Standard Protocols for Working with Bacteria

Preparation of Electrocompetent *E. coli*

Day 1

1. At the end of the day, streak out cells from the original stock in the -80°C onto an SOB (or LB) plate with no antibiotics. Use a sterile needle. Leave to grow overnight at 37°C

Day 2

2. Prepare SOB media (1.6 L – 3 x 500 mL + 100 mL)
   SOB recipe – 32 g of tryptone, 8 g of yeast extract, 0.928 g of NaCl and 0.304 g of KCl. Adjust to pH 7.4-7.5
   Autoclave the media, as well as glass bottles for the glycerol solution, 6 centrifuge bottles (half-filled with water) and 3 cans of 1.5 mL Eppendorf tubes.
   Note: NEVER use white tubes.

At the end of the day, pick a colony and add it to the 100 mL culture. Grow overnight at 37°C

Day 3

3. Inoculate each of the three 500 mL cultures with 7 mL of bacteria from the 100 mL subculture. Leave to grow in the 37°C shaker for 3.5 hours.
4. Dilute 350 mL of glycerol into 3 L of ddH_2O. Filter sterilize and store at 4°C during the incubation.
5. Store three cans of autoclaved tubes at -20°C.
6. After 3.5 hours, place the three 500 mL cultures on ice for 30 minutes. Turn the centrifuge on and allow it to cool down to 4°C.
7. Spin the cells down at 4000 rcf for 8 minutes using the A-10 rotor.
8. Remove the supernatant and add ~50 mL of the chilled glycerol solution to each centrifuge bottle. Shake on ice until the cells are fully resuspended.
9. Top up the glycerol (+ ~200 mL) and spin the cells down again at 4000 rcf for 8 minutes.
10. Remove the supernatant and repeat re-suspension and washing with glycerol again.
11. Get liquid nitrogen in the 1 L dewar.
12. After the second wash, remove the supernatant and place the bottles on ice. Gently re-suspend the cells in the leftover liquid using transfer pipettes.
13. Aliquot 50 uL of cells into the 1.5 mL tubes. Snap-freeze the tubes in liquid nitrogen and store at -80°C.

LB Media Preparation

Note: Recipe prepares 1 L of media. Scale up as needed.

1. Weigh out 20 g of LB broth into a 1 L plastic beaker with a magnetic stir bar.
2. Add 800 mL of ddH_2O and stir until fully dissolved.
3. Adjust the pH to 7.3-7.4 with 10 M NaOH.
4. Top up to 1 L with ddH_2O.
5. Pour into appropriate liquid containers (bottles with loosened caps, flasks covered with foil).
6. Autoclave on the 15 minute liquid cycle.

7. Add additives to correct final working concentrations. See list below.

Common Additives' Final Concentrations

0.02 to 0.4% arabinose
0.5 to 1 mM IPTG
1x Ampicillin (0.1 mg/mL)
1x Chloramphenicol (25 µg/mL)
Spectinomycin (50 µg/mL)

LB Agar Plates Preparation

Note: Recipe prepares 2 L of media, which, in our hands, makes ~150-180 100 mm diameter plates. Scale up or down as needed.

1. Weigh out 40 g of LB in a 2 L plastic beaker with a magnetic stir bar.
2. Add 1.5 L of ddH_2O and stir until fully dissolved.
3. Adjust the pH to 7.3-7.4 with 10 M NaOH.
4. Top up to 2 L of ddH_2O.
5. Measure 15 g of agar (**NOT AGAROSE)** into two 2 L Erlenmeyer flasks with stir bars.
6. Add 1 L of LB to each Erlenmeyer flask. Cover with foil.
7. Autoclave on the 15 minute liquid cycle.
8. Allow solution to stir until warm to the touch (i.e., you must be able to hold the flask with your bare hands comfortably). Ensure that stirring is performed gently enough that no bubbles form; otherwise, plates will have air pockets when incubated.
9. Add additives, as needed. See list below.
10. Spray the bench down with 70% ethanol. When dry, light the Bunsen burner.
11. Pour warm solution to plates such that half the plate is covered with agar. Gently shake to evenly distribute agar.
12. Turn the Bunsen burner off. Leave plates to solidify. Plates can be used after an hour on the bench, but for minimal condensation after incubation, leave plates overnight at room temperature. Store at 4°C the next day until use.

Common Additive Amounts

1 mL 20% arabinose for 0.02% arabinose plates
1 mL 0.5 M IPTG for 0.5 mM IPTG
4 mL 1000x ampicillin for 4x ampicillin plates (note: in our hands, a final concentration of 4x ampicillin allows for longer storage of plates and a delay in the appearance of satellite colonies without much negative effect on the growth of desired colonies)
1 mL 1000x chloramphenicol

Mueller-Hinton Broth and Plates Preparation

Media – see **LB Media Preparation** for more detail.

