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Let me know if you have questions.

iGEM Lab Manual 2015

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Table of Contents:

Laboratory Information Management System

Sample Entry Forms

Click to enter a new **DNA** sample: **Sample Entry Form**

Click to enter a new **Strain**: **Sample Entry Form**

Queries

Click to perform a **DNA** query: **Query Form**

Click to perform a **Strains** query: **Query Form**

Spreadsheets

Click to view the **DNA LIMS** spreadsheet: **LIMS Spreadsheet**

Click to view the **Strain LIMS** spreadsheet: **LIMS Spreadsheet**

Tools

Dilution & Solution Calculator
Ligation Calculator
Intensity Conversion Tool

Lab Accounts and Passwords

Chow Lab Computers

NanoDrop/PCR Bench Computer

Username: BChow
Password: optogenetics

Tecan Computer:

Password: flyers87

Picasa Album:

Email/Username: brianchowlabimages@gmail.com
Password: optogenetics

Chemical Stockroom:

Username: upbchow
Password: fisher

Lab Protocols

Molecular Cloning Methods

Available Protocols:

- Colony PCR
- DNA Digestion
- DNA Gel Electrophoresis
- DNA Purification
- E-Gel Electrophoresis
- Glycerol stock preparation
- Inoculating cultures
- Ligation
- Making LB Media
- Miniprep
- Measuring DNA Concentration
- PCR
- Pouring and preparing selective plates
- Plasmid Transformation & Strain Choice
- Resuspending Primer stocks from IDT
- Storage and Usage Temperatures for Molecular Cloning
- Submitting Sample for Sanger Sequencing at Penn

Experimental Methods

Available Protocols:

- Back Dilution
- Experimental Design
- Fitting Curves
- Graphing Data
- Induction

Colony PCR

Goal: *PCR Amplification from a template inside a colony rather than an isolated and purified DNA molecule. This can be used for many purposes, but we will commonly use it for one of the following two reasons:*

(1) Can be used to verify that ligation of two or more pieces of DNA to form a plasmid and subsequent transformation were successful. If a sizeable chunk of DNA were inserted into a vector, then PCR amplification of the insertion region should result in a longer (by the length of the inserted DNA segment) amplicon than for the original empty vector. By using primers where one binds upstream or downstream of the insertion site and another that binds to the inserted sequence (or two primers that bind on either side of the insertion) - one can perform colony PCR for the purpose of validating a cloning reaction. If the amplicon is the expected length and that length is distinct from the length that would be expected for the original vector without the inserted segment, then one can be reasonably confident that cloning was successful. However, sequencing is still required to identify mutations and additions/deletions on the single base scale, which cannot be resolved for PCR amplified DNA run on a traditional gel.

(2) Can be used to amplify a gene off of the E Coli genome of the colony being PCR amplified. Note that other species (i.e. yeast), the cell wall may be too tough for this protocol to be successful and the genome must be extracted and isolated from a colony before PCR can be performed.

Protocol:

Template Preparation

First, one must have a *monoclonal* E Coli colony source. This may be one of the following three things:

(1) A glycerol stock made from a SINGLE colony on an agar plate
(2) An overnight culture in LB media (+ antibiotics if appropriate) started from a SINGLE colony on an agar plate
(3) A SINGLE colony on an agar plate

Label a sterile eppendorf with the strain number and assign a unique colony number for each

colony picked. Pipet 20 uL of sterile water into the tube. If the e coli is coming from sources (1) or (3), use a clean, sterile pipet tip to scrape a tiny bit of bacteria (even the very smallest amount is okay) and then pipet up and down into the waiting 20 uL. If the e coli is coming from source (2), pipet 1 uL of the LB culture into the water. Mix well.

This water/colony mixture will be the “template” for your PCR reaction. Next, set up your reaction mixture and run the cycler protocol. Follow this up by running a gel to examine the PCR amplicon lengths.

Reaction Preparation

Reaction Mixture (20 uL):

1 uL Template
10 uL 2x Taq Master Mix (Long term storage in freezer, will last one month at 4C)
1 uL FWD Primer (@ 10 uM)
1 uL REV Primer (@ 10 uM)
7 uL H2O

Cycler Protocol

The cycler protocol is fixed except for the extension time. Set the extension time for 1 min per kb of desired amplicon at 68C, plus a little bit of buffer time. For example, for a 2 kb amplicon, set the extension time to 2 min 30 seconds.

95ºC for 6 minutes
30x [95ºC for 30 sec, 55ºC for 30 sec, 68ºC for 1 min/kb amplicon]
68ºC for 20:00 min
Hold at 4ºC

Notes

(1) You will have leftover template mixture (19 uL left). You may use this to start a culture growing in 3.5 mL of liquid media to optimize timing. By the time the Colony PCR has run and a gel has been visualized, the liquid media culture will be approaching an OD sufficiently high for miniprepping. At that time, save 500 uL for a glycerol stock and use the rest (3 mL) for a miniprep. This will be perfect for submitting sequencing and future transformations/manipulations.
(2) Colonies in water may be stored at 4C. In general, do not leave colonies for longer than 12 hours in water before growing them in selective media.

Troubleshooting

No bands on the gel where you expected a band

a. No DNA gel stain in the gel – dna gels must have stain in them to be successfully visualized. If you just poured an agarose gel and forgot to add the SYBR Safe stain, then you won't be able to image your gel. SYBR safe is red in color and when added to a gel, the gel turns pinkish. If you forgot to add it, the gel will be colorless.
b. Didn't run enough DNA - there must be enough DNA in the gel for it to be visible to the human eye. Typically, you need > 100 ng of DNA for a very obvious band and smaller amounts will be faint. I recommend loading the entire 20 uL colony PCR reaction. If you are amplifying a very very small fragment that has little mass, it may be hard to see on the gel. Try to run as much DNA on the gel as possible, or consider running multiple Colony PCRs with the same template and concentrating the DNA before running the gel.
c. Primer doesn't bind - one of your primers may not bind to the DNA template. Double check that you used the right primers and that you used primers at the right concentration (10 uM). Consider diluting the primers again fresh from stock, using the primers in a normal PCR to verify that they perform adequately, or trying a different primer. Note that if your primer was supposed to bind to an insert region, and the insert region is not in the template plasmid, then it will not bind to anything and no band will result - see below.
d. Extension time too short - if your extension time is too short, no bands will show up. Make sure it is sufficiently long to amplify the desired fragment.
e. Unsuccessful cloning reaction - If you don't get an amplicon of the desired length, then cloning may have failed - especially if one of the primers was supposed to bind to the insert region. If the insert region isn't present because cloning failed, no band will show up. Before concluding that cloning was unsuccessful, make sure you have run a positive Colony PCR control showing that your PCR is working and that your primers do bind. This may be done with the original vector and primers that bind before and after the insertion region.

