Protein analysis by SDS-PAGE

Safety notes: Many of the chemicals involved here are toxic (acrylamide is a neurotoxin, APS is an irritant and TEMED is foul smelling). **Make sure to wear gloves + glasses and remember that anything in the acrylamide area stays in the acrylamide area**. All wastes should go in the acrylamide waste bag for separate disposal. The precast gels from BioRad are recommended as a way to minimise safety risks of handling acrylamide monomer, and come in a range of acrylamide concentrations including gradients (the "Any kD" gradient gels are good general-purpose gels).

Making the gel (starting from scratch): <If you are using **precast gels,** skip to the next section>

As a rule, whenever you make these gels, a few will leak no matter how well you think you've sealed it so aim to make twice as many as you need. Any successful gels you don't need today can be kept for a few days in the cold room wrapped in wet paper towel and gladwrap or you can run a gel at the same time with a different amount of protein per lane in case one comes out nicer than the other.

The trickiest part is assembling the gel former and will take practice to get right. For each gel pick a short plate (the thin, evenly rectangular ones) and a back plate (have raised sides and come in either 1.0 or 0.75mm depths). If they look dirty you can clean them by dipping wet fingertips in Ajax powder and rubbing it into the plates and rinsing it. A few plates have chips – these are a major cause of leaks so either avoid them or make sure the chips aren't at the bottom of the gel.

Get a green plastic cassette holder from the big plastic beaker (they have folding wings to clamp in on the plates) and slide the plates in. Before you clip them into place make sure the short and back plates are completely flush with each other at the bottom, if they are slightly out you will get a leak and your gel won't be very nice. Once they're flush clamp the wings down and check again to make sure they're still neatly together.

Put a grey foam rubber strip down on the clear plastic gel holders in the groove at the bottom and carefully push your gel down onto it. It matters much more that the plates form a seal with it than that the green part is resting on the bottom or straight. Hold the gel former in place using the plastic clip at the top.

To check that your gel is sealed get a plastic pipette and drip water between the plates to the top and watch closely over a few minutes to see if the level drops. If it does, start again and check for chips or cracks. If it doesn't you can pick up the whole assembly and tip the water out. Don't move the glass plates out of the housing at all. The last bit of water can be wicked out with the corner of a paper towel but leaving a little bit doesn't matter too much.

Get two 50mL falcon tubes and assemble the reagents you'll need. Acrylamide solutions are in the cold room, TEMED in the flammables cupboard and APS in the common freezer. Use the electric pipetteboy and the glass 10mL tips for the buffers, water, SDS and acrylamide and the acrylamide area pipettes for the other reagents. You can make up everything but the APS and TEMED for both the stacking and resolving gels but only add APS and TEMED right as you're ready to use the mix.

NOTE → APS solution must be prepared fresh from powder, immediately before use.

Resolving gel	**Stacking gel**
30% Acrylamide……3.3 ml	30% Acrylamide……..670 μl
1.5 M Tris (pH8.8)……2.5 ml	0.5 M Tris (pH6.8)…….1.25 ml
R.O. water…….4.1 ml	10% SDS……..50 μl
10% SDS……..100 μl	R.O. water…….3 ml
10% APS……100 μl	10% APS……...50 μl
TEMED……10 μl	TEMED……..10 μl

Add the APS and TEMED to the resolving gel mix, and use a plastic 'squeeze bulb' pipette to drip the gel into the cassette. Fill to approx. 80% of the glass plate height…leave enough space at the top for the green well former, plus another 5-10 mm. Wait a minute to see if the level drops and if it holds reasonably steady, pipette a layer of MilliQ water over the top to hold it level. Leave a bit of gel in the pipette and/or falcon tube – this can be used to test if it has set (usually 15-30 min).

Pour off the MilliQ, add the APS and TEMED to the stacking gel mix and pipette that on top of your now set resolving gel and fill it up nearly to the top. Slide in the plastic well comb, making sure to pick the correct size for your gel (0.75 or 1.0mm). Leave some of the stacking gel mix in the pipette and leave it for approx. 15-30 min.

Preparing the pre-cast gel): <skip this section if you made your own gel>

Open the sachet the gel comes in. The gels are sitting in a storage buffer so there will be a bit of excess liquid; discard this to acrylamide waste. It's a good idea to mark the location of the bottom of the wells with a permanent marker at this stage to make it easier to see where the wells are later.

