

Prather Lab Advanced Protocols

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This document contains several advanced molecular biology protocols that you may need to do from time to time.

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1. Preparation of Bacterial Lysates by Freeze-Thaw

Note that this protocol only works on bacteria. This protocol will not work on yeast.

This protocol requires liquid nitrogen. Our lab usually has a tank of liquid nitrogen around for use in this protocol. If our lab does not have liquid nitrogen, the Gleason lab almost always has some. You will need ~300 mL of liquid nitrogen for this procedure.

1. Prepare and grow the desired culture ensuring sufficient volume and appropriate conditions to express the protein of interest (10 mL of culture will yield ~1 ml of lysate)
2. Pellet your cell culture at 1,000 x g for 10-20 minutes at 4°C. Discard the supernatant.
3. Re-suspend the pellet in ~1/10 culture volume of 1 mg/mL lysozyme in 10 mM Tris-HCl (pH = 8.0) (you may add 1 Roche protease inhibitor cocktail tablet (found in the 4°C refrigerator) to 10.5 mL of lysozyme solution to mitigate protein degradation if desired). Transfer the re-suspended cell solution to a 1.7 mL tube. Using more lysozyme solution at this stage yields a larger volume of more dilute lysate, while using less yields a smaller volume of more concentrated lysate.
4. Incubate the re-suspended cells on ice for 30 minutes. While the cells are incubating on ice, pre-heat the lab's water bath to ~37°C.
5. Add ~300 mL of liquid nitrogen (from a Dewar or the lab's liquid N₂ tank) to a small Styrofoam container placed on the floor. Use blue cold gloves to do this.
6. Take your 1.7 mL tube containing your cell suspension and place it in a white 4x4 floating tube holder.
7. Using cold gloves and the tube holder, submerge your tube in the pool of liquid nitrogen for ~5 seconds (or until suspension is completely frozen – the liquid N₂ should stop bubbling when samples are frozen). After the 5 seconds have elapsed, submerge the tube in the 37°C water bath for about 30 seconds (or until suspension is completely thawed). Be very careful while freezing your cells, as the blue cold gloves will not protect your fingers from the liquid nitrogen for very long.
8. Submerge your tube again in liquid nitrogen for 5 seconds, followed by another 30 seconds in 37°C water. Repeat this until your tube has been through a total of 5 of these freeze-thaw cycles.
9. Centrifuge your sample at >14,000 x g for 15-20 minutes at 4°C. Make sure the sample is thawed before centrifuging.

10. Transfer the supernatant (your lysate) to a fresh tube. You may have problems removing the supernatant with a pipet due to the slimy white pellet getting sucked up into the pipet tip. If this is a problem then simply pour the supernatant into a fresh tube. Discard the pellet.

You may wish to aliquot your lysate out to several tubes to avoid having to repeatedly thaw your lysate as you use it. Store your lysate at -20°C . Lysates should generally be thawed at 4°C . Bacterial lysates prepared by this freeze-thaw method (using 50 mL of culture) will typically contain 1-5 $\mu\text{g}/\mu\text{L}$ of total protein (BSA equivalent by Bradford assay) if you used 1 mL of lysozyme solution in Step 6.

You may notice that your supernatant is viscous. Depending on how concentrated the lysate is, the solution may be so viscous that you may have trouble pipeting or otherwise manipulating the sample. If this is a problem you can dilute your sample with 10 mM Tris-HCl (pH = 8.0, with or without protease inhibitors) to lower the viscosity. Remember though that by diluting your sample you are decreasing the concentration of protein within it.

2. Bradford Total Protein Assay

The Bradford total protein assay measures the total amount of protein in your sample using a special dye that changes color from amber to sapphire blue in the presence of protein. The dye interacts non-specifically (on a mass basis) with all proteins and does not distinguish between different kinds of proteins. The blue form of the dye absorbs light at 595 nm, thus an OD_{595} measurement can be taken of the dye in contact with protein to determine the total mass of protein in a sample.

