GENERAL PROTOCOLS

Preparing LB Media

Note, do not add antibiotics before autoclaving

1. In a 1 L autoclave bottle add:
    a. 25 g LB broth powder
    b. 1000 mL miliQ water
2. Swirl to mix. Powder may not dissolve completely, that is ok but avoid clumps
3. Autoclave 121 C for 30 min on liquid cycle. Be sure to add water to autoclave basin
4. Cool to room temp before storing in the 4 C fridge. Do not tighten the cap until cool.

Preparing Agar Plates

Note: before adding your antibiotics, create a 1000x stock solution. For example, if you are preparing plates with a final concentration of 100 ug/mL ampicillin, you should make a stock solution of 100 000 ug/mL (100 mg/mL). Simply measure out 100 mg of ampicillin powder, add it to 1 mL of water, dissolve by vortexing, and filter sterilize.

*Plates will contain one or more antibiotics, LB broth with agar, and an inducer if applicable.

1. Calculate final agar volume (same as required Miliq water volume):
   # of plates desired × 10 mL × 1.5 = total volume of LB agar required
    a. E.g. 10 plates × 10 mL × 1.5 = 150 mL
       **Some plates are larger/smaller. Make sure you double check the volume*

NOTE: The largest bottle you can use for making LB plates is a 500 mL bottle, DO NOT USE A BIGGER BOTTLE and only make enough agar to fill the bottle half way (about 300 mL).

2. Use the calculated final volume to calculate the grams of LB agar powder needed
    a. Final agar concentration: 40 g/L
    b. Using the previous example: 40 g × 150 mL / 1000 mL = 6 g

2. Add calculated volume of Miliq water from step 1 to calculated grams of LB agar powder
    a. Using the same example: add 150 mL Miliq water to 6 g LB agar powder
3. Stir/shake until no clumps remain
    a. If there are clumps: use a glass rod to break them apart
4. Cover the opening with aluminum foil or lightly screwed on lid, and tape with autoclave tape (**DO NOT make air-tight seal**)
    a. **DO NOT ADD ANTIBIOTICS** (do not autoclave antibiotics)
5. Autoclave using liquid cycle at at least 121 C under 20 psi for at least 30 min
6. After autoclaving, either store bottle at 4 C or proceed to pouring plates

Pouring Plates

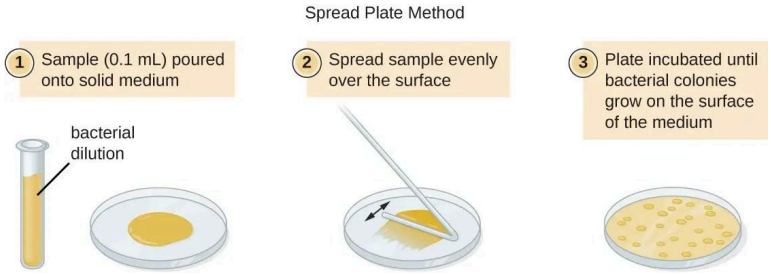
1. Prepare plates for pouring:
    a. Spray down bench with 70% ethanol and wipe down
    b. Label appropriate number of plates with medium, antibiotic they will contain, the date, and inducer if applicable
    c. Position the flame next to to where the plates will be poured
2. IF AGAR HAS SOLIDIFIED: Either use a water bath at 60 C or microwave agar bottle to reheat agar
    a. ONLY HEAT IF ANTIBIOTICS HAVE NOT BEEN ADDED
3. Wait until you can safely touch the agar bottle before adding antibiotics or pouring plates
4. Light flame at plate pouring station, and add antibiotics to agar bottle. **Make sure final concentration is correct:**
    a. Ampicillin - 100 ug/mL
    b. Kanamycin - 50 ug/mL
    c. Chloramphenicol - 25ug/mL
    d. Gentamicin - 20 ug/mL
    e. DO NOT ADD SOLID ANTIBIOTICS TO THE LB AGAR, ONLY ADD ANTIBIOTICS FROM LIQUID STOCK
        i. Meaning: must use prepared 1000x stocks of the antibiotics dissolved in a solvent (located in -20 C small black freezer in biozone room 403)
        ii. When preparing antibiotic stocks, be sure to check the **appropriate solvent** and **filter sterilize**
            1. For chloramphenicol: the solvent is 100% EtOH

5. Swirl agar bottle to ensure even distribution of antibiotic throughout the mixture (be careful to minimize creating bubbles when swirling)
6. Open plate one at a time and begin pouring
    a. If antibiotic has been added: work fast and try to plate all of it as reheating it in the autoclave/microwave would denature the antibiotics and render it ineffective
7. Leave the plates out on the bench to solidify (should take roughly 30 min)
8. Package the plates in the bags they came in and store upside down at 4 C (ensure clear and proper labelling of the bag prior to storage)

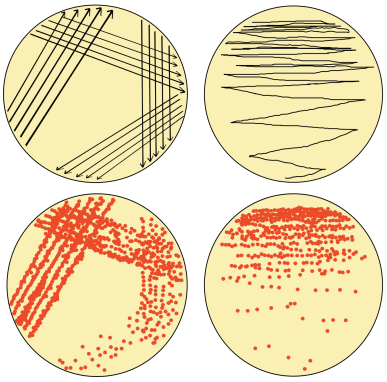
Inoculating, Plating, and Streaking

Inoculating - Introducing bacteria to liquid culture.

Plating - Spread bacteria over the surface of an agar plate.



Streaking - Plating so that an isolated colony can develop.