The green well comb needs to be removed gently, evenly and straight out to avoid displacing the wells. You may find your own way that feels natural but a good place to start is bracing your thumbnails against the ridges on the front of the comb, with your index fingers against the top of the clear plastic back panel of the gel and slowly pinching them together. Gently but forcefully sliding the comb out evenly is ideal but inching each side out a couple of millimetres at a time can also work.

Once the comb has been removed, peel the strip of green tape off the bottom of the gel, and place the gel into the side of the unfolded green and white electrophoresis cassette. If running one or two gels, use the cassette with the connector pins, if running a third or fourth gel then the second cassette should be the one without pins at the top. It should naturally rest at a ~45' angle with the taller plastic side facing outwards. If running only one gel then use the clear plastic 'buffer dam' on the other side of the cassette. If running two gels then the second will mirror the first. Fold both the hinged green wings of the cassette up, they will press the gels/buffer dam up against the green rubber gasket.

There is a notch in the gasket that should fit snugly into the step between the inner and taller outer pane of the gel. If these aren't aligned, the inner buffer reservoir will leak until eventually the circuit is broken and your gels will run slowly or not at all.

Preparing samples

First decide what kind of samples you should load based on what kind of experiment you are doing and what you expect the protein(s) you are interested in to do in your cells. The three main categories are **Soluble Protein**, **Whole Cells** and **Purified Protein**, each are prepared differently and best in different situations. **Soluble Protein** assumes that your protein of interest is soluble in your buffer which may not always be true but loading can be easier to standardise between samples. **Whole Cell** doesn't require your protein to be soluble and needs less equipment to prepare, but the presence of every cellular protein in the lane can make it harder to pick out specific bands. **Purified Proteins** and other fractions from protein purification are the most straightforward.

In general, prepare more sample than you think you need for one gel and keep the excess until you know that your gel ran successfully and with the right amount of protein in each lane. Larger volumes reduce the error from pipetting and volume lost while boiling, and if you need to re-do the gel with adjusted amounts of protein in each lane you can base that off of the first gel.

Soluble Protein - One of the simplest ways to do this is by bead-beating a cell suspension. Prepare beadbeating tubes as follows: Get glass beads plus the scoop and funnel from the chemicals cabinet (under 'G' for Glass) and put 2 large beads and two scoops each of the medium and small size beads into each tube. Pellet your cells from broth or scrape off a plate and resuspend in 500µL of TE and 5µL of protease inhibitor cocktail (optional, but keep your samples on ice). How much culture to use is tricky to know, more concentrated is usually better, but too many cells and suspension may be gloopy and hard to work with. The pellet from ~ 2 ml of broth culture or a large loopful from a plate is a good starting point. Keep in mind some proteins will not dissolve well in some buffers, depending on charge and pH. TE is a good starting point but consider using a buffer with NaCl (250-500mM) or Triton X-100 (0.1%) if there's a strong band when looking at whole-cell protein but not after bead-beating.

Take your tubes over to the bead beater and unscrew the tube-holder. Space your tubes around the disc (make sure the caps are on tight!) and replace the cover as tight as you can. Beat for 30 seconds. If results suggest poor lysis, or if you are working with Gram-positive bacteria or yeast etc, you will need to do multiple cycles of beating – in these cases, chill your tubes on ice for 1 min between beatings. You may need 5 x 30 sec beatings to fully lyse tough cells like mycobacteria.

Centrifuge at ~10,000 g or more for at least 1 minute (ideally at 4 °C) to remove beads and any remaining whole cells. Retain supernatant. Keep on ice. Check the absorbance on the Nanodrop using the protein A280 setting. Ideally we want around 5 mg/ml for nice PAGE gels. (between 2 and 10 mg/mL may be OK) If it's less than 2 mg/ml, either repeat with more cells or bead beat for more cycles. If it's more than 10 mg/ml dilute down to about 5 mg/ml and remeasure to improve accuracy. Measuring the protein concentration between cycles of beadbeating in this way is an easy way to tell if your cells are completely lysed or if there is more protein to be gained by further cycles.