Bands on the gel that are a different length than expected

f. Extra restriction site - if you cloned with restriction enzymes, double check that there isn't a cut site somewhere you didn't expect. If there were, cutting at that site could result in a product of a length other than the expected one
g. Primer dimers - sometimes, forward and reverse primers may bind to each other (form a heterodimer) and amplify each other to create low molecular weight amplicons. These will run fast - i.e. be at the bottom of your gel and will often look like a big blob. If this happens, run your forward and reverse primers through IDT's "oligo analyzer" tool add use the "hetero-dimer" function to examine possible heterodimer formation. Redesign or switch primers.
h. Incorrect primers - check to make sure your forward and reverse primers bind where expected

i. Unsuccessful cloning reaction - If you don't get an amplicon of the desired length, then cloning may have failed. Before concluding that cloning was unsuccessful, make sure you have run a positive Colony PCR control showing that your PCR is working and that your primers do bind and give an expected length for a control. This may be done with the original vector and primers that bind before and after the insertion region.

Multiple bands for a single colony

j. Picked multiple colonies – when you picked a "monoclonal" colony, you may have actually scraped two colonies with two different plasmids. These might give two amplicons. If one of the bands is correct, then consider taking that mixed population colony/water mixture and restreaking it out on an agar plate. Pick single colonies and repeat colony PCR to find the colonies with the correct plasmid that give the right single band.
k. Multiple primer binding sites - make sure primers only bind to one site on the plasmid.

2DNA Digestion

Goal: *Digestion involves cutting double stranded DNA molecules with enzymes. Enzymes may recognize a specific DNA sequence and cut at that site (i.e. restriction enzymes) or have general nuclease activity and chew on DNA at the ends of DNA molecules (i.e. nucleases). Both of these activities are useful for creating single stranded regions at the ends of a double stranded DNA molecule. These single stranded regions are "sticky" - meaning that they will readily bind matching single stranded regions to form a stable double stranded molecule. Therefore, digesting DNA and creating single stranded sticky sites is useful for cloning where we want to paste together multiple sequences in a specific order.*

Protocol:

Reaction Mixture Preparation

Reaction Mixture (50 uL reaction volume):

5 uL 10X Digest Buffer
1 uL of each digestion enzyme up to maximum of 2.5 uL
2000 ng of desired cut fragment up to max volume of 50 - enzyme volume (cutsmart+digestion enzyme volumes)
Fill with milliQ water to 50 uL

Digestion Protocol

Incubate the reaction at 37ºC for 1 hour.

Notes:

(1) The digest buffer is specific to the enzyme and it stated on the enzyme product description - look it up on NEB's webpage for each enzyme. It is generally "Cutsmart." For digests with multiple enzymes, use NEB's tool to identify the right buffer.
(2) Enzymes are stored in glycerol, but glycerol can inhibit digestion reactions. To keep the volume to 2.5 uL, use 1 uL of 2 enzymes. For three enzymes, consider using just 0.5 uL of the most active one or reducing the volume of all three to 0.8 uL.
(3) You want there to be at least 2000 ng of the fragment you want to isolate in the end. This is because DNA digestion products must be purified, a process that is only about 50% efficient. Isolating 2000 ng ensures you have enough DNA in the end for cloning. To calculate the volume, use the DNA concentration and the relative fraction of the plasmid that is the fragment you want. For example: for a 10 kb plasmid at 1000 ng/uL with a 1kb fragment I want to cut out, I'd want to do the following calculation:

    (1kb /10kb)*(1000 ng/uL) = 100 ng/uL fragment I want
    2000 ng/(100 ng/uL) = 20 uL

    In this case, I'd digest 20 uL of plasmid to get 2000 ng of the fragment I want in the end.

(4) Some enzymes cut quickly and only require a 5 min digestion - see NEB's website for details. In general, I leave everything for 1 hour to ensure completeness.

Troubleshooting

Bands on the gel that are a different length than expected

a. Extra restriction site - if you cloned with restriction enzymes, double check that there isn't a cut site somewhere you didn't expect. If there were, cutting at that site could result in a product of a length other than the expected one
b. Correct enzymes and buffers - verify that you used the right restriction enzymes in their appropriate digestion buffer
c. Multiple bands might indicate incomplete cutting - especially if one band is what you expect and another band is the pre-cut length. Consider cutting for longer times or digesting less DNA.
d. Enzymes might have gone bad. Note that enzymes are very sensitive to temperature changes. Stocks must be kept at -20ºC or on ice at all times. Consider purchasing a fresh enzyme aliquot and repeating.

DNA Gel Electrophoresis

Goal: *A method for rapidly resolving DNA fragments of different lengths by applying an*

electric field across an agarose gel. May be used analytically (to assay for DNA molecule lengths) or preparatively, to separate DNA molecules of different lengths and then isolate a single product of interest - this is called "gel extraction" since DNA constructs are cut directly out of an agarose gel and then chemically treated to remove the agarose and other contaminants.

Pouring the Gel:

(1) Make up the agarose gel mixture - 1% agarose seems to work well for constructs between 0.5-3 kb. Typically make 50 mL (mix 500 mg of solid agarose in 50 mL of TAE Buffer)
(2) Heat in the microwave for 5-10s bursts until the agarose has completely dissolved. In between microwave bursts, mix well by gently swirling. Careful - the glass may get quite hot. Do not heat too much such that the mixture boils and spills out of the vessel
(3) Once the agarose has completely dissolved, add SYBR SAFE DNA gel stain at a 1:10,000 ratio - i.e., for a 50 mL gel, add 5 uL. Gently swirl until the dye is uniformly distributed (should be a pinkish color).
(4) Pour the liquid agarose mixture into a gel mold with a comb in place (choose between different well arrangements). Careful not to introduce bubbles. If bubbles do form, pop or move them out of the way with a pipet tip.
(5) Allow the gel to cool until it solidifies - generally takes 30-45 min. Gels may be wrapped in saran wrap and stored at 4C for a short time before use.