The protocols below always include a negative control, but think about the experiment you are conducting and decide if you really need a negative control. The negative control is there to ensure that you do not have contamination. They are included because we are not working with a flame or in a laminar flow hood/BSC so there is a risk of contamination if there are any mistakes in handling the samples (ie. accidentally sneezed on the sample). If you are confident with your sterile technique, feel free to forgo the negative control when you don't need one.

When is a negative control needed? - When you need a clean sample (ie. for making a glycerol stock)

Inoculating from glycerol stock

1. Prepare two 15 mL culture tubes and label one tube with [iGEM + STRAIN + ANTIBIOTIC + DATE + INITIALS] and the other tube with negative control [-]
2. Add 5 mL of LB into each 15mL Falcon culture tube.
3. Take glycerol stock of the [sample you are inoculating].
4. Scrape some E. coli from the glycerol stock into the one falcon tube (You can use a clean P10 pipette tip and eject it into the falcon tube after picking up the colony). Make sure that you do not touch the glycerol tube for too long and that it goes back on ice and into the -80C freezer as soon as possible.
5. In the negative control tube, eject a clean tip.
6. Place the caps back onto the tube. If you are using a falcon tube instead of a culture, do not tighten the lids all the way so the culture can be supplemented with air.
7. Place both tubes in the shaking incubator at 37 C and 200 rpm.

Inoculating from plates

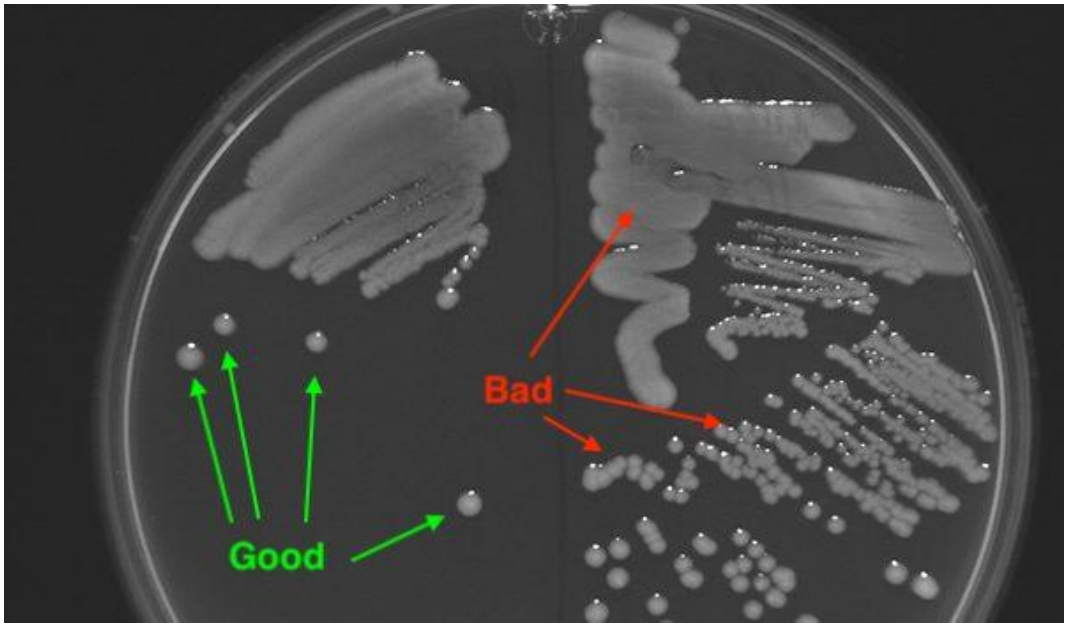
1. Prepare 2 15mL culture tubes and label one tube with [iGEM + STRAIN + Antibiotic + date + initials] and the other tube with negative control [-]
2. Add 5 mL of LB into each 15mL culture tube.
3. Add the appropriate antibiotic from the stock into each culture tube. Stock antibiotics should be at 1000x working concentration. This means you add [culture volume]/1000 m of stock volume

a. E.g., if you are culturing 5ml, add 5ml/1000 of stock antibiotics (i.e., add 5ul). It doesn't matter which antibiotic you are using, because the stock should be at 1000x working concentration

Antibiotic	Recommended Stock Concentration	Recommended Working Concentration
Ampicillin	100 mg/mL	100 µg/mL
Carbenicillin*	100 mg/mL	100 µg/mL
Chloramphenicol	25 mg/mL (dissolve in EtOH)	25 µg/mL
Hygromycin B	200 mg/mL	200 µg/mL
Kanamycin	50 mg/mL	50 µg/mL
Spectinomycin	50 mg/mL	50 µg/mL
Tetracycline	10 mg/mL	10 µg/mL

***Note:** Carbenicillin can be used in place of ampicillin.*

4. Grab the plate with [the sample you want to inoculate] and look for a colony that is well isolated.



5. Use an inoculation loop or a P10 pipette tip to gently scrape the colony.
    a. For inoculation loop: Open the culture tube and lower the inoculation loop with the bacteria into the culture, then swirl the loop around a little, then dispose of the inoculation loop (only for single use inoculation loop).
    b. For P10 tip: Eject the tip into the falcon tube after picking up the colony.
6. In the negative control tube, eject a clean tip or swirl a clean inoculation loop.

7. bbvPlace the caps back onto the tube. If you are using a falcon tube instead of a culture, do not tighten the lids all the way so the culture can be supplemented with air.
8. Place both tubes in the shaking incubator at 37 C and 200 rpm.