Keep in mind this Nanodrop reading is only an estimate of the protein concentration, since there is other junk in the cell extract that will interfere, DNA and RNA absorb strongly at 280nm and make up about 20-30% of the absorbance that the nanodrop is assuming is protein, but for the purposes of calculating how much to load on a gel this can be ignored.

Whole Cell – for the 10 lane BioRad gels we use, a good rule of thumb is to aim for the sample you load to contain the cells from about 20uL of a saturated broth (OD600 ~3) of E. coli condensed into a final volume of 10uL, though these numbers are a starting point and may need to be adjusted depending on your specific experiment. A suspension of cells scraped up from a plate will also work, but measure the OD and aim to load a similar number of cells.

As a starting point: Take 200uL of an overnight culture and spin down in a 1.5mL tube, 10000g for 30sec. Discard supernatant and for best results wash away residual media by resuspending cells in an equal volume of EB or TE before spinning again. Resuspend the cell pellet in 100uL of 1X SDS-PAGE loading buffer and boil in the heating block set to 100°C for 5 minutes. Chill on ice or in the cool room for a couple of minutes to condense any water vapour in the tube. Vortex each tube for 30 seconds to redistribute condensation and help shear the long chromosomal DNA that can make whole cell samples gloopy and difficult to load. Centrifuge at max speed for 5 minutes to pellet any remaining insoluble junk and aim to pipette your sample from the very top of the liquid in the tube. Whole cell lysates can sometimes be very viscous and hard to handle due to the DNA content. This can be fixed by 1. adding DNase or 2. by sucking up and expelling your sample a few times through a narrow gauge disposable needle (25G works well) with a 1mL syringe. In the latter case, spin for 5 min at max speed in microfuge to collapse all the foam and leave you with a sample ready to load. Resuspending cell pellets in too small a volume make this problem more likely to occur so adding more 1X SDS-PAGE Loading Buffer and pipetting up and down vigorously before spinning again can often be enough.

Purified Protein/Purification fractions – The main thing to keep in mind when loading samples of purified protein on a gel is the amount of protein that can be resolved in a single band. Beyond ~1ug the band will be spread out and diffuse and difficult to size accurately. Conversely, if you want to know if there are any contaminating proteins in your sample then you can load more to increase the visibility of less concentrated proteins. Loading a range of dilutions, or different volumes of the one sample can be worthwhile if you're not sure of the concentration or purity and want to see both.

The nanodrop can be used to give a rough estimate of protein concentration but be careful to blank with the same buffer as your sample in case a component also absorbs at 280nm (for example imidazole used in his-tag purifications). If you know the amino acid sequence of your protein you can improve the accuracy of your estimate by finding the extinction coefficient using the ExPASy ProtParam tool as this can vary substantially between proteins, for example the A280 of a 1mg/mL solution of sfGFP is around 0.76, whereas lysozyme would be ~2.5. So, a solution with an A280 of 1, which the Nanodrop would call as 1mg/mL, would *actually* be 1.3mg/mL for sfGFP but only 0.4mg/mL for lysozyme.

Cleaning the wells

There will be some residual glycerol storage buffer in the wells and likely some small bubbles, both of which will interfere with how your samples sit in the wells and run. To remove both, fill each of the wells with milliQ water until they've all overflowed to begin diluting the storage buffer. Using a yellow P100 tip pipette a further ~50uL up and down into each well, angling it slightly to shoot water into the corners of the wells to dislodge the bubbles. Be careful not to add further bubbles, going to the second stop on the pipette when sucking up the water at this stage will give you some insurance against this. Don't wedge the tip in between the plates too hard or they'll separate, and be careful not to damage the wells by pipetting too forcefully or dragging the tip sideways between wells.

Make and add the running buffer

Make up a bottle of 1X SDS PAGE running buffer from the 10X stock in milliQ or RO water. Running two gels will use ~800mL of buffer. Do this before beginning to load the samples, since you should run the gel as soon as your samples are in the wells to prevent them from diffusing. Pour 1X running buffer into the space in the cassette between gels (ie. the inner buffer reservoir) until it reaches the point at which it overflows the sides of the gels. Avoid pouring the buffer directly into the wells, as this might damage them or dislodge your samples if you chose to load them first. Fill the outer tub to the appropriate 2 or 4 gel line.