1. Dissolve 21 g of MHB mix in 1 L of ddH_2O.

2. Adjust the pH to 7.4 using 10 M NaOH or 6 M HCl.
3. Autoclave.

Plates – see **LB Agar Plates Preparation** for more detail.
Use 42 g of MHB mix instead of 40 g of LB.

Transformation of *E. coli*

1. Get ice. Wipe the bench down with 70% ethanol. Turn on the Bunsen burner.
2. Retrieve 50 µL aliquots of competent cells from -80°C freezer and put on ice to slowly thaw.
3. Place cuvettes on ice to cool.
4. Add 1 µL of plasmid to the competent cells. For reaction transformations, please see the specific reaction for the reaction volume to be transformed.
5. Turn on the electroporator, and use the "bacteria" setting. Press Measurement button to "ms".
6. Transfer the competent cells (all of it) to the cuvette.
7. Thoroughly dry the outside of the cuvette using a Kimwipe or paper towel.
8. Place cuvette into the holder with metal plates positioned to make contact with the electrodes.
9. Press pulse. Measurement readings should be 5 ms or higher without arcing.
10. Add 900 µL LB with no antibiotic to the cuvette to resuspend the bacteria. Move the resuspended bacteria into a labelled 1.5 mL tube (usually the original comp cells tube)
11. If the plasmid contains an ampicillin resistance gene, proceed to step 12. If not, shake the cells at 37°C for at least an hour.
12. For plasmids, use a sterile inoculation loop, streak out the resuspended bacteria on a plate with the appropriate additives. For reaction transformations, plate 60 µL of the transformed bacteria, add ~5 sterile glass beads to the plate, and gently shake the plate to spread the cells evenly over the surface of agar before dumping out the glass beads. Incubate the plate upside down at 37°C.
13. Turn the Bunsen burner. Store transformed competent cells at 4°C for up to a week.
14. Clean the cuvettes.
    a. Fill with 10% bleach and let the cuvettes stand for 5-10 minutes.
    b. Rinse with distilled water.
    c. Add 1 M HCl to the halfway mark and let stand for a minute. (Note: leaving HCl in the cuvettes for an extended period of time will corrode the metal.)
    d. Neutralize with 1 M NaOH. Shake cuvette vigorously.
    e. Rinse with excess distilled water to remove salt.
    f. Add 70% ethanol and let stand for 5-10 minutes. Empty ethanol and leave to dry upside down.

Inoculating Cultures

1. Identify target colonies on plates by circling them with a marker.
2. Wipe the bench down with 70% ethanol. Turn on the Bunsen burner.
3. Prepare liquid media with appropriate additives (see **LB Media Preparation** for LB).
4. Aliquot 4 mL of media into Falcon culture tubes.
5. Using a sterile small pipette tip, pick a target colony and drop it into the 4 mL culture.
6. Incubate in the 37°C shaker for at least 10 hours.

Note: If the objective is to grow and collect plasmids and there is no need for a visible phenotype such as fluorescence (e.g., when growing up a previously sequenced stock of plasmid), we recommend skipping any inducers for better yield.

Plasmid Minipreps (ThermoFisher Column-based Method)

1. Spin down bacteria cultures (usually 4 mL – see **Inoculating Cultures**) in a 2 mL tube for 1 minute using at least 12,000 rpm. Discard supernatant.
2. Resuspend bacteria pellet in 250 µL of Resuspension Buffer (with RNAse added) by vortexing.
3. Add 250 µL of Lysis Solution and mix for ~1 minute. Do not leave cells in Lysis Solution for more than 5 minutes as it will degrade the DNA.
4. Add 350 µL of Neutralization Solution and mix.
5. Centrifuge for 5 minutes using at least 12,000 rpm.
6. Transfer the supernatant to the column (blue BioBasic or ThermoFisher's GeneJet columns). Centrifuge the column for 1 minute.
7. Discard the flow through in the collection tube.
8. If the plasmid is in BL21(DE3) or other *endA+* strains, add 500 µL of Buffer PB from Qiagen. Spin for 1 minute with at least 12 000 rpm. Otherwise, skip to step 9.
9. Add 500 µL of Wash Solution (with ethanol added). Spin for 1 minute with at least 12,000 rpm.
10. Repeat steps 7 and 9.
11. Centrifuge again for 1 minute to dry.
12. Transfer the column to a clean and labelled 1.5 mL centrifuge tube.
13. Add 50 µL of *hot* elution buffer into the center part of the column, making sure not to touch the actual column. Incubate at room temperature for 2 minutes.
14. Centrifuge for 2 minutes using at least 12,000 rpm.

