Protein analysis by SDS-PAGE

General 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.

Making a gel:

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 so that you'll know when it's set, about 15-30 min later.

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 samples

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. Sterilise by autoclaving (prob. not essential if you are in a hurry or forget, but that would be 'best practice'). Pellet your cells from broth or scrape off a plate and resuspend in 500µL of TE and 5µL of protease inhibitor cocktail. 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.

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 for 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 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 (unlikely!) dilute down to 5 mg/ml.

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. For some applications like enzyme assays, you may need to use a different protein quantitation methods.

Loading and running the gel

Calculate how much of each sample to load to get a constant amount of protein in each lane. Its very important to try to match the total protein in each lane so that we can compare e.g. induced vs. uninduced samples. Between 25 and 50µg per lane usually works out well. For example if your sample is 5mg/mL that's equal to 5µg/µL so for a 40µg lane you would need 8µL of it.

Add an appropriate amount of SDS-PAGE loading buffer (in this case ~3µL of 4x buffer for a total sample volume of 11µL) checking that the buffer has had β-mercaptoethanol added (you'll know if it has because it will smell sulfurous and awful). Make up 50% more sample + loading buffer than you actually need to prevent loss in the next step. 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!

Take your prepared gel cassette(s) and remove the holders and well combs. Put them into the housing with the short plate sides facing inwards and clip in place. It's a good idea to mark the location of the wells with a permanent marker at this stage to make it easier to see where the wells are later. Put the assembly into the clear tank and check that the top part makes contact with the terminals. Fill the space between the gels with 1x SDS PAGE running buffer and fill the tank up to the 2 or 4 gel line depending on how many you are running. If you only have one gel, use the "buffer dam" on the second side so that you get a distinct pool of buffer at the top.

Load 5uL of protein standards (these don't need to be boiled or have buffer added) and the appropriate amounts of each of your samples. 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).

Run gel at 200V for about an hour 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.

Staining and visualising

Carefully pry open the plates using the green plastic wedge tool and peel out the gel with tweezers. It will be reasonably tough but may still tear or break so be careful. Place it in a plastic dish and pour over enough coomassie blue solution to cover. Put some gladwrap over the top and put on the rocker for half an hour. Pour the stain into the methanol waste bottle and cover with high-destain solution and return, covered, to the rocker for another half an hour. Pour off into the methanol waste and replace with low-destain solution and rock, covered overnight. The high-destain step may be omitted and done the next morning if there is still a lot of background stain. Don't worry about a bit of background stain, that can be taken out in the image software but less is better as it will enhance the contrast.

To image the gel the best thing to use is the Typhoon scanner on Level 6. The instructions are printed on a piece of paper stuck to the scanner for which software to use. 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. Something you may want to try is spritzing the gel with water and pressing a clear plastic sheet onto the gel to squeeze out any air and prevent the gel from curling up while you image it.

Start a new protocol and use the preview tool to focus on your gel. Adjust the image settings to the highest resolution if you like and you can also get the software to automatically annotate lanes, bands and even sizes of markers. Press “RUN” to take the image. Adjust the contrast and high/low settings to define the bands more clearly. The 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 different imaging and analysis tools as they are quite powerful. If it’s not essential to do all of this you can also use the GelDoc on white light transillumination (there’s a movable tray in the housing at the back for this) for a simple black and white image. In either case save the file and export at maximum quality and save 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.