1. Turn on the visible lamp on the spectrophotometer and set the spectrophotometer up to measure absorbance at 595 nm.
2. In a clean 20 mL culture tube, add 3 mL of Bio-Rad protein dye reagent to 12 mL of water and mix. The color of this solution should be dark red with bluish bubbles.
3. Using a 10 mg/mL (100x) protein standard of Bovine Serum Albumin (BSA, found in the 4°C refrigerator), prepare standard solutions in 1.6 mL microtubes as follows:

Tube	BSA (μL)	Water (μL)	Total (μL)	Final [BSA] (mg/mL)
Standard 1	0	100	100	0
Standard 2	2	98	100	0.2
Standard 3	4	96	100	0.4
Standard 4	6	94	100	0.6
Standard 5	8	92	100	0.8
Standard 6	10	90	100	1.0

You can also use ten times as much of a 1 mg/mL (10x) BSA solution to prepare the standards. If you do, adjust the amount of water you add to each standard such that the total volume of the sample is 100 μL .

4. Add 1 mL of diluted Bio-Rad dye in cuvettes, one for each standard and for as many experimental samples as you have to measure. Add 5 μL of each standard and 1-5 μL of each experimental sample. Vortex briefly to mix.
5. Let each sample stand for 10 minutes at room temperature.
6. After the 10 minutes have elapsed, measure the OD_{595} of each sample. Blank the spectrophotometer to the Standard 1 solution or to water.
7. Build a standard protein concentration curve using the standard samples. If you blanked to Standard 1, do not include this data point on your standard curve. Generally you should obtain an R^2 value greater than 0.97 when your data is fit with a linear function.
8. Use the standard curve to calculate the protein concentration of your experimental samples (in μg BSA equivalent / μL). For very dilute samples, you may get an unreliable result from your experimental sample (i.e. a very low or negative OD_{595}), while for very concentrated samples you may saturate the OD_{595} . Such data should be discarded when calculating the final concentration of total protein in your experimental sample, and the amount of protein added should be adjusted to obtain a reading within the range of the standard curve.

Note that you should account in your calculations for the volume of experimental sample you use. Using a larger volume of experimental sample in the Bradford assay will yield a higher OD_{595} reading.

3. Addition of Chemicals to Pre-Made Plates

Sometimes you may find it necessary to add chemicals like antibiotics or nutrients to already-made agar plates. To do so is fairly straightforward. Note that anything you add to a plate must be sterile.

1. Set the plate(s) out in the biosafety hood for ~15 minutes to remove condensation. If you need to apply a large ($>50 \mu\text{L}$) volume of liquid to the plates you should leave the lid off of them during this time to dehydrate them slightly. This helps them absorb more liquid.
2. Add an appropriate amount of sterilized chemical solution to the top of your plate(s). The amount of chemical you should add is equal to the amount of that chemical you would add to a 20-25 mL of liquid culture. For example, you would add 20-25 μL of stock (1000x) ampicillin to your plates to turn them into Amp plates.

If needed, you can sterilize your chemical solution by passing it through a 0.2 μm sterile filter.

3. Spread the chemical evenly and thoroughly over the surface of the plate(s). Failing to spread an antibiotic over the entire plate surface, for instance, may result in the growth of non-resistant cells on the plate. If the volume of chemical you added is too small to spread it over the surface of the plate (generally $<30 \mu\text{L}$), a small volume ($\sim 50 \mu\text{L}$) of sterile water can be added to your chemical prior to addition to the plate to aid in spreading.
4. Let the plates stand in the biosafety hood at room temperature for 15 minutes to allow the chemical to soak into them.
5. You may now use your plates. Any unused plates should be refrigerated at 4°C .

4. Boiling-Lysis Preparation of Plasmid DNA

The boiling-lysis plasmid prep is used to isolate certain plasmids (like pMMB206) from their host cells. This plasmid prep should be used when the normal Qiagen miniprep kit does not work on the desired plasmid. This protocol works only on bacterial cells. It does not work on yeast cells.