Standard Q5 PCR Protocol

1. In a thin-walled 200 µL PCR tube, add the following:

Enhancer	10 µL
ıction Buffer	10 µL
dNTPs	1 µL
Forward Primer	2.5 µL
Reverse Primer	2.5 µL
ıte	1 µL
ıved ddH_2O	22.5 µL
yme	0.5 µL

Note 1: To set up multiple reactions, we recommend preparing a master mix of the invariable components (usually the enhancer, buffer, dNTPs and ddH_2O) by multiplying the required volumes by (n+1), where n is the number of reactions needed, and aliquoting to reduce pipetting errors and speed up set up times.

*Note 2: If running a scaled up reaction (e.g., 100 µL), we recommend scaling up all the components **except the template** and just adding ddH_2O to make up the difference.*

2. Place the reaction in a thermocycler and run the following program:

1.	98°C	30 sec
2.	98°C	10 sec
3.	T_m + 0-3°C*	30 sec
4.	72°C	30 sec/kb
5.	Go to step 2 and repeat 34 more times.	
6.	72°C	2 min**
7.	4-12°C	Hold

**Note: The T_m used for this calculation is the lowest T_m of the primers used. Optimization may be needed to find the best annealing temperature.*

***Note: For genes $\leq$ 2 kb, a final extension time of 2 min should suffice. However, for longer genes, we recommend increasing the final extension time.*

3. OPTIONAL. We recommend performing this step when preparing linearized vectors for Gibson assembly or InFusion to minimize background. Add 1 µL of Dpn1 per 50 µL reaction and run the following thermocycler program:

37°C	Minimum 1 hour
80°C	5 min
4-12°C	Hold

Agarose Gel Electrophoresis and Extraction

Preparing 1% agarose gels

Note: This procedure makes two gels.

1. Set up the gel mold apparatus with the right-sized wells.
2. Weigh out 500 mg of **agarose** in a 250 mL Erlenmeyer flask.
3. Add 50 mL of 1X TAE buffer.
4. Microwave the mixture for a minute to dissolve the agarose.
5. Let the solution cool until bearably warm to the touch.
6. Add 2 µL of ethidium bromide. Swirl.
7. Pour solution into mold. Remove any air bubbles. Leave gel to completely solidify before removing combs.

8. Store unused gels in a box with TAE buffer to prevent dehydration. Stored gels are good for ~5 days, depending on how much ethidium bromide was added.

Running gels

1. If there is no dye in the reactions, add 1 µL of DNA loading dye for every 5 µL of reaction. Mix thoroughly by pipetting.
2. Ensure that you have enough wells for all your samples plus DNA ladder. Pipette the full volume of each reaction (or the maximum volume your wells can hold) into a well.
3. Load ~5 µL of DNA ladder into a well.
4. Ensure that the gel is fully submerged in TAE buffer in the gel box.
5. Close the lid. Set the power supply to 100 mV, 400 mA and constant voltage. Run for 20 minutes.
6. Check the gel using the UV trans-illuminator or the gel imaging apparatus. *Note: It is important to MINIMIZE the exposure of DNA to UV light.*
7. If necessary, run the gel for longer with 5 or 10 minute intervals.

Extraction

1. Excise the DNA band from the agarose gel with a clean, sharp razor blade. Minimize the size of the gel by removing excess agarose. Place the excised fragment into a 2 mL centrifuge tube.
2. Add ~3 volumes of Binding Buffer to 1 volume of gel – usually ~500 µL.
3. Incubate at 70°C to melt the agarose and allow the binding buffer to bind the DNA. Once the gel slice is fully melted, allow the tube to cool down to room temperature.
4. Transfer the solution into a gel extraction column (purple for ThermoFisher).
5. Spin with at least 12,000 rpm for 1 minute.
6. Add 700 µL of Gel Wash Buffer (with ethanol added).
7. Spin with at least 12,000 rpm for 1 minute. Discard flow through.
8. Spin for another minute with at least 12,000 rpm to dry the column.
9. Transfer the column to a clean and labelled 1.5 mL centrifuge tube. Add 50 µL of *hot* elution buffer to the center of the column, making sure not to touch the column.
10. Elute by spinning for 2 min with at least 12,000 rpm.

Quantifying DNA using the Nanodrop

1. Wipe the sensor on the Nanodrop with a clean Kimwipe.
2. On the computer, open the NanoDrop software and choose Nucleic Acid Analysis.
3. Initialize the Nanodrop by adding 2 µL of ddH_2O on the stage.
4. Dry the Nanodrop with a Kimwipe.
5. Blank the Nanodrop by adding 2 µL of elution buffer on the stage and hitting “Blank” on the software. When blanking is done, dry the Nanodrop with a Kimwipe.
6. Add 2 µL of your sample to the stage and press “Measure”. Record the concentration, the A260/A280, and the A260/A230 values. Clean the nanodrop by dabbing ddH_2O on a Kimwipe and gently wiping off the sample from the stage.
7. Repeat step 6 for all samples.
8. Close the software. Clean the Nanodrop for the next user by dabbing ddH_2O on a Kimwipe and gently wiping the stage down.