Running the Gel:

(1) Mix each DNA sample with 6x loading buffer (5ul for 50ul of DNA) . Mix well.
(2) Carefully arrange plastic gel bed (with gel) in the gel running setup. Fill to the line with 1x TAE buffer.
(3) Carefully and slowly pull out the comb. Can gently wiggle back and forth to help - watch out for ripped wells!
(4) Load the gel by slowly pipetting the DNA into each well. The DNA sample should sink to the bottom of the well and be clearly visible thanks to the loading buffer. Do not overfill wells or else they will spill into neighboring wells. Generally, 8-well combs leave wells that can accommodate 50 uL DNA and 15-well combs leave wells that can accommodate 20 uL DNA.
(5) Set the voltage to 120V and the time to 35 min. Once the bands have migrated ⅔-¾ of the way down the gel, stop it and place the gel on a blue-light imager.
(6) Take a picture for your notebook with the gel camera.
(7) For preparative gels - use a razor to cut out desired bands and put them in eppendorf tubes (no more than 400 mg of gel in each tube).

For 8-well, load 15 uL of DNA 2LOG ladder

For 16-well, load 10 uL of DNA 2LOG ladder

Gel extraction:

Follow the Qiagen QiaQuick DNA Gel Extraction protocol that accompanies their kits. Below are the essential steps:

(1) Add 3 volumes of QG buffer per 100 mg of agarose gel (i.e. add 450 uL of QG buffer for a 150 mg agarose gel chunk)
(2) Heat at 50ºC for ~10 minutes until the gel completely dissolves. Vortex to mix.
(3) Add 1 volume of isopropanol per 100 mg of agarose gel (i.e. add 150 uL of Isopropanol to a 150 mg agarose chunk dissolved in 450 uL of QG buffer)
(4) Vortex to mix.
(5) Add up to 700 uL isopropanol-QG-DNA-Agarose mixture to a gel extraction column (purple with lids). Spin down at 13000 rpm for 1 minute. Discard the flow through.
(6) Repeat step 5 until you have spun down all the dissolved gel you have
(7) Pipet 750 uL of PE buffer (**Make sure 200 proof Ethanol has been added) onto the column
(8) Spin down at 13000 rpm for 1 min. Discard the flow through.
(9) Air dry by spinning down at 13000 rpm for 1 min.
(10) Move the column to a fresh eppendorf tube. Pipet 35 uL of EB buffer above the column's membrane - careful not to puncture it!
(11) Let the EB buffer sit on the membrane for 1 min.
(12) Spin at 13000 rpm for 1 min to elute the purified DNA into the eppendorf tube.
(13) Consider measuring concentration on the nanodrop (see separate protocol)

Notes

(1) You make make more 1x TAE buffer by dilution 1:10 from a 10x concentrated stock
(2) I find that yields are higher for DNA extracted from chunks with less agarose - so don't cut a giant circle around your band. Better to cut it out as tightly as possible with no excess.
(3) Try to get rid of any bubbles that appear when pouring the gel. You can do this by popping them with a pipette tip. If you can't pop them, push them to the side.

DNA Purification (aka PCR cleanup)

Goal: *To isolate long DNA molecules from short oligonucleotides and proteins and perform a buffer exchange. This is often necessary to do after PCR or DNA digestion since you have done those procedures with enzymes around in specific buffers and will need to remove everything but the DNA of interest before you proceed to downstream cloning steps.*

Protocol:

It is best to follow the QiaQuick PCR Purification protocol supplied with their kit. Below are the essential steps:

All buffers and columns are supplied with the QiaQuick PCR purification kit.

(1) Add 5 volumes of PB (binding) buffer to the DNA sample you'd like to purify. For example, if you have a 50 uL completed PCR reaction to cleanup, start by adding 250 uL of PB buffer and mixing well.
(2) Add the PB + DNA mixture to a PCR cleanup column (purple columns, same as gel extraction). Spin at 13000 rpm for 1 min. Discard the flow through.
(3) Pipet 750 uL of PE buffer onto the column. Spin at 13000 rpm for 1 min. Discard the flow through.
(4) Air dry the column by spinning at 13000 rpm for 1 min. Transfer the dried column to a fresh eppendorf tube.
(5) Add 35 uL of EB buffer to the column and allow it to stand for 1 min.
(6) To elute, spin at 13000 rpm for 1 min. The solution that flows into the eppendorf collection tube is your purified DNA. Consider measuring its concentration with the nanodrop.

Option: Skip the gel extraction, and just run a digest with DpnI.

- DpnI template digest
    - cuts methylated DNA sequence at every GATC
    - minimalizes contamination of template plasmid that will also give transformed bacteria antibiotic resistance

- 2000 ng Backbone (x uL) ****2000 ng MAX***
    - If you need any more, do double
    - (40 uL of DNA) ^
    - 1 uL RE #1
    - 1 uL RE #2
    - 5 uL buffer (usually CutSmart)
    - 0.5 uL of dPn1
    - 42.5-x uL H2O
- TOTAL: 50 uL

So if you are worried about your yields with gel extraction, use DpnI for your restriction digest post-PCR. **NOTE:** You will only need to column purify the PCR product before digestion with your restriction enzymes + DpnI if the enzymes you are using are not compatible buffer you used for your PCR reaction. In many cases with iGEM stuff, your restriction enzymes will NOT be compatible with the 5X HF buffer you use for phusion PCR reactions, so you will need to column purify before digesting.

To decide if you need to column purify, check the activity of your restriction enzymes in PCR buffers here.

P.S. DpnI is compatible with phusion HF buffer :)

E-Gel Electrophoresis

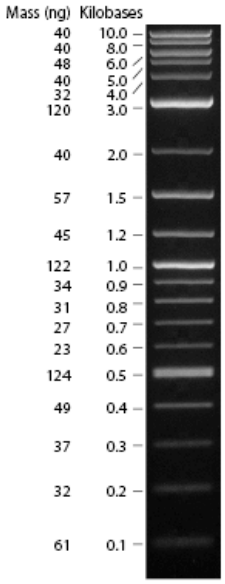
Goal: *A method for rapidly resolving DNA fragments of different lengths for analytical purposes only by applying an electric field across a commercially prepared gel.*

Protocol:

(1) Unwrap a new E-Gel and snap it into the E-Gel Holder. Make sure the gel holder lights up to indicate that the E-Gel is properly connected.
(2) For the 1.2% 12 well E-Gels, add 15 uL of H2O to each well that will receive a sample, and add 5 uL of sample (for a total of 20 uL). You may load all 20 uL for a colony PCR reaction to ensure bright bands for even the smallest of amplicons. NO loading buffer is required - do not add any!
(3) Add 20 uL of 2-log DNA Ladder (at 1 ug/20 uL) to one lane
(4) Run for 15 minutes-30 minutes and visualize on the system itself (SAFETY: Only visualize the gel when the orange protective cover is on! Do not look directly into the blue light)
(5) Capture image on gel camera for long-term preservation of the result

Notes:

(1) You may make up a stock of 2-log DNA ladder by adding 1 uL of DNA log ladder at 1000 ug/mL for every 19 uL of water (i.e. to make 1 mL stock add 50 uL 2 log ladder at 1000 ug/mL to 950 uL water).
(2) Most DNA fragments can be resolved after 15 minutes. If you image the gel and length differences are hard to see clearly, then pop the gel back into the system and run for another 10-15 minutes. You can start and stop the gel without issue.
(3) See the 2-log ladder standard below - use this as a rubric for identifying the lengths (and approximate masses) of your fragments



2-Log DNA Ladder visualized by ethidium bromide staining on a 1.0% TBE agarose gel. Mass values are for 1 µg/lane.