Plating liquid culture

1. Grab the culture plates from the incubator or the fridge.
2. Grab [x] Agar Plates [with the correct antibiotics] and label them [iGEM + STRAIN + Antibiotic + date + initials].
3. Prepare [x] L-shaped spreading sticks.
4. Pipette 25 uL of [strain] liquid culture into the respectively labeled plate.
5. Grab an L-shaped spreading slick and open the plate lid slightly with the other hand.
6. Spread the culture around the plate, make sure to get the entire surface.
7. Close the lid and dispose of the spreading stick.
8. Place the plates into the 37C incubator overnight. Make sure that the plates are turned with the lid-side down and do not wrap the plates in parafilm while in the incubator.

Streaking from glycerol stock

1. Spray bench with 70% ethanol, soak for 30 seconds, and wipe down with paper towel.
2. Grab [x] Agar Plates [with the correct antibiotics] and label them [iGEM + STRAIN + Antibiotic + date + initials].
3. Prepare 3 sterile inoculation loops.
4. Take the glycerol stock of the [sample you are inoculating].
5. Scrape some E. coli from the glycerol stock with the inoculation loop. Make sure that you do not touch the glycerol tube for too long and that it goes back on ice and into the -80C freezer as soon as possible.
6. Gently, but firmly, spread the bacteria onto the plate with the inoculating loop with the following pattern (streak #1 below):



7. Using another inoculating loop, gently but firmly drag through the first streak, and spread the picked up bacteria in another section of the plate. (streak #2 above)
8. Repeat step 7 another time to create streak #3.

Incubate the plate at 37℃ overnight. Make sure that the plates are turned with the lid-side down and do not wrap the plates in parafilm while in the incubator.

Preparing Glycerol stock

Reagents

- 50% glycerol
- Overnight grown E.Coli in tube
- A 2mL cryovial tube

Procedure

- Label your tube with [Plasmid Name+Date+Antibiotic resistance+iGEM 2025].
- Add 500µL of your cultured media with growing E.Coli in the 2mL cryovial tube, add 500µL 50% glycerol stock and gently mix it.
- freeze the glycerol stock in -80.

Mix & Go Transformation (if we can get it to work)

ALL STEPS (until spreading/outgrowth) should be done on ice.

1. Earlier in the shift, place plates in the incubator to pre-warm them. (Chilled plates decrease efficiency severely, but will not absolutely ruin the protocol.)
2. Once ready to transform, move the competent cell aliquot from the -80, immediately put on ice. (Should be 50 uL, if not, that's okay.)

3. Thaw comp cells 2-5 minutes ON ICE (I typically do 3 minutes).
    - At the same time, I like putting the plasmid on ice (especially if it came fresh out of an assembly), but that's just a superstition of mine.
4. Pipette 1-2 uL of the desired plasmid DNA directly into the thawed aliquot. Quickly mix, then put back on ice
5. Wait 2-5 minutes for the bacteria to take up the DNA, or even longer (at Garton, I often wait 7).
6. FOR NOT AMPICILLIN (kan or chlor): add 200 uL SOC and put in the shaking incubator for 1 hour at 37 degrees C for outgrowth. Just like the other protocol below.
7. Spread the transformation mixture on the prewarmed plate, dry by flame if wet, label, and put the plate back in the incubator.

Transformation

Note, make sure your heat block is preheated to 42°C before starting!
ALL STEPS should be done on ice.

1. Label 1.5ml tubes. Fill ice bucket with ice, and pre-chill 1.5ml tubes (one tube for each transformation).
2. Thaw competent E. coli DH5a cells on ice for 5–10 min.
    a. Do not refreeze thawed comp cells.
3. Pipette 50uL of competent cells into 1.5 mL tube. Keep tubes on ice.
4. Pipette 2ul (1pg-100ng) of the reconstituted plasmid; gently mix by pipetting. Keep tubes on ice. Do not vortex.
5. Incubate on ice for 30 min.
6. Heat shock at 42°C for exactly 30 sec. **Timing is critical**. Do not mix.
7. Immediately place back on ice for 5 min.
8. Add **200 µl** of SOC media (warming SOC to room temp is not absolutely necessary).
9. Incubate at 37°C for 60 min, at 250 rpm.
10. Warm LB agar plates containing appropriate antibiotic at 37°C for 15 min.
11. Mix the cells thoroughly by inverting, then pipette 100uL of each transformation on plates. Spread with a sterilized spreader. If the plate looks wet, let it dry before flipping it over (see below).
    a. Make sure the plates are labelled, and supplemented with the correct antibiotics.
    b. Pour the leftover culture into liquid bacteria waste and discard the tube in solid biological waste.

    c. Drying the plate: If the plate is too dry, simply keep the lid on, and after a few minutes it'll dry up. Or you can have the lid slightly off, and dry it under a flame.
12. Incubate the plates overnight at 37°C (or 30°C for unstable plasmids).
    - MAKE SURE TO WAIT FOR THE CULTURE TO DRY NEXT TO A FLAME **BEFORE FLIPPING UPSIDE DOWN**
    - AND STORE THE PLATES UPSIDE DOWN
13. Remaining transformation samples can be poured out into the liquid culture waste bottle. Discard the 1.5 mL tubes in the solid waste.