Preparative DNA Digestion

1. In a thin-walled 200 µL PCR tube, add the following:

	ł µg
een FastDigest Buffer	
ɜved ddH_2O	ł8 µL
tion enzymes	ɔtal volume (usually 3 µL of each enzyme for a two-enzyme digestion)

2. Place the reaction in a thermocycler and run the following program:

37°C	ɜ0 min – 1 hour*
85°C**	10 min**
4-12°C	Hold

**Note: For digestions with linear PCR products, ~30 minutes is normally sufficient. For plasmid digestions to collect the insert, we usually run the reactions for an hour. When digesting plasmids to prepare a stock of vectors for ligation, we recommend digesting for at least four hours if possible.*

***Note: Change these settings according to the inactivating conditions for your enzymes. For enzymes with different inactivating conditions, run the more extreme conditions.*

3. For plasmid-based digestions, the bands must be separated by **Agarose Gel Electrophoresis** followed by **Extraction** of the correct bands. For linear PCR-based digestions, the agarose gel can be skipped and the **Extraction** performed directly by adding 500 µL of Binding Buffer to the column, adding the digestion reaction to the Binding Buffer and mixing before proceeding with the rest of the **Extraction** protocol.

DNA Ligation

1. In a thin-walled 200 µL PCR tube, add the following:

*	1 µL
	16 µL
se Buffer	2 µL
se	1 µL

Note: For efficient ligation, the vector must have a concentration of at least 100 ng/ µL.

2. Place the reaction in a thermocycler and run the following program:

22°C	20 min

70°C	5 min
	Hold

3. To transform, see **Transformation of E. coli.** Transform 4 µL of the ligation reaction.

InFusion Assembly

1. In a thin-walled 200 µL PCR tube, add the following:

Vector*	1 µL
Insert	1 µL
5X InFusion Mix	1.5 µL

2. Place the reaction in a thermocycler and run the following program:

50°C	20 min
	Hold

3. To transform, see **Transformation of E. coli.** Transform 2.5 µL of the InFusion reaction.

Diagnostic Digestion

1. In a thin-walled 200 µL PCR tube, add the following:

.0X Green FastDigest Buffer	2 µL
Autoclaved ddH_2O	Up to 14 µL
Restriction enzymes	1 µL total volume (usually 0.5 µL of each enzyme for a two-enzyme digestion)

2. Place the reaction in a thermocycler and run the following program:

37°C	15 min
4-12°C	Hold

3. Run on gel. See **Agarose Gel Electrophoresis**.
   Note: If using older gels, we recommend running only 10 µL of the reaction in case the EtBr has diffused out of the gel to avoid having to re-run the reaction.

In-House Single-Primer Q5 Site-Directed Mutagenesis Protocol

1. In a thin-walled 200 µL PCR tube, add the following:

Q5 Reaction Buffer	10 µL

10 mM dNTPs	1 µL
10 µM Primer	2.5 µL
Template	1 µL
Autoclaved ddH_2O	32.5 µL
Q5 Enzyme	0.5 µL

2. Place the reaction in a thermocycler and run the following program:

98°C	30 sec
98°C	10 sec
T_m + 0-3°C*	30 sec
72°C	30 sec/kb
Go to step 2 and repeat 34 more times.	
72°C	10 min
4-12°C	Hold

**Note: The T_m used for this calculation is the lower T_m on either side of the mutation. The mutation must be flanked by at least 15 bp on either side. Optimization may be needed to find the best annealing temperature.*

3. Add 1 µL of Dpn1 to the reaction and run the following thermocycler program:

37°C	Minimum 1 hour
80°C	5 min
4-12°C	Hold

4. To transform, see **Transformation of E. coli**. Transform 2.5 µL of the Q5 SDM reaction.

Agilent Quikchange Lightning Multi

1. In a thin-walled 200 µL PCR tube, add the following:

QCM Buffer	2.5 µL
QuikSolution	0.75 µL
dNTP Mix	1 µL
10 µM Primer	0.5 µL for 2-3 primers
Template	0.5 µL
Autoclaved ddH_2O	Up to 25 µL

QCM Enzyme	2 µL

2. Add 1 µL of Dpn1 to the reaction and run the following thermocycler program:

37°C	Minimum 1 hour
80°C	5 min
4-12°C	Hold

3. To transform, see **Transformation of E. coli**. Transform 2.5 µL of the QCM reaction.

PBS Buffers Recipes

10x PBS (per L)

80 g NaCl (1.37 M)
2 g KCl (27 mM)
14.4 g Na_2HPO_4 (100 mM)
2.4 g KH_2PO_4 (18 mM)

Dissolve in 900 mL of ddH_2O. Adjust pH to 7.4 with 10 M NaOH or 6 M HCl. Top up to 1 L. Filter sterilize using a 0.2 µm filter.