Glycerol Stock Preparation

Goal: *To save a monoclonal culture of a bacterial strain - especially one with a transformed plasmid of interest - for long term usage.*
Protocol:

(1) Grow a saturated culture of a bacterial strain of interest in LB (plus antibiotics if appropriate)
(2) Pipet 560 uL of the saturated culture into a sterile eppendorf tube
(3) Pipet 140 uL of sterile filtered 50% glycerol into the tube
(4) Be sure to clearly label the tube with the strain ID and date you made the glycerol stock
(5) Mix well by pipetting or vortexing
(6) Store at -80°C and avoid freeze/thaws!

Notes:

(1) Make up the 50% glycerol by adding 25 mL of pure glycerol to 25 mL of MilliQ water. Sterile filter through a 22 um membrane to remove any non-sterile contaminants. I particularly like the SteriFlip filters.
(2) Be careful not to leave the 50% glycerol out, uncovered. If that gets contaminated, then ALL of your precious stocks will be contaminated. I recommend streaking out the glycerol on LB plates (no antibiotic) from time to time to make sure nothing is living in it.

Inoculating Cultures

Goal: *To grow up a large volume of bacteria from a monoclonal source for use in cloning (i.e. miniprep to obtain plasmid), protein purification (express protein of interest, lyse cell and purify) or experimentation (monitor how genetic circuit of interest is behaving).*

Protocol:

(1) Obtain a sterile SNAP top culture tube and label it with the strain ID of the bacteria you'd like to grow
(2) Fill with 3-5 mL of LB (plus antibiotic if appropriate) using a sterile serological pipetter and sterile technique

If inoculating from a glycerol stock:

(3) Put a sterile pipete tip on a P200 pipetter as if you were to pipet a liquid. Touch the tip to the surface of the glycerol stock so a tiny bit of it is on the tip. Minimize time the glycerol stock is out of the -80ºC freezer to prevent freeze/thaw.

If inoculating from a single colony on an agar plate:

(3) Put a sterile pipete tip on a P200 pipetter as if you were to pipet a liquid. Touch the tip to the surface of the colony so a tiny bit of it is on the tip. I find holding the plate above a dark surface helps to visualize the colony and reduce glare. It is optimal to come at the plate with the tip from a roughly 45° angle and to just touch the surface of the colony without gouging the agar underneath.

Then for both glycerol stock/agar plate colony cultures:

(4) Pipet up and down with the bacteria-stabbed tip to mix.
(5) Replace Snap Cap top on culture top such that it is down a single notch (snap cap tops may be pushed to two stops. The first maintains the top on, but allows it to wiggle up and down. The second stop, achieved by pushing the cap further down, locks it into place). You want to have to cap on, but NOT in the locked position, such that the culture may be aerated.
(6) Place the tube in a shaking incubator set to 250 rpm and your growth temperature. For most E Coli work, 37ºC is appropriate for growing cultures. See the storage and experimental temperatures section for more detail.

Ligation

Goal: *To stitch or glue together one or more DNA molecules to themselves or each other. This*

is extremely useful for putting manipulated DNA sequences into a defined order and circularizing them to form a transformable plasmid that may be propagated in bacteria.

Protocol:

Reaction Mixture:

To calculate the volumes to be used, try this ligation calculator.

5 fmols backbone
30 fmols insert
1 uL 10x T4 ligation buffer
0.5 uL T4 ligase
Fill to 10 uL with MilliQ Water

Mix everything together.
*If you get a negative value for water, add 2 uL of ligase buffer and 1 uL ligase and make a 20 uL ligation reaction. Add 2 uL of ligation product for transformation
Reaction Conditions:

Quick Ligation
Incubate mix at 37ºC for 30 min
Chill to 4ºC until ready to use

Overnight Ligation
Incubate mix at 16ºC overnight
Chill to 4ºC until ready to use

Notes:

(1) Ligase is very sensitive to elevated temperatures. Be careful to keep it in the freezer or on ice at all times
(2) I find it helpful to use the ligation calculator if you get stuck on the fmol calculations.
(3) Include a no-insert negative control and an insert only negative control. This will help make it clear how much background you are getting from your individual parts and allow you to troubleshoot.

Making LB Media

Goal: *Prepare sterile liquid growth media for E Coli cultures*

Protocol:

(1) Measure out 25 grams of LB broth powder and add it to a 1L vessel
(2) Fill to 1L with RO water
(3) Shake and allow to sit until most of the LB broth dissolves
(4) Loosen cap and mark with autoclave tape
(5) Autoclave on the liquid setting ("9" on floors 3,4 in Skirkanich)
(6) Tighten cap and allow to cool to room temperature before use

Notes:

(1) BE SURE TO LOOSEN THE CAP ON THE BOTTLE BEFORE AUTOCLAVING TO ALLOW FOR PRESSURE RELEASE
(2) Once you add the LB powder to water, you must autoclave right away (within an hour). Otherwise, any microorganisms that were in the air when you dissolved the powder in water and got in the bottle will grow and ruin your media

Making M9 Media

Protocol

For 1L of media

- **500ml 2xM9 salts** ∗
- **30ml 10 mg/ml thiamine hydrochloride**
    1. **Dissolve 10 mg per ml of H_2O**
    2. **Filter-sterilize using a 0.22µm filter**
    3. **Light-sensitive: store covered**
- **10ml 40% glycerol** ∗
- **20ml 10% casamino acids** ∗
- **20ml 0.1M $MgSO_4$** ∗
- **200µl 0.5M $CaCl_2$** ∗
- **419.8ml sterile deionized H_2O** ∗

Set up a sterile filter column and add each component separately into a 1 L bottle