1. Prepare the following solutions (or make sure they already are prepared). Note that the solutions do not need to be sterilized.

STET Solution

10 mM Tris-HCl (pH = 8.0)
 0.1 M NaCl
 1 mM EDTA
 5% (v/v) Triton X-100
 Store at 4°C .

100% Isopropanol

Store at Room Temperature.

2.5 M Sodium Acetate, pH = 5.2

20.51 g $\text{NaC}_2\text{H}_3\text{O}_2$ per 100 mL Solution.
OR 34.02 g $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ per 100 mL Soln.
 Adjust the pH of the Solution to 5.2.
 Store at Room Temperature.

70% (v/v) Ethanol in ddH₂O

Store at 4°C .

1x TE Buffer with RNase A

10 mM Tris-HCl (pH = 8.0)
 1 mM EDTA
 100 $\mu\text{g}/\text{mL}$ RNase A
 Store at Room Temperature.

Lysozyme Solution

1 mg/mL Lysozyme in ddH₂O
 Store at -20°C .

2. Grow up cells containing the plasmid in 3-5 mL of rich media (LB) overnight. Do not try to prep more than 5 mL of culture in one tube.

3. Prepare a boiling water bath by filling a water dish with ~350 mL of water. Place a small stirring bar in the water bath to facilitate heat transfer. Put the water bath on a hot plate and set the heating dial to 7. The water should be steadily boiling after about 15 minutes.
4. Pellet the cells at 1000 x g for 5 minutes at room temperature. Remove as much of the supernatant as possible by aspiration.
5. Re-suspend the bacterial pellet in 350 μ L of 4°C STET solution. Transfer the mixture to a 1.7 mL tube.
6. Add 25 μ L of 1 mg/mL lysozyme solution and mix by inversion.
7. Place the tube in a 4x4 floating white tube holder. Use the tube holder to submerge your sample in boiling water for **exactly 40 seconds**.
8. Centrifuge the sample at >14,000 x g for 15 minutes at 4°C and pour the supernatant into a fresh 1.7 mL tube.
9. Add 40 μ L of 2.5 M sodium acetate (pH = 5.2, room temperature) to the supernatant.
10. Add 420 μ L of pure, room temperature isopropanol. Mix the solution by inversion.
11. Let the solution stand at room temperature for 5 minutes. The solution should gradually become cloudy, as nucleic acids are being precipitated during this stage.
12. Centrifuge the sample at >14,000 x g for 15 minutes at 4°C.
13. Remove as much supernatant as possible by aspiration of the nucleic acid pellet. You may wish to stand the tube on top of a paper towel to help remove any residual liquid.
14. Rinse the pellet with 1 mL of 70% ethanol at 4°C. Do not break up the pellet.
15. Remove as much of the ethanol as possible by aspiration/towel treatment as described in Step 13.
16. Stand the open tube in the 37°C incubator for ~30 minutes to evaporate any residual ethanol.
17. Add 50 μ L of 1xTE buffer with RNase A (100 μ g/mL). You may break up the pellet at this point. The pellet may or may not dissolve in the TE solution.
18. If the pellet doesn't dissolve then let the mixture sit overnight at room temperature.

19. Assuming that the pellet dissolved, add 250 μL of Buffer PB (from the Qiagen miniprep kit) to the TE solution. At this point you can combine multiple tubes of plasmid-containing TE solution together to concentrate your plasmid.

If the pellet does not dissolve repeat the boiling-lysis prep using less culture volume per tube (if you tried to prep 5 mL of culture try prepping just 1-2 mL).

20. Apply the solution to a Qiagen miniprep column and centrifuge for 1 minute at 13,200 rpm and room temperature. Discard the flow-through.

21. Add 750 μL of Buffer PE (with ethanol) to the column and centrifuge again at 13,200 rpm at room temperature for 1 minute. Discard the flow-through.