E. coli Electroporation

1. Pick an isolated colony from an LB agar plate and grow it in 3 mL of LB at 37 °C and 250 r.p.m. to an OD600 of 0.50–0.60.
    a. **CRITICAL STEP:** E. coli cells should be collected in exponential-growth phase.
2. Transfer 2 mL of the culture to a 2.0-ml microcentrifuge tube. Spin it at ≥10,000g for 2 min at 4 °C.
    a. **CRITICAL STEP:** E. coli cells must be kept ice-cold.
3. Remove the supernatant. Resuspend the cell pellet in 1 mL of ice-cold 10% (vol/vol) glycerol. Spin it at ≥10,000 rcf for 1 min at 4 °C.
4. Remove supernatant and wash the cells with 1 mL of ice-cold 10% (vol/vol) glycerol, and then resuspend the cell pellet in 20 µL of ice-cold 10% (vol/vol) glycerol.
    a. Washing cells: add glycerol, vortex, spin down (10 000 rcf, 1 min), remove glycerol
5. Mix ~100–200 ng of plasmid with the electrocompetent cells. Transfer the cells to a pre-chilled 0.1-cm cuvette.
6. Introduce the DNA into the cells by electroporation (1.8 kV, 25 µF capacitance and 200 Ω resistance). After electroporation, immediately add 0.8-1 ml of LB and transfer the cells to a sterile culture tube.
7. Incubate the cells for 1 h at 37 °C and 250 r.p.m.
8. Spin down and resuspend in 100µL LB.
9. Spread on plate with appropriate antibiotics and incubate overnight at 37 °C.

Miniprep

Note, this is for the Thermo Fisher GeneJET miniprep kit. If you are using another kit, follow their protocol
PUT ALL MINIPREP WASTE IN THE BOTTLE LABELLED "MINIPREP-WASTE". If miniprep waste is bleached, chlorine gas is released.

1. Spin down the culture tubes in the large centrifuge in 301.
    a. Spin for 10 min at 5300 RPM (not RCF).
    b. The centrifuge in 301 is only rated to spin at a MAX of 5300 RPM. If you find a rotor that is **capable of spinning faster**, **please use it instead**.
2. Once pelleted, pour out the media (be careful to not pour out the pellet).
    a. Pour out the media into the liquid waste bottle. You can use a 50 mL falcon tube to temporarily store the media if you don't want to go back up to 403.
3. Take a p1000, set it to 200ul, and pipette out the remaining media. **Make sure to change pipette tips each time.** Don't accidentally suck up the pellet!
4. Add 250ul of resuspension buffer (R3) into the culture tubes. Use your pipette tip to resuspend the pellet, pipette mix.
    a. Be careful to not introduce too many bubbles.
    b. R3 buffer has RNase, it must be stored in the fridge, do not leave it out once you're done with it.
5. Once resuspended, transfer the culture to a labelled 1.5ml tube. Make sure to transfer all of the liquid.
6. **Vortex the absolute shit out of the tube**. You can also scrape the tube along a rack.
    a. A good miniprep depends on how well you resuspend the cells.
7. Add 250 uL of lysis buffer, then mix by inverting the tubes. Do not mix too vigorously, but make sure it's well mixed. Let it incubate for 4.5 min.
    a. **DO NOT** vortex. It can cause the DNA to shear.
    b. **DO NOT** incubate for more than 5 min. This is very important!
8. After 4.5 min, immediately add 350ul of neutralization buffer. Mix by inverting the tube. You want the result to be homogenous (don't worry if two layers form after a while).
    a. This step is important! You want to make sure the lysis buffer is fully neutralized.
9. Centrifuge for 10min at 15,000 rcf. MAX SPEED
    a. If your centrifuge is capable of a higher spin speed, use it!
    b. 1,000 rcf = 1000 g

10. Pipette the supernatant (if your lysis step is good, you should get 850 uL) into the spin column, careful to not disturb the white lysate. MAKE SURE to not pipette any of the white lysate. This will clog the filter.
11. Centrifuge at 15,000 rcf for 1 min.
12. Discard the flow-through in the miniprep-waste bottle.
13. Pipette 500 uL of wash buffer I (for EndA+ strains) onto the column. Incubate for 1 min, then centrifuge at 15,000 rcf for 1 min. Discard the flow-through.
14. Pipette 500 uL of wash buffer onto the column. Centrifuge at 15,000 rcf for 1 min. Discard the flow-through.
15. Repeat the wash procedure using 500 uL of wash buffer again. Centrifuge and discard flow-through.
16. Centrifuge the column at 15,000 rcf for 1 min. Discard the flow-through.
17.
18. Put the column into a clean and labelled 1.5ml tube. Add 50ul of nuclease free water (ultrapure) onto the filter. Incubate for at least 5 min, max 1h.
    a. Longer incubation = better results
    b. Be careful not to touch the membrane with pipette tip.
19. Centrifuge the column for 2 min at 15,000 rcf. The resulting liquid that flows through into your 1.5ml tube contains the plasmid.
20. Nanodrop, write the concentration on the side of the tube.
    a. If the purity is bad, put an asterisk beside the concentration number (e.g., 180 ng/ul*)
21. Store plasmid at -20C.

PureLink Quick Plasmid Miniprep Kit

1. Centrifuge 1–5 mL of the overnight LB-culture. (Use 1–2 ×109 E. coli cells for each sample.) Remove all medium.
2. Add 250 µL Resuspension Buffer (R3) with RNase A to the cell pellet and resuspend (vortex) the pellet until it is homogene ous.
3. Add 250 µL Lysis Buffer (L7). Mix gently by inverting the capped tube until the mixture is homogeneous. Do not vortex. Incubate the tube at room temperature for 5 minutes.
4. Add 350 µL Precipitation Buffer (N4). Mix immediately by inverting the tube, or for large pellets, vigorously shaking the tube, until the mixture is homogeneous. Do not vortex. Centrifuge the lysate at >12,000 × g for 10 minutes.
5. Load the supernatant from step 4 onto a spin column in a 2 mL wash tube. Centrifuge the column at 12,000 × g for 1 minute. Discard the flowthrough and place the column back into the wash tube.