Add antibiotic as appropriate and store at 4°C

∗ **Can be obtained from the media room**

*Add 1 ml of desired antibiotic to media

Measuring DNA Concentration

Goal: *To quantify the concentration of DNA you have in a given sample - useful for preparing for downstream cloning applications such as ligation, transformation, digestion, etc.*

Protocol:

(1) Squirt some water onto the nanodrop pedestal and wipe with a kimwipe
(2) Open the nanodrop software and chose the “Measure DNA” option
(3) Pipet 1.5 uL of EB buffer onto the nanodrop pedestal and hit the “blank” button to subtract off the background absorbance
(4) Pipet 1.5 uL of the EB buffer onto the pedestal and hit the “measure” button. The concentration should be between 0-2 ng/uL. If not, repeat steps 1-3 until the concentration is between 0-2 ng/uL.
(5) Vortex your sample and briefly spin in a minifuge. Pipet 1.5 uL of your DNA sample onto the nanodrop pedestal. Hit the “measure” button. See your yield and 260/280 ratio - which should ideally be between 1.8-2.0

Notes:

(1) Your 260/280 ratio may not be between 1.8-2.0 for DNA that has been gel extracted since some of the gel and qg residue can skew the shape of the absorbance curve
(2) Yields may be low for low-copy plasmids (~100 ng/uL)
(3) Yields will likely be low for gel extracted products (~10-20 ng/uL)
(4) Any concentration higher than 4 ng/uL is believable. Lower concentrations are beneath the detection limit of the machine

Miniprep

Goal: *To isolate plasmid DNA from a monoclonal bacterial culture*

Protocol:

Follow the Qiagen QIAprep Spin Miniprep protocol that accompanies their kits. Below are the essential steps - all buffers and columns are supplied with the Qiagen QiaPrep Spin Miniprep kit:

(1) Grow 3-5 mL of saturated culture. The larger the volume, the more plasmid you’ll get in the end. For a normal cloning strain, grow the culture for ~12-16 hours. For NEB Turbo, 5-8 hours is sufficient, with yields increasing towards 8 hours.
(2) Spin the tubes at 3000 x g for 5 min or until all bacterial has pelleted and the remaining culture is clear. Discard the supernatant (spent media). You may do this by fully inverting the tube - the pellet will remain secure in the bottom of the tube. Note that spent media should be bleached before being disposed of in the sink.
(3) Pipet 250 uL of P1 buffer (make sure RNAse buffer has been added) into the tube and pipet up and down to resuspend the bacterial pellet. Return the P1 buffer to 4C storage. Transfer the resuspended pellet to a fresh eppendorf tube.

(4) Pipet 250 uL of P2 buffer into the tube. Invert 5-6 times to mix. Contents should become gooey.
(5) Pipet 350 uL of N3 buffer into the tube. Invert 5-6 times to mix. White precipitate should form.
(6) Spin at 13000 rpm for 10 min. White precipitate should form a pellet or deposit along the sides of the tube.
(7) Pipet the supernatant onto a miniprep spin column, careful not to dislodge any of the white precipitate. Discard the white precipitate pellet.
(8) Spin the column at 13000 rpm for 1 min. Discard the flow through by inverting the collection tube.
(9) Pipet 750 uL of PE Buffer (Make sure 200 proof ethanol has been added) onto the column. Spin the column at 13000 rpm for 1 min. Discard the flow through.
(10) Air dry by spinning at 13000 rpm for 1 minute. Transfer the column to a fresh eppendorf tube.
(11) Pipet 35 uL of EB buffer onto the column. Allow the column to stand for 1 minute.
(12) Spin the column for 13000 rp m at 1 min to elute the DNA. Consider measuring the concentration by nanodrop.

Notes:

(1) It is VERY easily to mixup which tubes are which. Make sure to label all collection tubes AND miniprep columns AND eppendorf tubes. At every point in the protocol, make sure all labels line up. Careful when pipeting PE buffer, which contains ethanol since it may wash off your label if you get some on the tube. Proceed with a lot of attention and focus.

PCR

Goal: *To amplify a desired DNA sequence for cloning, sequencing, mutagenesis, etc.*

Taq Polymerase: General all-purpose polymerase that is VERY robust
Phusion Polymerase: For amplifying a sequence with high fidelity and very quickly
Pfu Turbo Cx Polymerase: For amplifying uracil-containing sequences with high fidelity

Taq PCR Protocol:

Taq Reaction Mixture (50 uL):

25 uL 2x Taq Master mix
2.5 uL Forward Primer (10 uM)
2.5 uL Reverse Primer (10 uM)
1 uL DNA template @ 10 ng/uL
19 uL H2O

Taq thermocycler conditions:

95ºC for 3 minutes (or 6 min for colonies)
Repeat 30 cycles of:
    95ºC for 5 seconds
    55ºC for 30 seconds
    68ºC for x seconds (should be 60s per kb of desired amplicon, i.e. 2 min for a 2 kb amplicon) Sometimes 72º, check for the enzyme/ manufacturer that you're using.
68ºC for 20 minutes
Hold at 4ºC

Phusion PCR Protocol

Phusion Reaction Mixture (50 uL):

0.5 uL Phusion Polymerase (Note: polymerase and other enzymes should be stored on ice until immediately before adding. Once polymerase has been added to the tube, it should also be set on ice.)
10 uL 5X HF Phusion Buffer
2.5 uL Forward Primer (10 uM)
2.5 uL Reverse Primer (10 uM)
1 uL 10 mM dNTPs
1 uL DNA template (@ 10 ng/uL)
32.5 uL H2O

Phusion thermocycler conditions:

98ºC for 30 seconds (or 6 minutes for colonies)
Repeat 30 cycles of:
    98ºC for 10 seconds
    58ºC for 30 seconds
    72ºC for x seconds (should be 30s per kb of desired amplicon, i.e. 1 min for a 2 kb amplicon)
72ºC for 10 minutes
Hold at 4ºC

Troubleshooting:

No bands or faint bands show up on the gel

a. No DNA gel stain in the gel – dna gels must have stain in them to be successfully visualized. If you just poured an agarose gel and forgot to add the SYBR Safe stain,

then you won't be able to image your gel. SYBR safe is red in color and when added to a gel, the gel turns pinkish. If you forgot to add it, the gel will be colorless.

b. Didn't run enough DNA - there must be enough DNA in the gel for it to be visible to the human eye. Typically, you need > 100 ng of DNA for a very obvious band and smaller amounts will be faint. I recommend loading the entire 20 uL colony PCR reaction. If you are amplifying a very very small fragment that has little mass, it may be hard to see on the gel. Try to run as much DNA on the gel as possible, or consider running multiple Colony PCRs with the same template and concentrating the DNA before running the gel.
c. Primer doesn't bind - one of your primers may not bind to the DNA template. Double check that you used the right primers and that you used primers at the right concentration (10 uM). Consider diluting the primers again fresh from stock, using the primers in a normal PCR to verify that they perform adequately, or trying a different primer. Note that if your primer was supposed to bind to an insert region, and the insert region is not in the template plasmid, then it will not bind to anything and no band will result - see below.
d. Extension time too short - if your extension time is too short, no bands will show up. Make sure it is sufficiently long to amplify the desired fragment.
e. Annealing temperature is incorrect, resulting in non-optimal primer binding and amplification. Run a temperature gradient for the annealing temperature
f. Verify that you added all the right components to the reaction mixture with a positive control PCR. If that amplifies, then you know your dNTPs, enzyme, buffer, etc are not the issue.