1x PBS (per L)

100 mL 10x PBS
900 mL ddH_2O
Adjust pH to 7.4 with 10 M NaOH or 6 M HCl. Filter sterilize using a 0.2 µm filter.

PBS Wash Buffer (per L)

100 mL 10x PBS
1.36 g imidazole
900 mL ddH_2O
Adjust pH to 8.0 with 6 M HCl. Filter sterilize using a 0.2 µm filter.

PBS Elution Buffer (per L)

100 mL 10x PBS
17.02 g imidazole
900 mL ddH_2O
Adjust pH to 7.8 with 6 M HCl. Filter sterilize using a 0.2 µm filter.

Protein Expression and Purification in LB Media

1. Wipe the bench down with 70% ethanol and light a Bunsen burner.
2. Pick a single colony from a plate (the fresher the plate, the better) and inoculate a baffled flask (or less preferably, an Erlenmeyer flask) containing LB media supplemented with the

appropriate antibiotics and inducer. Common culture volumes are 50 mL, 150 mL, 200 mL, 250 mL and 500 mL. For volumes larger than 500 mL, we recommend using multiple flasks.

3. Shake overnight at 250 rpm in 37°C. If incubating for one day, ensure that the culture is given at least 24 hours to incubate before proceeding to step 4. If incubating for two days, move the culture to a 30°C incubator for at least 24 hours following the overnight 37°C incubation.
4. Transfer the cultures into large centrifuge bottles (250 mL).
5. Spin at 8000 rpm for 10 minutes at 4°C using rotor A-10. Repeat as necessary to pellet all the cells down.
6. Discard supernatant and resuspend in ~25 mL of 1x PBS on ice.
7. Use the sonicator to mechanically lyse the cells.
    a. Turn on sonicator.
    b. Sonicate each protein sample on ice for 2.5 minutes total run time, with alternating 1 sec ON pulses and by 2 sec OFF pulses.
    c. Clean the sonicator tip in between runs and at the end by wiping with ddH_2O, 70% ethanol, and drying with paper towels.
8. Transfer the sonicated samples into the small centrifuge bottles (40 mL).
9. Centrifuge the samples down at 14,000 rpm for 35 minutes at 4°C using rotor A-14.
10. Pour supernatant into 50 mL conical tubes through a Kimwipe.
11. Add ~1-4 mL of Ni-NTA beads (depending on the quantity of protein produced) and shake on ice for at least 1 hour.
12. Set up vacuum filtration apparatus with appropriately-sized columns.
13. Slowly transfer the Ni-NTA containing solution to the column, making sure that the beads remain wet.
14. Wash the beads with at least 50 mL of cold wash buffer (1x PBS + 20 mM imidazole pH = 8.0). For best results, cap the column, detach from the vacuum manifold and resuspend the beads.
15. Move the column to a protein collection tube on ice and elute the protein with ~2-8 mL of elution buffer (1x PBS + 250 mM imidazole, pH = 7.8). The volume of elution buffer used is dependent on how much protein needs to be eluted off the resin.

Modified Terrific Broth Recipe

This media is best suited for very high levels of protein expression.

Note: This recipe makes 1 L. Scale up as needed.

1. In a plastic beaker, add the following:

Tryptone	14 g
Yeast Extract	7 g
Glycerol	8 mL

2. Add 900 mL of ddH_2O. Adjust to pH = 7.2.
3. Aliquot 90% of the total culture volume desired to baffled flasks (**NOT Erlenmeyer flasks**). Common total volumes of TB for culturing are 50 mL, 100 mL, 150 mL, 200 mL, 250 mL. For example, if the desired total TB volume is 250 mL, add 225 mL of the media to the baffled flask.
4. Cap with foil and autoclave for 15 minutes on the liquid cycle.

5. Make a 10x Phosphate Buffer solution (per L):
    a. 94 g K_2HPO_4

    22 g KH_2PO_4

    Dissolve in 1 L. Adjust to pH = 7.2. Filter sterilize using a 0.2 µm filter.
6. Add 10% Phosphate Buffer solution of the total culture volume (i.e., diluting to 1X). For example, for a total desired TB volume of 250 mL, add 25 mL of the 10X Phosphate Buffer solution to 225 mL of media in the baffled flask.
7. Store at room temperature until use.