6. Add 500 µL Wash Buffer (W10) to the column. Incubate the column for 1 minute at room temperature. Centrifuge the column at 12,000 × g for 1 minute. Discard the flowthrough and place column back into the wash tube.
7. Add 700 µL Wash Buffer (W9) with ethanol to the column. Centrifuge the column at 12,000 × g for 1 minute. Discard the flowthrough and place the column into the wash tube. Centrifuge the column at 12,000 × g for 1 minute. Discard the wash tube with the flowthrough.
8. Place the Spin Column in a clean 1.5-mL elution tube. Add 50 µL of preheated UP H2O to the center of the column. Incubate the column for 1 minute at room temperature.
9. Centrifuge the column at 12,000 × g for 2 minutes. The elution tube contains the purified plasmid DNA. Discard the column. Store plasmid DNA at 4°C (short term) or store the DNA in aliquots at −20°C (long term).

PCR

Check the "DNA and Oligos" spreadsheet.
Make sure to use the 10uM aliquots, not the 100uM stock

- Diluting primers: do c1v1 = c2v2

  (100 µM)(v1 µL) = (10 µM)(100uL)

  v1 = 10 µL

  100 µL - 10 uL = 90 uL

  So add 10 µL of stock primers (100uM) to 90 µL ultrapure water (UP H2O) to make a 100uL volume worth of primers diluted to 10uM. pipette mix VERY WELL, and use in the PCR reaction.

For each 25 uL reaction:

- 2X Q5 mastermix: 12.5uL
- 10 uM Forward Primer: 1.25uL
- 10 uM Reverse Primer: 1.25uL
- Plasmid (DNA): 1uL (diluted to 25 ng/uL using water)
- Nuclease free water: up to 25ul

Procedure

1. Once everything is mixed (see formula above), put your tubes in the thermocycler.
2. Thermocycling profile:
   - 98C for 30s

- 30 cycles: 98C for 10s, 62C for 30s, 72C for 30s/kb
- 72C for 2min
- Hold 4C

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C *50–72°C 72°C	5–10 seconds 10–30 seconds 20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

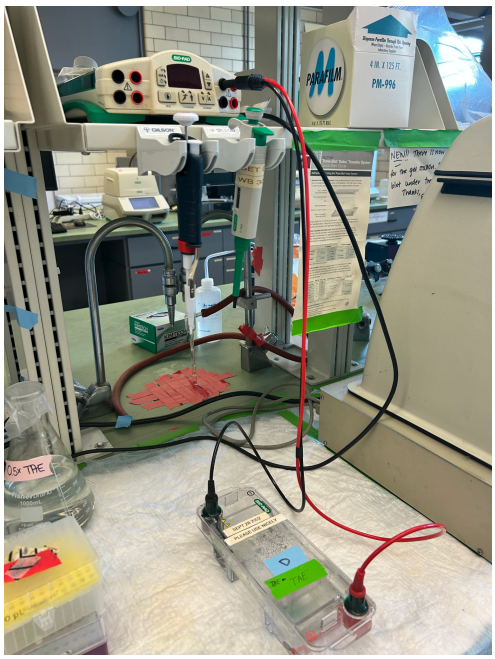
Note, if the protocol offers a range for the time. Use the upper option.

Gel Electrophoresis

Gel Electrophoresis Procedure

1. Turn on scale, place weighing boat on top, and TARE.
2. Pour and measure 0.5g of agarose powder using a spatula.
    a. Note: ensure you are careful with measuring and pouring so as to avoid getting fine agarose granules on the scale or stuck on spatula.
3. Transfer measured 0.5 gram of agarose to a microwavable flask.
4. Add and mix 50mL of 1x TAE.
    a. Note: it is often useful to measure using a graduated cylinder – nothing has to be sterile.
5. Mix thoroughly using a stir rod or by swirling.
    a. Note: use a **125 mL flask at a minimum** to ensure effective swirling and so that no bubbles overflow when heating in microwave.
6. Microwave 1-3 minutes until agarose is completely dissolved. CHECK EVERY 20 SECONDS TO AVOID OVERFLOW OF BUBBLES; SWIRRRRLLL. Start with 1 minute to prevent overboiling and potentially ruining the concentration.
7. Allow agarose to cool down to 50°C, which is when you can comfortably wrap your hand around it. This usually takes 5 minutes after microwaving until dissolved.

    a. Note: running the flask under cool water will speed up the cooling process! Just ensure that you don't solidify the agar by keeping track of the flask temp.
8. Use P20 to add 4µL of sybr safe.
    a. We also have RedSafe, but use sybr safe!!
    b. We are adding 4ul because the biozone visualizer is trash.
9. Pour agarose gel into a gel tray with comb in place and let it sit for 20-30 minutes at room temperature. Optionally, if you need it to harden quicker, place it at 4°C for 15 minutes.
10. Add loading dye into the sample. Loading dye is 6x, meaning for every 5µL of sample, add 1µL of loading dye. Load 3uL of sample + dye into the well.
    a. **For PCR products**: you can directly add (around 4ul) of loading dye into the entire 25ul PCR product. Mix well, then take 2ul and load it into the well.
        i. This is ok because we'll be doing purification on the product anyways.
11. Once the gel is solidified, place it into electrophoresis box.
12. ALWAYS RUN TO RED, make sure the negatively charged DNA has to run towards the red side.
13. Fill the box with 1X TAE until the gel wells are covered, use this one in 303:

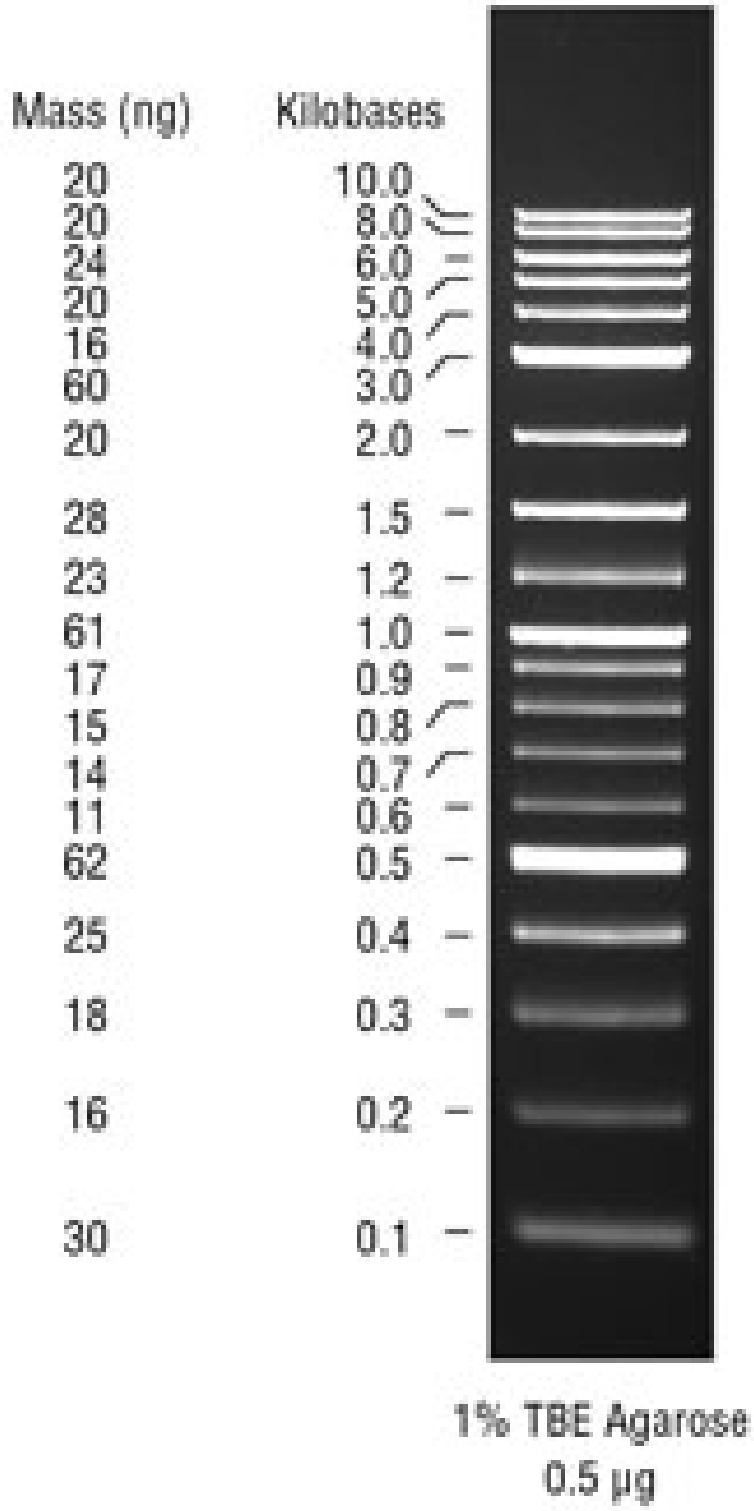


14. Using P20, carefully load molecular weight ladder into first lane of gel, typically 5 uL is good, but DOUBLE CHECK with manufacturer.
15. Using P20, load your samples into the other lanes of the gel (P200 also works, just ensure you have the correct pipette for your desired volume and gel well size).

16. Run gel at 150V for 25-30 minutes.
    a. Note: you can run for longer if the loading dye has not moved far enough – a general rule of thumb is to ensure that the bottom dye front band is 2⁄3 down the gel.
17. Turn off power supply.
18. Use a visualizer to observe gel. BE CAREFUL WHEN TRANSPORTING GEL!

Visualizing Gel Procedures/Settings

1. Change settings to **"gel red"**
2. Click on “position gel” - make sure to place gel in center
3. Image the gel
4. Send image to yourself (Log onto email on browser)


Mass (ng)
Kilobases
20
10.0
20
8.0
24
6.0
20
5.0
16
4.0
60
3.0
20
2.0
28
1.5
23
1.2
61
1.0
17
0.9
15
0.8
14
0.7
11
0.6
62
0.5
25
0.4
18
0.3
16
0.2
30
0.1
1% TBE Agarose
0.5 µg

[1kb+ from NEB]

Restriction Enzyme Digestion

Note. Most companies want you to do a 50 uL reaction. We will do 10 uL to save money - gives you the same result anyways.

Protocol (applies to all NEB enzymes)

1. Find all your enzymes and buffers, keep them on ice.
    a. If you are using NEB enzymes, use rCutSmart buffer. If you are using thermo fisher enzymes, use their FastDigest buffer (for fast-digest compatible enzymes).
    b. Do not mix and match buffers from different companies
2. (optional) If you are digesting multiple plasmids with the same enzymes, you can make a master mix.
3. For each 10 uL reaction, add the following into a 0.2 mL PCR tube :
    - *Note, this is applicable for ALL NEB enzymes. Protocol might be different for thermo fisher.*
    - 1 uL rCutSmart buffer
        - Not all enzymes use cutsmart. Be careful
        - E.g., BsmBI uses NEBuffer 3.1
    - 200 ng of plasmid DNA
        - You need to do some calculations. Show your work on the lab notes.
        - E.g., if your plasmid stock concentration is 100 ng/uL, you need to add 2 uL of your plasmid DNA to get 200 ng.
        - **Stronger band: 400 ng of plasmid and 0.4 uL of enzyme**
    - 0.4 uL of each enzyme (if you are using multiple enzymes, add 0.4 of each).
    - Nuclease free water: add enough to reach a final volume of 10 uL.
4. Incubate for 30 minutes at the correct temperature. Check experiment calendar
    a. Ideally you incubate for AT LEAST 30 min. 1h is optimal.
    b. NEB says 5-15 min is enough for some enzymes, but there's no harm in doing it for longer.