Smear or extra band higher than expected

g. Primer dimers - sometimes, forward and reverse primers may bind to each other (form a heterodimer) and amplify each other to create low molecular weight amplicons. These will run fast - i.e. be at the bottom of your gel and will often look like a big blob. If this happens, run your forward and reverse primers through IDT's "oligo analyzer" tool add use the "hetero-dimer" function to examine possible heterodimer formation. Redesign or switch primers.
h. Used too much template DNA and it is showing up on the gel

Preparing selective plates

Goal: *To make custom selective LB agar plates beyond the LB-amp and LB-kan plates that the UPenn Cell Center offers.*

Protocol:

First, you need to determine your antibiotic working concentration. You may find working concentrations for several commonly used E Coli antibiotics here. With that number, calculate

how much of your concentrated stock solution you need to add to the plate such that it is at the correct final concentration. You may assume that the plain LB agar plates have a volume of 25 mL. Feel free to use the dilution calculator to help you.

Reaction mixture:

x uL concentrated antibiotic stock
Fill to 150 uL with Sterile Water

Example - Kanamycin comes from the cell center in a concentrated stock of 10 mg/mL. It is used at a working concentration of 20 ug/mL. This is a 500 fold dilution. (10 mg/mL)*(x mL) = (0.02 mg/mL)*(25 mL). I'll need to add 0.05 mL or 50 uL. Note, this is also 25 mL/500 = 50 uL. My reaction mixture will be 50 uL conc. kanamycin stock and 100 uL sterile water.

Chloramphenicol- (Working concentration of chloram: 25 ug/ml; stock concentration chloram: 34 mg/ml; 10-15 mL plates from stock center)

10LB chlor: 1 mL per 1000 mL LB
LB Kan: 2 mL per 1000 mL LB
LB Amp: 3 mL per 1000 mL LB
LB Amp + Chlor: 735 uL Chlor (assuming stock 34 mg/mL) + 4 mL Amp (assuming stock 25mg/mL)

Spreading protocol:

(1) Prewarm an LB agar plate at 37ºC for at least 30 min
(2) Spread all 150 uL of the reaction mixture on the prewarmed LB agar plate
(3) Allow to dry face up (agar side down) for 1 hour
(4) Once dry, the plate is ready for transformation

Pouring protocol (for 500 mL of LB-Agar):

(1) Weigh out 12.5 g of LB powder + 8 g of Agar
(2) Fill to 500 mL with water
(3) Autoclave on liquid setting (remember to loosen cap before autoclaving)
(4) After removing from autoclave, cool to 55ºC by placing in a water bath at 55ºC
(5) Add chloramphenicol from 34 mg/mL stock to 25 ug/mL final working concentration by diluting 1,360 fold [i.e. add 367.6 uL of chlor stock at 34 mg/mL to 500 mL LB agar cooled to 55ºC]
(6) Swirl to mix
(7) Pour or use a serological pipet to transfer ~20- 25 mL of lb agar + antibiotics into an empty petri dish [try to maintain as sterile of an environment as possible - ideal to do this in a sterile hood, or by a flame/bunsen burner, bench is okay if you've sprayed

down with ethanol first)
(8) Allow plates to cool until solidified (30-60 min). Invert the plates and let sit overnight/several more hours.
(9) Store in plastic bags at 4C.

Notes:

(1) Always include positive and negative controls when making your own plates to ensure that you made your plates selective. Spread something that should definitely and should definitely NOT grow.
(2) You may notice that you get a ring of growth around the outer edge of your homemade plate. This is because you likely pipetted the reaction mixture in the center of the plate and spread outward (which is correct technique!). That means that the antibiotic is most concentrated at the plate's center and less so at the edges - hence, allowing for non-selective growth in that region. ALWAYS pick colonies towards the center of the plate for follow up work to ensure that they have the resistance you seek.

Plasmid Transformation & Strain Choice

Goal: *To transform a plasmid into a chemically competent cell. This will enable the plasmid to be effectively stored, maintained and multiplied by bacteria. One may find that useful for (1) copying vast amounts of that original plasmid and isolating it via miniprep for use in another application (i.e. mammalian gene expression) (2) Distinguishing between ligation products for ones that are circular and contain functional antibiotic resistance markers and origins of replication OR (3) expressing genetic circuitry or a protein or interest in E Coli.*

Protocol:

Competent Cell Choice

<u>E Coli Strains good for Cloning</u>

•Neb Turbo - accelerated growth for faster cloning turnaround times. Can see colonies on a plate after 9 hours and can miniprep from a single colony after 4.5-5 hours.
•NEB10
•Top10
•DH5a

<u>E Coli Strains good for Expressing Protein - especially if driven by a T7 promoter</u>

•BL21 (DE3)

Transformation Protocol

(1) Obtain chemically competent cells from the -80ºC freezer. These MUST be kept on ice since their membranes are very very fragile. Do not vortex! Allow to completely thaw while on ice.
(2) Add 100 ng of DNA or 1 uL of ligation product to a labeled eppendorf tube that will be used for transformation.
(3) Pipet competent cells onto the DNA. For commercially made cells (i.e. NEB Turbo), I'd use 10 uL of cells. For homemade cells, I'd use 50 uL of cells. For BL21, homemade cells, use 100uL of cells.
(4) Mix the DNA with the cells by gently flicking the tube.
(5) Incubate the DNA/cells on ice for 30 minutes.
(6) Heat shock the sample by placing it in a float and lowering it into a water bath at 42ºC for exactly 30 seconds.
(7) Place the tube back on ice for an additional 2 minutes.
(8) For commercially made cells, add 1 uL of SOC media to the tube. For homemade cells, add 200 uL.
(9) Incubate at 37ºC while gently shaking for 60 minutes.
(10) Plate the entire reaction on an LB-Antibiotic plate.