Loading samples

Calculate how much of each sample to load to get a constant amount of protein in each lane. It's very important to try to match the total protein in each lane so that we can compare e.g. induced vs. uninduced samples. Using the nanodrop A280 for lysed cells, between 25 and 50 µg per lane usually works out well. For example, if your sample is 5mg/mL that's equal to 5µg/µL so for a 30µg lane you would need 6µL of it. Alternatively, if you are confident your cells are more or less completely lysed, then the soluble protein from ~30uL of saturated broth or 45uL of IPTG-induced BL21 cells with pET-type plasmids is a good amount to aim for. E.g. 6uL of the supernatant from 2mL of culture (or 3mL of induced cells) lysed in 400uL of TE. This method works better to keep lanes comparable when the induced protein makes up a significant fraction of the soluble protein – if 25% of the cell is now one protein, your "40ug" lane is now really only 30ug and will look faint next to the uninduced lane.

Add an appropriate amount of SDS-PAGE loading buffer to your protein samples, in the above case this would be 6µL of 2x buffer for a total sample volume of 12µL, but if you need to load a different volume of each of your samples then it can be easier to add extra buffer to the more concentrated ones so that when it's time to load the gel they are all equal volumes. If you have enough sample to spare, then making up 4-5x as much as you need for each sample reduces losses in the boiling step and allows you to re-run samples more easily if needed. Draw up a table like this example

Sample	**Concentration**	**uL for 30ug (4x)**	**plus TE (4x)**	**2X dye (4X)**
A	5 mg/mL	6uL (24uL)	1.5uL (6uL)	7.5uL (30uL), **Total = 60uL, load 15uL on gel**
B	6 mg/mL	5uL (20uL)	2.5uL (10uL)	
C	4 mg/mL	7.5uL (30uL)	0uL	

First check that the loading dye has had β-mercaptoethanol added (you'll know if it has because it will smell sulfurous and awful) or else add 50uL in the fume hood to a fresh 950uL aliquot. Heat samples at 100°C for 3min (heating block) and then allow to cool down before loading on gel. Remember to turn off the heating block!

Make sure you keep track of which samples are going into which wells. Especially note that the gel may get flipped over later, so ensure the gel is asymmetrical to prevent confusion later, e.g. only put standards on one side). Load 5uL of protein standards (these don't need to be boiled or have buffer added), then load the rest of your samples. Try to have equal volumes of sample in each well and equal concentrations of salt. If some lanes have more sample or salt, that can cause the electric field to warp, leading to ugly band patterns. If your samples and ladder add up to fewer than the number of lanes in your gel then this effect can also occur; in that case, loading an equal volume of diluted (1x) loading buffer to all the empty lanes helps to minimise these artifacts.

Running the gel

This step is optional but after all your samples have been loaded onto the gel and the lid placed on the tank, you can run it at 100V for five minutes before increasing the voltage to 200V for another 30min or until the blue tracking dye gets close to the bottom of the gel. This is to give the samples the best chance of migrating neatly through the stacking gel (the top part with the wells) and concentrating before being resolved. You can just run the gel for 200V the whole time to simplify things. It is worth checking on the gel a couple of times as it runs to make sure the inner chamber isn't leaking and that the dye is migrating well.

Run gel at 200V for about 30 minutes or until the dye front reaches the bottom of the gel. Some of the housings leak and this will reduce the amount of current that can flow, if this is happening just check the gel every so often and if it is transfer some buffer from the bottom part of the tank to the space between the gels to top it up. A 50mL syringe is very useful here. When the dye has reached the bottom of the gel, stop and disassemble it. Used buffer can go down the sink and should not be re-used as the pH and solute concentrations are essential for running properly and are changed after running.

The most common cause of the gel not running properly (or stopping part-way through) is a leak of buffer from inside the cassette; it is critical that the inner buffer level stays above the level of the wells to ensure a complete electrical circuit.

Staining and visualising

After the gel has finished running, turn off the power, pour the running buffer out and disassemble the cassette. Give the outside of the gel a quick rinse to get rid of some of the soapy SDS. For pre-cast gels, pry open at each of the 4 points on the gel case with triangles (there is a special tool for this, but a small screwdriver or metal spatula will also work); be gentle and careful since the gel is thin and fragile. For home-made gels, slowly pull apart the glass plates to free the gel inside.