Protein Expression and Purification in Modified TB

1. Wipe the bench down with 70% ethanol and light a Bunsen burner.
2. Pick a single colony from a plate (the fresher the plate, the better) and inoculate a baffled flask (or less preferably, an Erlenmeyer flask) containing TB supplemented with the appropriate antibiotics (see **Inoculating Cultures**).
3. Shake in 37°C for 5-7 hours.
4. Add the inducer (e.g., 0.1% L-arabinose) to the desired final concentration and shake the culture at 30°C for two days.
5. Transfer the cultures into large centrifuge bottles (250 mL).
6. Spin at 8000 rpm for 10 minutes at 4°C using rotor A-10. Repeat as necessary to pellet all the cells down.
7. Discard supernatant and resuspend in ~25 mL of 1x PBS on ice.
8. Use the sonicator to mechanically lyse the cells.
    a. Turn on sonicator.
    b. Sonicate each protein sample on ice for 2.5 minutes total run time, with alternating 1 sec ON pulses and by 2 sec OFF pulses.
    c. Clean the sonicator tip in between runs and at the end by wiping with ddH_2O, 70% ethanol, and drying with paper towels.
9. Transfer the sonicated samples into the small centrifuge bottles (40 mL).
10. Centrifuge the samples down at 14,000 rpm for 35 minutes at 4°C using rotor A-14.
11. Pour supernatant into 50 mL conical tubes through a Kimwipe.
12. Add ~1-4 mL of Ni-NTA beads (depending on the quantity of protein produced) and shake on ice for at least 1 hour.
13. Set up vacuum filtration apparatus with appropriately-sized columns.
14. Slowly transfer the Ni-NTA containing solution to the column, making sure that the beads remain wet.
15. Wash the beads with at least 50 mL of cold wash buffer (1x PBS + 20 mM imidazole pH = 8.0). For best results, cap the column, detach from the vacuum manifold and resuspend the beads.
16. Move the column to a protein collection tube on ice and elute the protein with ~2-8 mL of elution buffer (1x PBS + 250 mM imidazole, pH = 7.8). The volume of elution buffer used is dependent on how much protein needs to be eluted off the resin.

Buffer Exchange/Protein Concentrator

1. Add up to 5 mL of protein solution to the top of 10K Microsep Column (protein must be bigger than 10 kDa).

2. Centrifuge at 4°C for 20 minutes at 6000 *x g*. Discard flow through.
3. For buffer exchange, add 2 mL of the new buffer.
4. Centrifuge at 4°C for 20 minutes at 6000 *x g*. Discard flow through.
5. Repeat steps 3 and 4 at least twice.
6. Resuspend protein to desired volume and collect from the top of the filter.

Quantifying Protein using A280

1. Wipe the sensor on the Nanodrop with a clean Kimwipe.
2. On the computer, open the NanoDrop software and choose Protein A280.
3. Initialize the Nanodrop by adding 2 µL of ddH_2O on the stage.
4. Dry the Nanodrop with a Kimwipe.
5. Blank the Nanodrop by adding 2 µL of elution buffer (or the buffer the protein is in, if exchanged) on the stage and hitting "Blank" on the software. When blanking is done, dry the Nanodrop with a Kimwipe.
6. Add 2 µL of your sample to the stage and press "Measure". Record the concentration. Clean the nanodrop by dabbing ddH_2O on a Kimwipe and gently wiping off the sample from the stage.
7. Repeat step 6 for all samples.
8. Close the software. Clean the Nanodrop for the next user by dabbing ddH_2O on a Kimwipe and gently wiping the stage down.

SDS-Polyacrylamide Gel Electrophoresis

Note: Ammonium persulfate solution must be made and used as soon as possible. Additionally, please note that the following recipe makes two gels.

Preparing the Gels

1. Set up the gel pouring apparatus. Ensure that there are no leaks.

Running Gel

1. Add 2.6 mL of ddH_2O to a beaker.
2. Add 3.2 mL of 30% acrylamide/bis-acrylamide solution.
3. Add 2 mL of 1.5 M Tris (pH = 8.8) and mix.
4. Add 80 µL of 10% SDS
5. Right before pouring the gel, add 8 µL of TEMED and mix.
6. Immediately add 80 µL of **freshly made** 10% (w/v) ammonium persulfate solution.
7. Pour the gel immediately. Keep some of the gel in the beaker to test polymerization.
8. Add isopropanol at the top of the gel to level the gel.

Stacking Gel

1. Add 2.6 mL of ddH_2O in a different beaker.
2. Add 1 mL of 30% acrylamide/bis-acrylamide solution.
3. Add 1.25 mL of 0.5 M Tris (pH = 6.8) and mix.
4. Add 50 µL of 10% SDS.
5. Check that the separating gel has solidified (usually takes 30 minutes at RT). Once solid, dump out the isopropanol.

6. Right before pouring the gel, add 5 µL of TEMED and mix.
7. Immediately add 80 µL of **freshly made** 10% (w/v) ammonium persulfate solution.
8. Quickly add the gel on top of the separating gel until the apparatus is full, such that inserting the combs will cause some to spill. Leave some gel in the beaker to test polymerization.
9. Insert combs.
10. Wait for gel to solidify, which usually takes approximately 30 minutes to 1 hour.