22. After discarding the flow-through, centrifuge the column yet again at 13,200 rpm for 1 minute at room temperature.

23. Place the column in a clean, labeled 1.7 mL tube and add 30-50 μL of Buffer EB to the center of the column. Let the column stand for 3-5 minutes and then centrifuge at 13,200 rpm for 1 minute at room temperature.

24. Store the flow-through (your prepared plasmid) at -20°C . Discard the column.

5. Sample Dialysis

Dialysis is used to remove small molecules (like amino acids, glucose, etc.) away from larger ones like proteins. Typically dialysis is used to partially purify cellular lysates in which a small molecule or a surfactant is inhibiting the activity of an enzyme of interest in the lysate.

In dialysis, a porous semi-permeable membrane is used to separate small molecules from larger ones. Smaller molecules are able to diffuse out of the membrane into (typically) a very large pool of buffer (effectively removing it from the sample) while larger molecules are retained behind the membrane.

The following protocol uses Slide-A-Lyzer dialysis kits purchased from Pierce (Product #66380).

1. Determine and acquire the correct dialysis membrane cassette for dialyzing your sample. Things to keep in mind when choosing a cassette are:

-The Molecular Weight Cut-Off (MWCO) of the membrane: MWCO is typically defined as the molecular weight at which 95% retention of the molecule is achieved by the dialysis membrane. So a 10,000 MWCO membrane will retain 95% of a molecule that has a molecular weight of 10,000 g/mol. Ensure that the

large molecule (enzyme) you are trying to purify in a sample has a molecular weight significantly above the MWCO of the membrane you are using.

-The Membrane Capacity: The Pierce membranes can only hold a certain volume of sample. You should neither underload nor overload the dialysis cassettes.

2. Determine what buffer you will dialyze against. If you are trying to purify an active enzyme away from small molecules, you should use a buffer that you know the enzyme will not denature in. If you have no idea what buffer would be good to dialyze against, 10-50 mM Tris-HCl (pH = 8.0) is a fairly safe starting point. If your enzyme of interest uses an ion for catalysis (like Mg^{2+}), it is usually helpful (for enzyme stability) to include that ion in the dialysis buffer at a concentration that the enzyme likes.
 3. If your sample contains an active enzyme of interest, you should perform the dialysis at 4°C. In this case, move a stirrer (hot plate) into the 4°C refrigerator. Plug the stirrer into an electrical outlet located on the mid-right side of the refrigerator.
 4. Attach a Styrofoam floatie to the side of the dialysis membrane cassette you will use.
 5. Place the dialysis membrane cassette in a large (~800 mL) pool of dialysis buffer. A 1000-1500 mL beaker works well as a container for the buffer. Allow the membrane to hydrate (by placing it in the buffer pool) for at least 30 seconds before proceeding.
 6. Take your sample up gently in a syringe. Also take up 0.5-1.0 mL of air into the syringe. The extra air will help ensure that none of your sample is lost to the dead volume of the syringe when you inject it into the dialysis membrane cassette.
 7. At each of the 4 corners of the dialysis membrane cassette is a hole containing a septum that can be used to inject your sample into the cassette. Gently pierce one of these septums with the syringe until you can see the tip of your needle inside the cassette. As you will experience resistance as you pierce the septum, be careful not to force the needle into the cassette too quickly. Also be careful not to pierce the dialysis membranes with the needle once the needle has entered the cassette.
 8. Slowly inject your sample and the extra air into the dialysis cassette (at a rate of <0.2 mL/sec).
 9. Slowly draw up the extra air you injected into the sample until no air pockets or bubbles are present in between the dialysis membranes.
- The now-used syringe should be re-capped and disposed of in the small red syringe disposal box.
10. Place the membrane with its floatie into a large pool (~800 mL) of dialysis buffer.