Restriction Enzyme Digestion

Steps

1. Set up reaction as follows:

COMPONENT	50 µl REACTION
DNA	0.2 ug
10X rCutSmart Buffer	1 ul (1X)
XhoI	0.4 ul
Nuclease-free Water	to 10 ul

2. Incubate at XX°C for 15-30 minutes

DpnI Digestion For PCR products

Rationale: DpnI only digests bacterial plasmid DNA, leaving PCR products untouched. This allows us to get rid of any remaining plasmid DNA in the background, ensuring they won't affect the assemblies in downstream steps.

Protocol for FastDigest DpnI [slightly modified from the manufacturer]

1. Add 0.5 uL of DpnI to a 25 uL PCR volume. No need to add buffer (Keep the DpnI on ice).
    a. If you used some PCR product for a gel, still add 0.5 uL of DpnI.
    b. E.g., even if you have 22 uL or 23 uL, add 0.5 uL DpnI.
2. Pipette mix.
3. Incubate at 37°C in the **incubator in 301** for AT LEAST 30 min.
    a. You should not use the water-bath due to evaporation.
    b. You should not use the heat block since it is too big for the 0.2 mL PCR tubes.
    c. No need to inactivate the enzyme. You'll be doing PCR purification right after anyways.
4. After incubating for at least 30 min, you can proceed to PCR cleanup (as long as the gel results look good).

PCR Purification

Make sure to use the correct protocol depending on the kit you're using!

GeneJET PCR Purification kit Protocol (don't use this ours is sus)

(https://static.igem.org/mediawiki/2017/8/81/T--Chalmers-Gothenburg--Thermo_Scientific_GeneJET_PCR_Purification_Kit.pdf)

1. Add a 1:1 volume of Binding Buffer to the completed PCR mixture (e.g. for a 25ul PCR reaction, add 25ul binding buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
    a. Optional: if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 75 µL of isopropanol should be added to 25µL PCR reaction with 125 µL of Binding Buffer). Mix thoroughly. Note. If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
2. Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 60s at 15,000 RCF. Discard the flow-through.
3. If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 60 seconds and discard flowthrough. Repeat until the entire solution has been added to the column membrane.
    a. Close the bag with GeneJET Purification Columns tightly after each use!
4. Add 700 µL of Wash Buffer (make sure it has already been diluted with the ethanol) to the GeneJET purification column. Centrifuge for 60s at 15,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
5. Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. Note. This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
6. Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add 20 µL of Ultrapure H2O to the center of the GeneJET purification column membrane, incubate for 2 min
7. Centrifuge for 1 min.

a. Note For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended. If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column. If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation. 7 Discard the GeneJET purification column and store the purified DNA at -20 °C

Luna NanoTech PuroSPIN PCR Purification Kit Protocol

Do NOT MIX NaOH with anything other than itself! Do not pour it into the liquid waste.

1. Activate a new PuroSPIN MINI Silica Spin Column inserted into the provided 2 mL collection tube by adding 100 µL of 3M NaOH solution to the column. Incubate for 5 min at room temperature.
2. Add 2 volumes of Binding Buffer BB-PP to 1 volume of DNA product. For example, add 100 µL of Binding Buffer BB-PP to 50 µL of DNA sample.
3. Add 2 volumes of 100% Isopropanol to the sample. For example, add 100 µL of 100% isopropanol to 50 µL of DNA sample.
4. Centrifuge the columns from step 1 for 1 min at 15,000 x g. After centrifuging the NaOH you should pipette the liquid back out, not pour. **The waste NaOH goes back into the 3M NaOH tube to be reused.**
    a. DO NOT mix the NaOH with the binding buffer. You will produce chlorine gas.
5. Transfer up to 800 µL of the sample (from Step 2) into the equilibrated PuroSPIN DNA Purification Column. Centrifuge at 15,000 x g for 1 minute to allow DNA to bind to the column. Discard the flow-through in DNA purification waste tubes.
6. Add 700 µL of Wash Buffer WB-PP and centrifuge at 15,000 x g for 1 minute. Discard the flow-through DNA purification waste tube.
7. Centrifuge at 15,000 x g for 1 minute to remove the residual washing buffer. If there is still flow-through, repeat this step.
8. Transfer the column into a clean 1.5 mL microcentrifuge tube.
9. Add 20 uL of nuclease-free water. Incubate for 10 minutes at room temperature.
    a. The longer you incubate the better the results!
10. Centrifuge the column at 15,000 x g for 1 minute to collect the purified product.

Colony PCR

Background

- Main principle: colony PCR (cPCR) is similar to normal PCR, but you use the genomic DNA from a colony on a plate as your template (as opposed to purified DNA). The purpose is to verify if your plasmid of interest has been successfully transformed into the strain.
- READ THE WHOLE PROTOCOL BEFORE YOU BEGIN.

