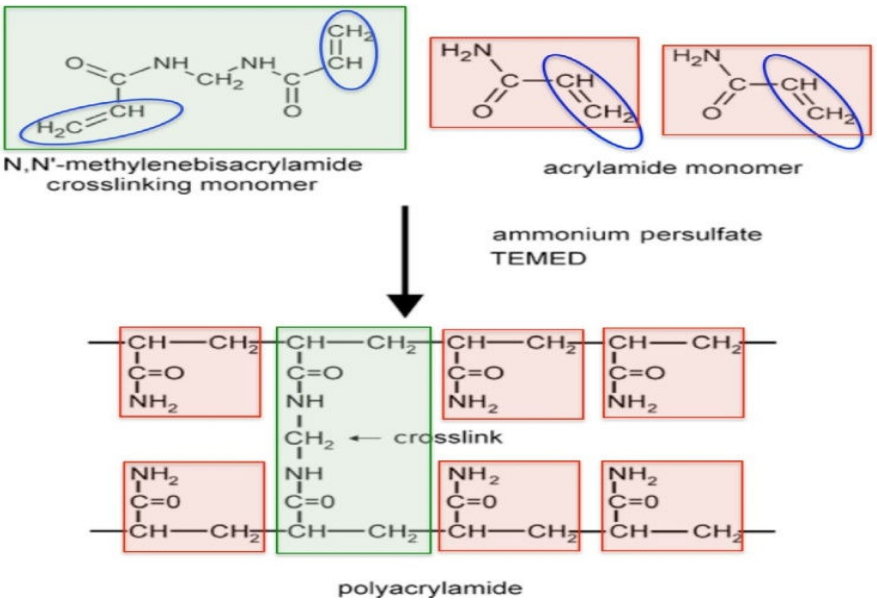
SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used method is SDS-PAGE system first described by Laemmli (1970). The system actually consists of two gels - a resolving (aka running) gel in which proteins are resolved on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions of the stacking gel, resolving gel and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their MWs.

Chemistry of acrylamide polymerization

The polyacrylamide gels used to separate proteins are formed by the chemical polymerization of acrylamide and a cross-linking reagent, N,N'methylenebisacrylamide (opposite page). Investigators are able to control the size of the pores in the gel by adjusting the concentration of acrylamide, as well as the ratio of acrylamide to bisacrylamide. Raising either the concentration of acrylamide or bisacrylamide, while holding the other concentration constant, will decrease the pore size of the gel. Polymerization occurs because of free oxygen radicals that react with the vinyl groups in acrylamide and bisacrylamide, as shown in the figure below. The oxygen radicals are generated from the catalyst, ammonium persulfate (APS), when it reacts with a second catalyst, N,N,N',N'-tetramethylethylenediamine (TEMED).



Proteins are denatured prior to electrophoresis

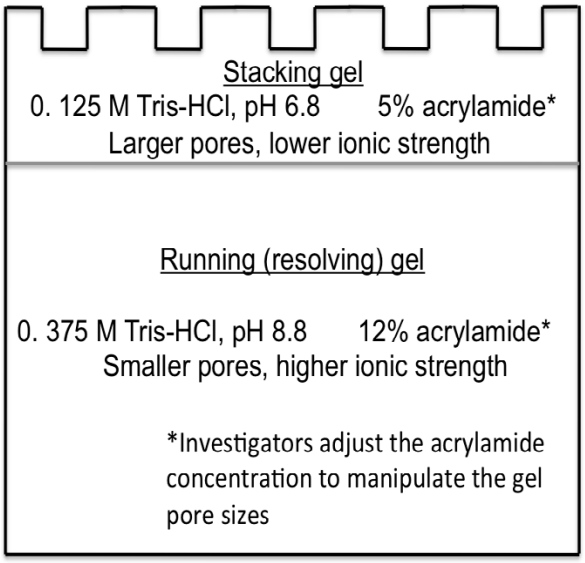
Because proteins are so diverse with respect to their surface charges and geometries, the molecular weights of ***folded*** proteins cannot be simply determined by their migration rate in an electric field. Postively and negatively charged proteins would migrate in different directions.

To resolve the proteins in a sample according to their size, investigators must convert the proteins to a uniform geometry and impart a uniform charge/mass ratio to the proteins. In SDS-PAGE, the solution is to denature the proteins by boiling them with the anionic detergent, sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. The combination of heat and detergent is sufficient to break the many noncovalent bonds that stabilize protein folds, and 2-mercaptoethanol breaks any covalent bonds between cysteine residues.