For stain free imaging:

This only works with gels from BioRad that come pre stained with their "stain free technology". It works by having a trichloromethyl compound in the gel react with tryptophan residues in the protein under UV light to create a fluorescent product. Thus proteins with no tryptophans will not show up at all, and comparisons between proteins with different numbers of tryptophans will be difficult to make. However, compared to other staining methods it takes a few minutes rather than hours or overnight, and doesn't preclude doing follow up staining with coomassie or blots.

Prestained protein ladders perform poorly with stainfree imaging. The BioRad Dual Colour ladder has pink bands with fluorescence that will wash out the surrounding parts of the gel, and the All Blue pre-stained ladder will obscure the fluorescence of the protein bands. It can still be worth imaging though, as if the rest of your gel is unusably bad it's better to know in 5 minutes than after overnight staining and destaining.

To take the image, lay the gel on the UV tray of the Chemidoc being careful not to tear or drag the gel as it can physically stretch leading to a warped image. It won't slide so lift it up with a spatula or similar and gently lay it down on the tray from one edge in a rolling motion to force out any bubbles. If it sticks, a little water will help release it. Select the Stain Free protocol under the Protein Gels menu, the automatic settings should work for most gels. Look at the image preview to make sure it is centred and zoomed in, though it can be easier to crop and rotate the image afterwards in

ImageLab than move the delicate gel around on the transilluminator. For increased sensitivity you can re-do the default 45 second activation or select a longer time and have a longer image exposure time. Save the image and export to USB, making sure to select the correct filetype. It can export images directly (JPEG and TIFF) or a .scf file that allows you to adjust exposure or do all kinds of analysis in ImageLab.

Coomassie Blue staining:

Place gel in a plastic dish and pour over enough coomassie blue solution to cover. cover tightly with gladwrap and put on rocker or shaker (gentle motion!) for half an hour or 15 minutes at 37°C if you're in a hurry. Pour the stain into the methanol waste bottle, cover with high-destain solution and destain with gentle shaking or rocking for another half hour or less at 37°C. Pour off into methanol waste and replace with low-destain solution and agitate overnight or for at least an hour. The high-destain step may be omitted and done the next morning if there is still a lot of background stain.

To image the gel, use the white light transillumination plate in the ChemiDoc but rinse it with water first as it often gets dusty and this will show up on your image. Carefully place the gel on the glass surface (don't use tweezers, you don't want to scratch the glass!) being sure to roll it out flat to prevent air bubbles. You can spray a little water on the gel and press a clear plastic sheet onto it to squeeze out any air and prevent the gel from curling up while you image it.

Change the image protocol to 'Protein Gel > Coomassie Blue' and check that your gel is roughly centred and zoomed in appropriately. Press the camera button to capture the image. Adjust the contrast and high/low settings to define the bands more clearly. The ImageLab software is capable of recolourising the image to look like a very nicely destained coomassie blue gel, which is helpful. It's worth familiarising yourself with the ImageLab analysis tools as they are quite powerful. Save the file on the GelDoc and export at maximum quality to a USB.

Gels can be stored in the cold room between two sheets of clear plastic sealed together with tape for later reference or mass-spec band excision. You can annotate the plastic directly but be aware the gel can shift in the plastic over time if not kept flat. If you want to discard the gel, this must be treated as contaminated waste, due to possible acrylamide monomer still present…discard into the acrylamide waste bag.

Coomassie Blue Staining Solution: 50% methanol, 10% glacial acetic acid, 0.1% Coomassie Brilliant Blue. Doesn't need to be sterile, but add methanol and acid to water in the fume hood as both are volatile irritants. The bottle will get quite warm as the liquids mix so go slowly. Be careful weighing out and transferring the dye, it has a tendency to get everywhere and even small specks will make a mess, wipe down area with 80% ethanol after you're done. Dye will take a while to dissolve, use a magnetic stir bar and check periodically for solids.

High Destain Solution: 40% methanol, 10% glacial acetic acid. Again doesn't need to be sterile but do mix methanol and acid into water gradually in the fume hood.

Low Destain Solution: 10% methanol, 10% glacial acetic acid. Not sterile, still make in the fume hood but don't need to worry about the solution getting too hot this time.