Protocol

1. Per 0.2 uL PCR tube:
    a. Nuclease free water: 10 uL
        i. Pipette this into the tubes first
    b. Bacteria (Pick ½ of a colony off of the desired plate—there needs to be bacteria left on the plate to grow into a liquid culture later if need be.)
        i. Do this in a sterile environment.
        ii. Use a P10 tip to pick ½ a colony, then swirl it around in the 0.2 uL tube with water in it to get the bacteria into the tube. Then discard the tip.
    c. 2X Q5 mastermix: 12.5 uL
    d. 10 uM Forward Primer: 1.25 uL
    e. 10 uM Reverse Primer: 1.25 uL
        i. We can use the PCR primers for GGA, just make sure you're using the correct one for each plasmid
2. Run PCR for each reaction, but change the initial denaturation step from 30 sec to 1 minute. Ensure you use the correct annealing temp for your primers and correct extension time for your amplicons.

After PCR is complete, proceed with gel electrophoresis to visualize your samples. Refer to other protocols for how to do gel electrophoresis if necessary. Load 10 uL of each sample (use the bigger comb).

Resuspending IDT gBlocks (Stock)

1. Before opening the tube, spin it down in a microcentrifuge for 3–5 seconds to ensure the DNA is in the bottom of the tube. The pellet can become statically charged and, without this step, can either fly out of the tube or remain in the cap, resulting in loss of yield.
    a. Try not to use the centrifuge on our bench in 403! It spins slowly.
2. Add ultrapure water to reach a final concentration of 10 ng/µL.
    a. Storage concentrations <1 ng/µL may result in loss of material due to adherence to the plastic tube. The addition of a carrier such as tRNA or poly A at a concentration of 0.1 to 1.0 mg/mL can help to avoid this issue.

3. Vortex briefly.
4. Incubate at approximately 50°C for 15–20 min. Heating the tube will ensure the solvent comes in contact with the tiny pellet, even if it is stuck to the side of the tube. Thus, this step will increase the likelihood that the entire pellet will be resuspended.
5. Briefly vortex and centrifuge.
6. Verify the final concentration using a Nanodrop.

Golden Gate Assembly

1. Use the Nanodrop spectrometer in 303 to record DNA concentration of vector inserts and plasmids. Update the “DNA Stock Conc. (ng/uL)” column of the GGA calculator with these values.
2. Make the ligation reaction mix according to the calculator.

Reagent	**10 uL reaction** (numbers vary - ignore this column and use calculator)
Plasmid backbone	75 ng/uL, 1 uL
Insert	75 ng each plasmid 2:1 molar ratio
T4 DNA Ligase Buffer	1 uL
NEB Golden Gate Assembly Mix	0.5 uL (For <10 inserts)
Nuclease-free H2O	To 10 uL

3. After making the ligation reaction mix, very gently pipette up and down a few times to mix well.
4. Incubate mixture in thermocycler with appropriate protocol:
    - Start with an initial hold for 2 min at 37 C.
    - Do 4 C infinite hold in the end if you want to leave it in the thermal cycler for a bit.

INSERT NUMBER	SUGGESTED ASSEMBLY PROTOCOL
For 1 Insert	37°C, 5 min (cloning) or 37°, 1 hr (library preparation) → 60°C, 5 min
For 2 -10 Inserts	(37°C, 1 min → 16°C, 1 min) x 30 → 60°C, 5 min
For 11 - 20+ inserts	(37°C, 5 min → 16°C, 5 min) x 30 → 60°C, 5 min

Gibson Assembly

Gibson Assembly Master Mix:

Prepare 6 ml of 5X ISO Buffer in a 15 ml falcon tube as follows:

- 3 ml 1 M Tris-HCl pH 7.5
- 150 µl 2 M MgCl2
- 240 µl 100 mM dNTP mix (25 mM each: dGTP, dCTP, dATP, dTTP)
- 300 µl 1 M DTT
- 1.5 g PEG-8000
- 300 µl 100 mM NAD+
- dH20 to 6 ml

Buffer Recipes (these do not need to be sterile, but should be made with nuclease free water):

Buffer	g in 1 mL (dH2O)
DTT	0.1542
MgCl2	0.4066
NAD+	0.0681

Store at -20 C in 320 µl aliquots.

Gibson assembly master mix Recipe (1.2mL, can make smaller volumes if necessary!)

- 320 µl 5X ISO Buffer
- 0.64µl 10 U/µl T5 exonuclease*
- 20 µl 2 U/µl Phusion polymerase
- 160 µl 40 U/µl Taq ligase
- dH20 to 1.2 ml

Store at -20 C in 15 µl aliquots.

*This is optimized for 20-150 bp sequence homology overlaps. Generally I aim for 20bp overlaps for my gibson assemblies.

Gibson Assembly Protocol:

1. Thaw a 15 µL aliquot of the Gibson assembly master mix, and keep on ice until use.
2. Measure the DNA concentration (ng/µL) of each assembly piece.
3. Add 100 ng of the linearized vector backbone and equimolar amounts of the other assembly pieces to the thawed 15 µL master mix in a 20 µl total volume assembly reaction mixture as follows:
    a. Linearized vector backbone (100 ng)
    b. Each additional assembly piece (to equimolar with backbone)
    c. 15 µL Gibson assembly master mix
    d. dH20 to 20 µL
4. Incubate the assembly reaction at 50 C for 60 minutes, and then place on ice.
5. Transform 5 µL of the assembly reaction into 100 µL of competent E. coli and/or run a diagnostic agarose gel to check for successful assembly.

Waste Disposal Procedure (Biozone)

- Dispose of any biological waste (eg. culture tubes, agar plates) in the biological waste bin (yellow bin). Dispose any liquid biological waste in a designated jar on the iGEM bench for autoclaving later.
- Dispose of tubes from PCRs and golden gate assemblies in the chemical waste bin (green bin).
- Dispose of gel from gel electrophoresis in the designated bin in room 314.