Notes:

(1) Step 9 may be skipped if you are transforming a plasmid that has ampicillin resistance. You will get fewer colonies, but the transformation will still work. If you skip step 9 for b plasmids with kanamycin or chloramphenicol resistance markers, the transformation will fail.
(2) If in a rush, step 5 may be shortened to 10 minutes for commercially competent cells and step 9 shortened to 45 minutes, but transformational efficiency will decrease.

Resuspending primers from IDT Stocks

Goal: *When you order oligonucleotides from IDT, they come "lyophilized" in a tube. This means that they have been freeze-dried such that only solid DNA is left at the bottom of the tube with no remaining solvent (water). In order to use the lyophilized oligonucleotide, you need to "resuspend" it in a solvent - typically we use "EB" buffer from the Qiagen Miniprep, Gel extraction or PCR cleanup kits, which is named because it is Elution Buffer in the Qiagen protocols. You may also hear it referred to as "TE" buffer - or Tris EDTA buffer, since this is the actual chemistry of EB buffer. This is a common storage buffer for DNA.*

Protocol:

Making a concentrated oligonucleotide stock from IDT's original shipment:

(1) When you get the tube from IDT (usually has a blue cap), you will be able to find some key information labeled right on the tube's side: The name of the sequence you entered when you ordered it, the sequence, the date, etc. The bottom line on the label contains information regarding how *much* DNA was sent to you in two different units - nanomols (nm) and milligrams (mg). Record the number of nanomols.
(2) You will need to add the right amount of EB buffer such that the final DNA concentration is 100 uM - this is the concentration at which we store oligonucleotides for long term use. Perform the following calculation (assume for this example, IDT sent you "x" nanomols and you want to solve for "y" microliters of EB to add:

    x *1E-9 mols DNA/(y *1E-6 liters EB buffer) = 100 * 1 E-6 mols/liter concentration

    y microliters EB buffer to add = (x*1E-9)/(100*1E-6*1E-6) = 10*x
    So, in the end, you need to add 10 times the number of nanomols in the tube in microliters of EB. For example, if IDT labels the tube saying it sent "28.3 nm", then you should add 283 uL of EB to resuspend.

(3) After pipeting in the 283 uL of EB buffer, vortex to mix and briefly spin down on tabletop minifuge.
(4) Store 100 uM stock at -20ºC for long term usage.

Making an oligonucleotide stock for PCR:

(1) For PCR, primers are used at 10 uM - however, you have prepared a 100 uM stock. You will need to dilute your primers 10 fold.
(2) To make 100 uL of 10 uM primers, add 90 uL of EB buffer to an eppendorf tube.
(3) Completely thaw your 100 uM oligonucleotide stock. Vortex to mix.
(4) Pipet 10 uL of your 100 uM stock into the 90 uL of EB. This is a 10 fold dilution.
(5) Vortex your eppendorf tube to mix, which is now at 10 uM. Store at -20ºC for long term storage.

Making an oligonucleotide stock for sequencing:

(1) For sequencing, primers are used at 1 uM - however, you have prepared a 10 uM PCR primer stock. You will need to dilute your primers 10 fold.
(2) To make 100 uL of 1 uM primers, add 90 uL of EB buffer to an eppendorf tube.
(3) Completely thaw your 10 uM PCR primer stock. Vortex to mix.
(4) Pipet 10 uL of your 10 uM stock into the 90 uL of EB. This is a 10 fold dilution.
(5) Vortex your eppendorf tube to mix, which is now at 1 uM. Store at -20ºC for long term storage.

Storage and Usage Temperatures for Molecular Cloning Products & Intermediates

Goal: *It is imperative that you are cognizant of the proper temperatures for storing the various products and intermediates of molecular cloning. Failure to adhere to these guidelines may be costly*

DNA

Of any kind- primers, plasmid, genes: Store long term at -20ºC. Stable at 4C too. Can be left out on the bench for 1+ days without worry, although this is not a good habit to get into.

Proteins

A lot of protein reagents are used in cloning (polymerases, ligases, restriction enzymes, nucleases). These are always stored at -20ºC. They must be kept cold at all times - use on ice when activity pipetting. Make an effort to minimize time spent outside of the freezer.

DNA Gels

Poured gels (that have not yet seen DNA or been run) may be stored at 4C for several days until use. Once run, DNA gels should be imaged or cut fairly quickly to minimize diffusion of the DNA away from clear bands. However, once a gel is sliced up to isolate a DNA fragment, that DNA fragment in gel may be stored at 4C indefinitely until use.

Bacteria

Glycerol stocks of bacteria are stored at -80ºC; this is how we store monoclonal cultures for long times. These should NOT be allowed to freeze thaw. Extreme effort must be made to minimize any time spent outside of the -80ºC freezer - it is NOT okay to let these sit on ice for longer than 1 minute.

Agar plates with bacteria may be stored for ~1 month at 4C. Parafilm the plate to minimize contamination/moisture buildup.

Saturated liquid bacterial cultures may be stored at 4C for 1-2 days. These cultures will still carry plasmid, but miniprep yields will be far lower than for a fresh culture.

Growing bacterial liquid cultures may be grown at a variety of temperatures between 16-40ºC.

16-30ºC: Lower temperatures slow all molecular processes. This can retard protein synthesis rates and enable better folding for hard-to-fold proteins. Consider reducing the temperature for better protein expression in Coli - especially for foreign proteins.

37ºC: This is the ideal temperature for E Coli growth. From a colony, cultures will saturate in roughly 16 hours. Note that ampicillin is easily degraded - if you leave ampicillin cultures longer than 16 hours, selective pressure on non-ampicillin resistant colonies will be diminished.

Submitting a Sample for Sanger Sequencing at UPenn

Goal: *Genetic circuitry is programmed at the DNA sequence level - whether it be a promoter, RBS, protein or terminator sequence, it is important for you to know the exact string of A's,T's,C's and G's that you are working with. Running a gel with a Colony PCR or PCR product can give you an idea of the length of a DNA sequence, but sequencing is the only way to give the exact letter sequence. Sanger sequencing is great for producing moderate length reads of the sort typically required for cloning.*

Reaction Mixture Preparation:

Reaction Mixture (9 uL):

DNA to be sequenced: 480 ng (up to maximum volume of 6 uL)
Water: 6 uL minus vol DNA to be sequenced
Sequencing Primer: 3 uL @ 1 uM (1:100 dilution from 100 uM stocks)

Sanger Reaction:

Drop the DNA sample off with a submission request form before 5 PM at the UPenn Sequencing Center at B1 Richards Building. A submission request form is required! See detailed submission instructions on the Penn Genomics Core Website. An advisor signoff is required to receive a sequencing submission form.