Preparing Sample

1. Mix protein sample with the buffer in a 4:1 ratio (protein:sample buffer).
2. Heat at 65°C for 10 minutes.

Running Gel

1. Set up gel apparatus.
2. Add a sample to each well. Do not forget to add the protein ladder!
3. Run samples at 250 V for 30 minutes, or until the ladder is fully separated and has travelled most of the gel.
4. Remove the gel from the apparatus.

Staining the Gel

1. Fix the gel for at least 30 minutes by gentle shaking in a 5:1:4 solution of methanol, acetic acid and ddH_2O.
2. Stain gel by shaking the gel using 60 mg/L of Coomassie Blue R-250 in 10% acetic acid for at least 30 minutes
3. Destain the gel overnight by gentle shaking in 10% acetic acid.

PPIX Stock Solution To Supplement Bee Sugar Water

*Note: PPIX is light-sensitive! Work with the lights off and wrap all containers in foil. PPIX should also be made fresh as much as possible, hence we recommend making as small of a batch as possible (~50 mL)**.***

Preparing the Solvent System

1. Prepare 500 mL of 0.1 M Tris base (pH uncorrected).
2. Prepare 500 mL of 10 mg/mL ethanol.
3. Mix the two solutions to make 1 L of 1:1 0.1 M Tris base: 10 mg/mL ethanol vol:vol.
4. Filter sterilize using a 0.2 µm filter.

Preparing the solution

5. Weigh out 9 mg of PPIX and dissolve in 100 mL of the Tris:ethanol solvent system.
6. Filter sterilize using a 0.2 µm filter.

EXPERIMENTS

MIC Assay

1. Wipe the bench down with 70% ethanol and light a Bunsen burner.
2. Steak out DH10B and Bl21(DE3) *E. coli* onto an LB plate each with no antibiotics. Incubate plates overnight at 37°C.
3. For DH10B and BL21(DE3), inoculate 3 separate cultures each in 4 mL of Mueller-Hinton broth (MHB). (see **Inoculating Cultures**).
4. Shake the cultures at 37°C and 250 rpm until the cultures are turbid.
5. Pipette 900 µL of fresh MHB into cuvettes (one per culture) and add 100 µL of a culture into a cuvette for a total volume of 1 mL per cuvette. Prepare a blank with 1 mL of fresh MHB.
6. Use the UV spectrophotometer to measure OD_{600}.
7. Using the measured OD_{600} values, calculate the volume of each culture required to dilute each culture to a final volume of 4 mL with an OD_{600} of 0.13 (equivalent to a density of 10^8 CFU/mL).
8. Prepare 1 mL of 32 mM PPIX stock solution in the dark. Wrap containers in aluminum foil.
    a. Dissolve 0.0043 g of PPIX in 1 mL of 1:1 0.1 M Tris base:10 mg/mL ethanol.
    b. Filter-sterilize the solution using 0.2 µm filter.
9. Dilute this PPIX stock solution by adding 9 mL of fresh MHB.
10. Dispense 1 mL of fresh MHB into ten 2 mL centrifuge tubes labelled “240 µM”, “120 µM”, “60 µM”, “30 µM”, “15 µM”, “7.5 µM”, “3.75 µM”, “1.875 µM”, “0.938 µM”, “0.469 µM”.
11. Serially dilute the diluted PPIX solution further by pipetting 1 mL into the “240 µM” tube, mixing and pipetting 1 mL from this tube to the “120 µM” tube. Repeat this dilution all the way down to the “0.469 µM” tube, and discard 1 mL from this tube.
12. In a sterile 96-well plate, transfer 50 µL of the diluted DH10B culture in triplicate to columns 1 to 11. In column 12, add 100 µL of MHB as a sterility control.
13. Transfer 50 µL if the diluted 240 µM PPIX in triplicate down the first column and mix the solution well. Repeat for the 120 µM PPIX solution in the second column, 60 µM in the third and so forth. In column 11, transfer 50 µL of MHB for growth control.
14. Repeat steps 11 and 12 for the diluted BL21 (DE3) cultures.
15. Cover plates with aluminum foil ensure minimal light exposure.
16. Incubate both plates at 37°C for 16-20 hours.
17. Measure OD_{600} of the plates.

Production of Protoporphyrin IX

1. Transform HemA in pBAD into DH10B (see **Transformation of E. coli**).
2. Inoculate 250 mL of modified TB media supplemented with 1x ampicillin with a single colony from the HemA/pBAD plate (see **Modified Terrific Broth Recipe** and **Inoculating Cultures**). Wrap the flask in aluminum foil to protect from light.
3. Shake at 37°C for 5-7 hours.
4. Add 500 µL 20% L-arabinose and shake at 30°C for two days.