The SDS hydrocarbon chain permeates the protein interior and binds to hydrophobic groups, reducing the protein to a random coil, coated with negatively charged detergent molecules all along its length.**Denatured proteins bind one SDS molecule for every two amino acids.**

Discontinuities between the stacking and running gels underlie the resolving power of the SDS-PAGE gels

The Laemmli (1970) SDS-PAGE system can be considered a 3-component system. The stacking and running (resolving) gels have different pore sizes, ionic strengths and pHs. The third component is the electrophoresis buffer (25 mM Tris, 192 mM glycine,, 0.1% SDS, pH ~8.3), which contains large amounts of glycine. The ionization state of the glycine is critical to the separation. At neutral pH, glycine is a zwitterion, with a negatively charged carboxyl group and a positively charged amino group. Consequently, very little glycine has a negative charge in the chamber buffer or stacking gel, and significant ionization does not occur until the glycine enters the more alkaline pH 8.8 environment of the running gel.



The sample buffer used for SDS-PAGE contains a tracking dye, **bromophenol blue** (BPB), which will migrate with the leading edge of the proteins being separated on the gel. The sample buffer also contains glycerol, which allows the protein samples to settle into the bottom of thegel wells.

Once a voltage is applied, the chloride ions in the sample buffer and stacking gel move rapidly toward the positive pole, forming the leading edge of a moving ion front. Glycine molecules have very little charge in the stacking gel, so they migrate at the rear of the moving ion front. This difference in chloride and glycine mobility sets up a steep voltage gradient in the stacking gel that sweeps along the negatively charged protein-SDS complexes. The large pores of the stacking gel present very little resistance to the movement of protein-SDS complexes, which then “stack up” into a very concentrated region at the interface between the running and stacking gels (right). Protein-SDS complexes remain concentrated at the interface until the slowly migrating glycine molecules reach the boundary between the two gels.

Dramatic changes occur as the glycine ions enter the running gel. The pH of the running gel is 8.8, so a significant fraction of the glycine molecules assume a negative charge. Negatively charged glycine molecules begin to move at the same rate as the chloride ions. The pores in the running gel are much smaller than those of the stacking gel, so the pores present frictional resistance to the migration of proteins. Proteins begin to migrate at different rates, because of the sieving properties of the gel. Smaller protein-SDS complexes migrate more quickly than larger protein- SDS complexes (right). Within a certain range determined by the porosity of the gel, the migration rate of a protein in the running gel is inversely proportional to the logarithm of its MW.

Proteins are visualized with stains.

To visualize the positions of proteins after electrophoresis is complete, investigators stain the gels with various dyes that bind non-covalently.

In our experiments, we will use a colloidal suspension of Brilliant Blue G-250, also known as Coomassie Blue G. Brilliant Blue G-250 bindsproteins nonspecifically through a large number of ionic and Van der Waals interactions. Protein bands appear rapidly, and when necessary, the gels can be destained with deionized water to lower the gel background. Brilliant Blue staining intensity is considered to be a quantitative procedure, because with some exceptions, the intensity of a stained band is directly proportional to the amount of protein in a band.

Casting SDS-PAGE gels

Assemble the gel casting apparatus

1. Assemble the components that you will need for casting the gel: a tall glass plate with attached 1 mm spacers, a small glass plate, a green casting frame and a casting stand.

2. Place the green casting frame on the bench with the green "feet"resting firmly against the bench and the clamps open (perpendicular to the frame) and facing you.

3. Place the two gel plates in the frame. Insert the taller spacer plate with the "UP" arrows up and the spacers facing toward you into the casting frame (the BioRad logo should be facing you). Insert the short glass plate in the front of the casting frame. ***There should be a space between the plates.***

4. Secure the plates in the casting frame by pushing the two gates of the frame out to the sides. IMPORTANT: the bottom edges of the two plates should be flush with the lab bench before you clamp the frame closed to ensure a watertight seal. T***o do this, rest the frame vertically on the bench BEFORE closing the gates.***

5. Clamp the casting frame with glass plates into the casting stand, with the gates of the casting frame facing you. ***Repeat steps 1-5 to prepare a second gel in the casting frame.***

6. Check to see if the assembled plates in the casting stand are sealed properly by pipetting a small amount of deionized water into the gap between the plates. If the glass plates hold water and don't leak, you are ready to make the gels. Pour the water out by holding the entire casting platform over a liquid waste container or sink. Use paper towels or tissues to absorb any residual water. If the gel leaks, disassemble the frame, dry the plates and go back to step 3.

Prepare resolving gels.

Assemble the chemicals that you will need to pour the gels. The table below shows the quantities of each chemical that you will need.Polymerization occurs rapidly, so be sure to follow the step-by-step instructions below.