11. Add a stirring bar to the buffer pool and put the beaker containing the buffer on a stirrer (if you are doing dialysis at 4°C then the stirrer should already be set up in the 4°C refrigerator). Set the stirrer to stir the mixture gently (a setting of 1-3 is sufficient, aggressive stirring can damage the membrane).
12. Allow the dialysis to proceed for at least 3-4 hours with gentle stirring. You can also let dialysis go overnight.
13. If desired, pour out the now used dialysis buffer and prepare another ~800 mL of fresh buffer. Add the dialysis membrane cassette to this fresh buffer and repeat dialysis as in Step 12. Each round of dialysis will dilute the concentration of small molecules in your sample by a factor equal to the volume of the dialysis buffer pool divided by the volume of sample dialyzed. So if you are dialyzing a 2.5 mL sample in 800 mL of buffer, the concentration of small molecules in your sample will be diluted $800 \text{ mL} / 2.5 \text{ mL} = 320$ -fold per round of dialysis. You do not need to transfer your sample to a new cassette after each round of dialysis.
14. To remove your sample from the dialysis membrane cassette, insert a fresh syringe containing 0.5-1.0 mL of air into one of the 3 unused ports on the cassette until you can see the needle sticking out between the membranes. Gently inject the air into the membrane. This air will help you remove more of the dialyzed sample from the cassette. Do not remove the syringe from the cassette yet.
15. Next, suck out the dialyzed sample (liquid) between the membranes with the syringe. It is ok to also suck out some of the air you injected in between the membranes. You should be able to recover 70-90% of the liquid between the membranes.
16. Spit your sample out of the syringe into a clean, labeled tube, or aliquot the sample out to a number of tubes. Aliquoting the sample is recommended if your sample contains an active enzyme of interest, as this will avoid having to repeatedly freeze/thaw the enzyme.

Again, the used syringe should be re-capped and disposed of in the small red syringe disposal box.

17. Store your dialyzed sample at an appropriate temperature. If you dialyzed a cellular lysate containing active enzymes, store the dialyzed sample at -20°C.

6. Running a Protein Gel

Protein gels are used to visualize proteins and to roughly estimate their size (by comparison to a protein ladder standard). The following protocol describes how to run a protein gel using Bio-Rad pre-cast polyacrylamide protein gels.

1. Pre-warm the heating block to 100°C.

2. Take a Bio-Rad pre-cast polyacrylamide protein gel out of the 4°C refrigerator. Check to make sure the gel has not expired. When you open the gel wrapper up, you will notice that there is liquid inside along with the pre-cast gel. This liquid contains sodium azide (NaN_3) and is somewhat hazardous. Place the wrapper in the sink and rinse the sodium azide off of the wrapper. Then place the wrapper in a white biohazard bin. Do not rinse the pre-cast gel.

It is generally ok to run proteins on *recently* expired gels, so long as the gels look normal by eye (i.e. nothing is growing on them, they aren't shriveled up or torn, etc.) Gels with expiration dates beyond a couple of weeks should be treated with suspicion and more ordered as soon as possible.

3. Using a knife, press down with the knife along a black "cut here" line at the bottom of the pre-cast gel. Run the knife along the entire length of this line.

4. Set up the protein gel electrophoresis apparatus. I

In setting up the apparatus, you must be extra careful when removing the comb from the polyacrylamide gel. Lift up on the comb slowly, gently, and uniformly. Failure to do this may bend the gel slightly, causing the proteins to run as a curved front down the gel rather than a straight line.

5. In the chemical hood, prepare the protein gel loading dye by mixing 237.5 μL of Laemmli buffer with 12.5 μL of β -Mercaptoethanol in a 0.6 mL tube. β -Mercaptoethanol has a strong unpleasant odor, so care should be taken to contain this odor in the chemical hood.

6. Mix x μL of each of your protein samples with 15 μL of the protein loading dye prepared in Step 5 and $15 - x$ μL of deionized water. The value of x should be chosen such that the total amount of protein in your sample is 15-20 μg of total protein (BSA equivalent determined by the Bradford assay). It is recommended that you do this in a 0.6 mL tube. Any extra loading dye should be disposed of by pipeting it into the aqueous waste container in the chemical hood.