Protoprorphyrin IX Extraction Protocol #1

Note: We performed the extraction on a 35 mL aliquot of the culture and stored the rest at 4°C. Given the light sensitive nature of protoporphyrin IX, this entire procedure is done in the dark and with foil-wrapped containers.

1. Transfer 35 mL of the culture to a small centrifuge bottle.
2. Add 140 mg of DEAE-Sephadex A-25 resin to the culture.
3. Shake on ice for at least 30 minutes.
4. Spin down at 21,000 *x g* for 15 minutes.
5. Discard supernatant. Add 35 mL of 10:2:1 acetone:6 M HCl:*N,N*-dimethylformamide.
6. Shake on ice for at least 30 minutes.
7. Spin down at 21,000 *x g* for 15 minutes.
8. Collect supernatant.

Protoporphyrin IX Extraction Protocol #2

Note: We performed the extraction on a 35 mL aliquot of the culture and stored the rest at 4°C. Given the light sensitive nature of protoporphyrin IX, this entire procedure is done in the dark and with foil-wrapped containers.

1. Transfer 35 mL of the culture to a small centrifuge bottle.
2. Spin down at 21,000 *x g* for 15 minutes.
3. Transfer supernatant to a clean small centrifuge bottle.
4. Add 350 mg of DEAE-Sephadex A-25 resin to the supernatant, and shake on ice for at least 30 minutes.
5. Spin down at 21,000 *x g* for 15 minutes.
6. Discard supernatant. Add 35 mL of 4:1 acetone:12 M HCl (vol:vol).
7. Shake on ice for at least 30 minutes.
8. Spin down at 21,000 *x g* for 15 minutes.
9. Collect supernatant.

Collecting the Fluorescence and Absorbance Spectra of Isolated Product.

Note: Acetone degrades most plastics (especially pipette tips and the attached pipette!) rapidly. As such, all the small volume transfers in this protocol were done with Pasteur pipettes. Given the light sensitive nature of protoporphyrin IX, this entire procedure is done in the dark and with foil-wrapped containers.

1. Prepare a stock of chemically-synthesized protoporhyrin IX in the same solvent system used during the extraction to use for comparison by dissolving a few mg of PPIX in the solvent system.
2. Approximately normalize the concentration of the stock PPIX solution to the extracted solution by diluting the stock solution with more solvent such that the intensity of both solutions' colours look the same.
3. Transfer ~100 µL of both stock and extracted solution to a 96-well plate.
4. On the Tecan Safire II platereader, perform an emission scan from 450 to 700 nm (ex: 400 nm) and an excitation scan from 350 to 580 nm (em: 605 nm).
5. Perform an absorbance scan from 380 to 630 nm.

TLC Protocol for Protoporphyrin IX

Note: Acetone and chloroform degrade most plastics (especially pipette tips and the attached pipette!) rapidly. As such, all the liquid handling, except for acetic acid, in this protocol were done with glassware. Given the light sensitive nature of protoporphyrin IX, this entire procedure is done in the dark and with foil-wrapped containers to minimize unnecessary light exposure.

1. Prepare the mobile phase system:
   Mobile phase system #1: *N,N*-dimethylformamide, methanol, ethylene glycol, acetic acid, chloroform (4:35:6:0.4::20 [vol/vol/vol/vol/vol])
   Mobile phase system #2: *N,N*-dimethylformamide, methanol, ethylene glycol, acetic acid, carbon tetrachloride, chloroform (4:35:6:0.4:18:20 [vol/vol/vol/vol/vol/vol])
2. On a TLC plate, use a pencil to draw a line 1 cm above the short edge of the plate as a baseline.
3. Line a 250 mL beaker with filter paper to saturate the beaker with the mobile phase's vapours. Add enough volume of the mobile phase such that the baseline remains above the level of the mobile phase. Cover the beaker with a watch glass to trap the vapors.
4. Use a capillary tube to spot the stock solution and the extracted sample side by side on the baseline.
5. Using tweezers, carefully position the plate in the beaker such that the baseline side with the spotted samples is closest to (**BUT STILL ABOVE)** the solvent line.
6. Capillary action will then pull up the solvent through the plate and allow for separation of the solutions' components. When the solvent is 1 cm from the top of the TLC plate, remove the plate from the beaker and mark the solvent front with a pencil. Measure the distance traveled by the solvent.
7. Use UV light (or blue light with red filters for fluorescence) to image the plate. Circle any visible spots and take note of any streaking. Measure the distance traveled by each spot.
8. Calculate the R_f values for each spot using the following formula:

$$R_f = \frac{\textit{distance travelled by spot}}{\textit{distrance travelled by solvent front}}$$