Typically, you will receive the results within 24-36 hours of dropping off the sample.

Notes:

(1) Sanger sequencing reads are usually high quality for 700-900 bp in length. Send off a template with more than one primer if you need to sequence a longer stretch. Be sure to tile multiple primers such that there is enough overlap between reads.
(2) The sequencing center already has some commonly used primers in its lab that it can add to the reaction mixture for you. See the Penn Genomics Core Website for a list of those primers. If you want them to add the primer for you, simply send off the DNA

and water - a total of 480 ng in 6 uL of water.

Troubleshooting:

Low Quality or Nonexistent Sequence

a. Not enough template DNA - if you sent off significantly less than 480 ng (i.e. less than 250 ng) because your DNA was at low concentration, repeat the miniprep and resubmit
b. Incorrect primer - verify that you sent off the right primer (at the right 1 uM conc.) or chose the sequencing center to add the right primer
c. Bad primer design - sometimes, primers don't work very well for sequencing. It isn't always easy to know if advance if this will happen. I tend to find that short (18-21) primers with 50% GC content work the best. Try to redesign the primer if it consistently gives bad results

Overlapping bases

d. If you see two bases that are called with high probability, that means you are getting two different reads from the same primer. The primer may be binding to two different sites (redesign primer) or you could have submitted a mixed DNA population (restreak colony, pick single colony, miniprep and resubmit)

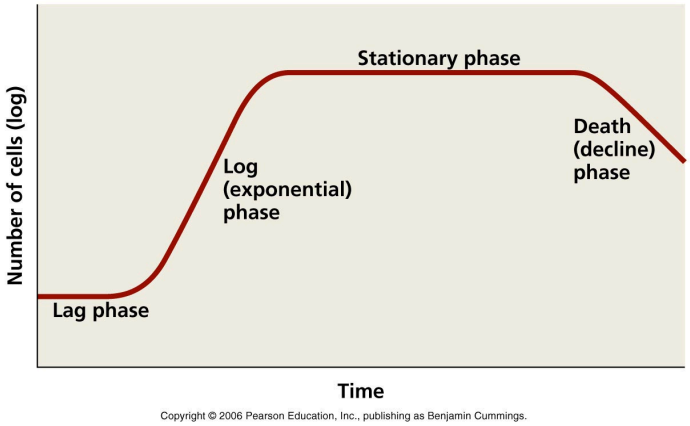
Don't match what you expected

e. Improper tube labeling - to try align the sequence against other DNA sequences you have worked with to make sure you didn't sequence the wrong thing by accident
f. Failed cloning reaction - Try to hunt for restriction enzyme sites, start codons, rbs's and stop codons in the sequence and use them as landmarks to identify what it is you did sequence. Most commonly, this will be the empty vector you started with.

Back Dilution

Goal: *Bacteria have 4 stages of growth: lag phase, log (exponential) phase, stationary phase, and decline phase (diagram below). At lag phase, the bacteria are adapting to growth conditions. They are synthesizing the necessary RNA and proteins to survive, but are not dividing. At log phase, the bacteria begin to divide and continue to double at a constant rate. At stationary phase, the bacteria have hit a limiting reagent (usually the amount of nutrients in the media) and have reached a steady state where the growth rate is equal to the death rate. Finally, after a certain amount of time the bacteria completely deplete an essential nutrient in the media and begin to die. Bacterial experiments are typically carried out in the log phase, because bacteria are the healthiest and most metabolically active. You must ensure that your bacteria are growing at log phase before doing any experiment. In*

order to do this, you often need to back-dilute cultures.



Protocol:

1. Start with an overnight culture inoculated from a glycerol stock (See: Inoculating cultures)
2. Depending on the volume of bacteria you need for your experiment, fill either a SNAP-top culture tube or sterilized, baffled culture flask with the appropriate media and antibiotic using sterile technique.
3. After the media is warm, pipet a small volume of your overnight culture into the new tube/flask. The amount of overnight culture you add is dependent on the volume of the tube/flask, and also the time at which you want to do your experiment. Generally, you can dilute the bacteria anywhere from 1:100 to 1:1000.

Notes:

1. SNAP-top culture tubes can be used for growing anywhere from 3-5 mL of bacteria
2. Flasks come in varying sizes (from 125 mL all the way to 4 L). A general rule of thumb is to fill a flask to approximately ⅕ of its total volume. For example, you can grow ~25 mL in a 125 mL flask and ~800 mL in a 4L flask.
3. Flasks need to be autoclaved before use, and can be covered with either specially designed tops or aluminum foil that has holes poked in it for aeration.Warm the media in the tube or flask to the temperature at which you want to grow the bacteria.
4. Pre-warming the media before adding the cells prevents the cells from experiencing an unnecessary temperature shock.
5. Back-diluting cultures can be a powerful tool when planning experiments. You can calculate the growth rate of a bacterial strain, and using that rate, dilute to a certain concentration of bacteria so that the bacteria are at the appropriate concentration that you want at the approximate time that you want.

Experimental Design

Goal:

Fitting Curves

Goal:

Graphing Data

Goal:

Induction

Goal: In biology, induction means to cause something to occur within a cell. When we refer to induction in the context of iGEM, we are usually referring to the process of turning on gene expression (and therefore protein production) within a cell. This is a useful tool within synthetic biology because it allows precise temporal control of gene expression. In general, we want to induce bacterial cultures when they are growing in log phase because they are the healthiest and most metabolically active. The two most common methods of induction we will use in iGEM are chemical induction and optogenetics.

Chemical Induction

(1) Start an overnight bacterial culture from either a plate or glycerol stock and grow it at 37C at 250 RPM
(2) Back-dilute the culture and allow it to grow to mid-log phase also at 37C and 250 RPM. With E. coli, this is generally around OD600 of 0.3-0.4.
(3)

Keeping Track of Samples

Goal:

Growing Cultures

Goal:

Pipetting

Goal:

Serial Dilutions

Goal:

Tecan Plate Reader

Goal:

Measuring Cell Concentration
Measuring Fluorescence
Measuring Luminescence

Other notes:
1M L-arabinose stock
MW = 150.13 g/mol
if need 5 mL of L-arabinose stock combine 750 mg of L-arabinose with 5 mL of mQ water

Stock nalidixic acid
stock is 10 mg/mL, induction concentration for SY104 is 10 mg/L
use 1:1000 dilution for induction!