7. Place the tubes containing your protein samples in the heating block (now at $\sim 100^\circ\text{C}$, as long as the temperature is $>90^\circ\text{C}$ it is safe to use). Incubate your samples at $>90^\circ\text{C}$ for 5 minutes. This heat treatment denatures (linearizes) your proteins samples.

This step is very important if you are trying to estimate the size of your protein. Non-denatured, globular proteins will run faster on the polyacrylamide gel than they otherwise would and will appear to have a smaller molecular weight than the actually do.

8. Let your protein samples cool for 5 minutes after the heat treatment. During this time take the protein ladder standard out of the -20°C freezer.

9. Carefully load all ~30 μL of your protein samples into the gel wells using special long pipet tips. Make sure there are no air bubbles trapped in the wells after loading your samples. Any air bubbles may be sucked out of the wells using the long pipet tips.

For wells in which you load protein ladder standard, use 15 or 30 μL of the standard (check the manual that comes with the standard for the exact amount). Do not heat treat the protein ladder standard. You do not need to add loading dye to the protein ladder standard, as it already contains its own loading dye.

10. Run your gel at 200 volts for ~35 minutes, or until the blue loading dye front reaches the bottom of the gel. While the gel is running, make sure that the level of SDS running buffer between the dams in the gel remains high. Sometimes the buffer leaks out from between the dams, causing the level of buffer inside to drop over time. If the buffer level gets low add more SDS buffer from the pool of buffer outside of the electrophoresis chamber. If the level of buffer drops below the upper electrode of the gel, your gel will stop running.

11. Once the gel has finished running, remove the gel cassette from the protein electrophoresis apparatus. Using a knife, carefully cut along the two sides of the cassette. Gently separate the two plastic plates holding the gel to free the gel from the cassette. You may wish to use a spatula to help remove the gel from the cassette to avoid breaking or damaging the gel. The polyacrylamide gel, while tougher than agarose gels, is still easily broken.

The SDS buffer that you used while running the gel can be disposed of by pouring down the sink with plenty of water. The protein electrophoresis apparatus should be washed only with deionized water to avoid damaging the unit with salt deposits on the electrodes.

12. Carefully place the liberated gel in a small tray filled with 200 mL of deionized water. Place this water tray on a shaker and gently shake the gel for 5 minutes at room temperature.

13. Remove the 200 mL of deionized water from the tray and place a fresh 200 mL of deionized water in with the gel. Shake the gel in this fresh water for another 5 minutes. Repeat this gel washing until the gel has gone through three 5 minute washes in deionized water.

The wash water can be disposed of by pouring down the sink, flushing with plenty of water.

14. Place the gel in a tray with 50 mL of Bio-Safe Coomassie. Place this pool on a shaker and gently shake the gel for 1 hour at room temperature. This stains the gel.

15. Remove the gel from the Bio-Safe Coomassie pool and place it in a pool of 200 mL of deionized water. Place this water pool on the shaker and let it gently shake overnight at room temperature. This de-stains the gel, allowing visualization of the protein bands.

The Bio-Safe Coomassie can be disposed of by pouring down the sink, flushing with plenty of water. Also you should be able to see definitive bands after about one hour of de-staining in deionized water. Letting the de-staining go overnight makes the bands sharper and easier to see.

16. You should now be able to see blue protein bands on your gel. You can take a picture of your gel using the lab's imager. To visualize protein gels on the imager, pull down the white gel table in the back of the imager, set your gel on the white table, and shine both reflective and transillumination white light on the gel. You will have to adjust the brightness, focus, and contrast of the image to get a good picture. Using false color scheme #9 also helps in visualizing the gel. Protein gel images are taken using camera setting #2 (same setting as ethidium bromide DNA gels).