strong clear peaks, where one of four different colors represents each possible base
    - Typically the beginning and end of the read will look sloppy, but the middle few hundred bases should look very pretty
    - If the read looks pretty clean, then open the .seq file and compare the sequence to the theoretical sequence.

iGEM and the Registry of Standard Biological Parts

Using iGEM Registry DNA

Detailed instructions for locating a particular part and reconstituting that DNA from the iGEM distribution plates can be found here:

- http://partsregistry.org/Help:Distribution_Kits

The plates are currently stored in the freezer in room 378
Important points:

- Transform the DNA into an E. coli cloning strain e.g. DH5α
- As always when generating a new strain: grow a liquid culture, cryostock the strain, and miniprep to have a source of the DNA

Creating a Registry Page for a New Part

The iGEM site guides you through this pretty well. From the main page for the Registry you will see a link for 'add a part' and go from there

- You will have options for submitting basic parts or composite parts; usually whatever functional unit you end up using in the iGEM projects will be a composite part, but for

every composite part you'll also want to create an individual part page for the basic parts from which it is made In fact, the basic parts pages should be made first, so you can reference them in creating the page for the composite part

- It's important to include as much information as possible on the parts
    - A lot of this will come late in the game of course as you get data on part functionality, etc. but sections like 'design considerations' can be filled out earlier
    - Be sure to take lots of pictures that can help represent results of part function
        - Parts pages with images look a lot more legit
    - Don't settle for hyperlinks to data or images elsewhere, actually embed them in the wiki page for the part
        - Information in links only won't be counted by the judges, we found out first hand

Submitting Physical Parts to the Registry

- You probably won't need to do this for several weeks at least, but the process is detailed on the iGEM site
    - http://partsregistry.org/DNA_Submission_Instructions
- Note that you can submit a part before testing it
    - This fulfills a medal requirement for the competition

At least 250ng at 25ng/uL.

3A Assembly

The power of the BioBrick format is that two bricks can be easily be combined together to form a new brick. We use the slightly modified protocol as follows:
http://parts.igem.org/Help:Protocol/3A_Assembly

Cultures

Bacillus subtilis

Bacillus subtilis Transformation

Phase 1

1.inoculate 25% (5mL) of desired final volume of LB with BS168 in the morning, in container >200% final volume (50mL falcon tube)
2.incubate @37C for ~6hrs, then top up to final volume (20mL) LB to incubate overnight
3.spin and pellet @3000g for 5 min
4.wash with cold (4C) sterile deionized water by resuspending (in 25-50% original volume) and spinning @3000g for 5min
5.discard supernatant, repeat twice more
6.finally resuspend in 1% of the original culture volume (from which the pellet

was formed) with cold (4C) 30% PEG solution
7.aliquot into 100µl samples (use pre-chilled tubes)
8.freeze immediately @ -80C
9.after waiting overnight, proceed to phase 2

Phase 2 (electroporation)
1.thaw cells @ 4C until liquid
2.transfer to cold .2cm electroporation cuvette
3.apply current with cuvette uncapped, @ 25µF, 2.5kV (12.5kV/cm), 400ohms
4.immediately add 2ml of prewarmed SOC to cuvette, cap, and mix by inverting several times
5.transfer cuvette contents to 15ml falcon tube by pipetting or decanting and incubate for 90 min @37C
6.plate on preheated selective agar (if unsure about efficiency, try 100µl, 15µl)

Escherichia coli (adapted from Dr. Shih's protocol)

Doubling time for E.coli in ideal conditions, 37ºC = 20 minutes
Media recipes

LB

1. 10 g tryptone
2. 5 g yeast extract
3. 10g NaCl
4. Autoclave for 20 minutes
5. To make LB agar, add 15 g agar or bacto-agar prior to autoclaving (makes ~ 25)

M9 media (minimal media useful for fluorescent measurements as LB is autofluorescent)
NOTE: if not growing in M9 but just measuring fluorescence, M9 salts is sufficient.

1. Autoclave ingredients as 10X-100X stock separately prior to mixing in sterile water
2. 1X M9 salt
3. 2 mM $MgSO_4$
4. 0.1mM $CaCl_2$
5. 0.4% - 2% carbon source (glucose, glycerol, etc)
6. To make M9 Agar, add 15g agar or bacto-agar to 1 L M9 salts prior to autoclaving, then add other ingredients (makes ~25 plates).

Antibiotic selection - Make stocks in sterile water, add to warm autoclaved media. Do not autoclave, as it will degrade the antibiotic.

- Ampicilllin - 100 ug/mL, 100mg/mL 1000X stock
- Kanamycin - 30 - 50 ug/mL, 30 mg/mL 1000X stock
- Chloramphenical - 20 ug/mL 20mg/mL 1000X stock in ethanol
- Streptomycin - 100 ug/mL

Storage - add 50% glycerol to stationary phase culture for final concentration of 15-25% glycerol, freeze at -80ºC.

Site Directed Mutagenesis

1. How to make primers (http://openwetware.org/wiki/Richard_Lab:Site_Directed_Mutagenesis)
    - Double primer method:

Design mutagenesis primers.

- The targeted mutation should be included into both primers.
- The mutation can be as close as 4 bases from the 5-terminus.
- The mutation should be at least 8 bases from the 3-terminus.
- At least eight non-overlapping bases should be introduced at the 3-end of each primer.
- At least one G or C should be at the end of each primer.
- Design your primers (including the mutations) to have a Tm >=78°C.

- Single primer method:

Design mutagenesis primer(s).

- The targeted mutation should be in the middle of the primer
- Design your primers (including the mutations) to have a Tm >=78°C.

2. How to use thermal cycling:
Specifics can be found in the QuikChange Lightning Site-Directed Mutagenesis Kit
http://www.chem.agilent.com/library/usermanuals/Public/210518.pdf
http://www.biomedcentral.com/1472-6750/8/91