Reagent	**Resolving gel**	**Stacking gel**
Deionized water	3.5 mL	2.1 mL
30% acrylamide:bis-acrylamide (29:1)	4.0 mL	0.63 mL
1.5 M Tris-HCl, 0.4% SDS, pH 8.8	2.5 mL	------
0.5 M Tris-HCl, 0.4% SDS, pH 6.8	------	1.0 mL
10% ammonium persulfate (catalyst)	100 µL	30 µL
TEMED (catalyst)	10 µL	7.5 µL

1. Label two 15 mL conical tubes “Resolving gel” and “Stacking gel”.
2. Prepare ONLY the resolving gels at this time. Mix the acrylamide solution, **pH 8.8** Tris buffer and water, as shown in the chart above. Mix the ingredients gently, trying not to introduce air. Oxygen inhibits polymerization of acrylamide gels.
3. To the resolving gel mixture, add 100 µL of a 10% ammonium persulfate (APS) solution. Gently mix the solution, trying not to introduce air. Oxygen inhibits acrylamide polymerization.
4. Add 10 µL of TEMED catalyst. Once again, gently mix in the catalyst trying not to introduce air bubbles. Cover the tube immediately after you aliquot this reagent.
5. Working quickly, use a plastic transfer pipette to fill the space between the two plates until the resolving gel solution reaches a height just above the green clamps on the gel casting frame.
6. Using a transfer pipet, add deionized water so that it gently flows across the surface of the polyacrylamide mixture. The water layer ensures that the polyacrylamide gel will have a level surface once it polymerizes.
7. Allow the gel to polymerize, which takes ~15-20 minutes. You will note that the interface between the polyacrylamide and water overlay disappears temporarily while the gel polymerizes. A sharp new interface then forms between the two layers, indicating that polymerization is complete.
8. When polymerization is complete, remove the water from the top of the resolving gel.

Pour the stacking gels

1. Prepare the stacking gels. Mix the acrylamide solution, **pH 6.8** Tris buffer and water, as shown in the chart above.
2. Add 30 µL 10% APS and 7.5 µL TEMED to the stacking gel acrylamide mixture. Mix the contents by gently inverting the tube twice.
3. Use a transfer pipette to pipette the stacking gel on top of the resolving gel between the two glass plates. Add enough stacking solution until it just reaches the top of the small plate.
4. Carefully, but quickly, lower the comb into position, being careful not to introduce air bubbles.

Running SDS-PAGE gels

Set up the electrophoresis apparatus

1. Retrieve one of the SDS-PAGE gels from the refrigerator.
2. Carefully remove the comb from the spacer gel.
3. Remove the casting frame from the gel cassette sandwich and place the sandwich against the gasket on one side of the electrode assembly, with the short plate facing inward. Place

a second gel cassette or a buffer dam against the gasket in the other side of the electrode assembly.

4. Clamp the green clamps on the sides of the electrode assembly (below).
5. Lower the chamber into the electrophoresis tank.
6. Fill the space between the two gels with Tris-glycine running buffer. This forms the upper chamber for electrophoresis.
7. Add Tris-glycine running buffer to the outer (lower) chamber until the level is high enough to cover the platinum wire in the electrode assembly.

Load and run samples on the SDS-PAGE gel

1. Retrieve your cell extracts from the freezer. Recall that the samples have already been mixed with a tracking dye and glycerol.
2. Using gel loading micropipette tips, load up to 15 µL of sample into each well. Load 5 µL of a molecular weight standard into one lane of the gel. Load samples slowly and allow the samples to settle evenly on the bottom of the well.
3. Connect the tank to the power supply
4. Turn on the power supply. Run the gel at a constant voltage of 120□150 V. Run the gel until the blue dye front nearly reaches the bottom of the gel. This may take between 45-60 min.

Staining SDS-PAGE gels

1. Remove the gel apparatus from the tank. Open the clamping frame and remove the gel cassette sandwich. Carefully, force open the two plates apart with a spatula.
2. Place the gel in a small plastic tray and label the tray with your initials on a piece of tape. To do this, fill the tray about halfway with deionized water. Gently free the gel from the glass plate, allowing it to slide into the water. The gel should move freely in the water. Place the gel and tray on a rocking platform. Rock the gel for 5 minutes.
3. Drain the water from the gel and add ~20 mL of Simply Blue. Cover the gel container and rock overnight.
4. In the morning, drain the Simply Blue stain.
5. Destain the gel by filling the container about half full with deionized water. Shake the gel in the water for ~2 minutes. Pour off the water and add new deionized water. Repeat, if necessary, until protein bands become visible.