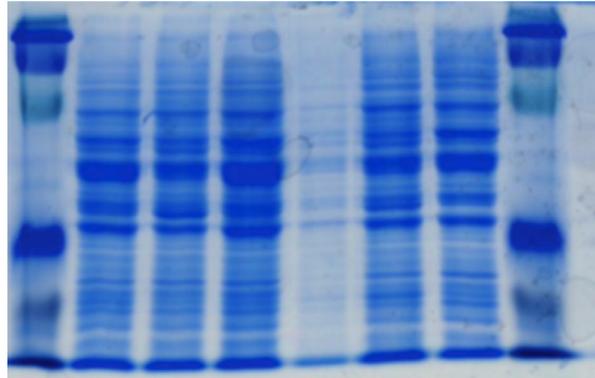


# Chapter 14

## SDS-PAGE



This lab will introduce you to SDS-PAGE, a simple and inexpensive method for resolving proteins in complex mixtures. SDS-PAGE gels provide the starting materials for western blots and for some proteomic techniques. In this lab, you will use SDS-PAGE to analyze the protein extracts that you prepared from yeast strains overexpressing Met and LacZ fusion proteins.

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## Objectives

At the end of this lab, students will be able to:

- discuss the principles that govern protein separation on discontinuous SDS-PAGE gels.
- cast and run SDS-PAGE gels.
- analyze the pattern of bands on a stained SDS-PAGE gel
- estimate the molecular weight of a protein from its migration on SDS-PAGE gels

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This lab will introduce you to SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis), a simple and inexpensive method for resolving proteins in complex mixtures. You will use SDS-PAGE gels to analyze the yeast protein extracts that you prepared in the last lab. Each team will make two gels. One gel will be stained with Simply Blue to visualize all the proteins in the extracts. The second gel will be used for western blots (Chapter 15) that will specifically detect Met and LacZ fusion proteins in the extracts.

## *Background*

SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used methods are derived from the discontinuous 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.

### **Gel electrophoresis of macromolecules**

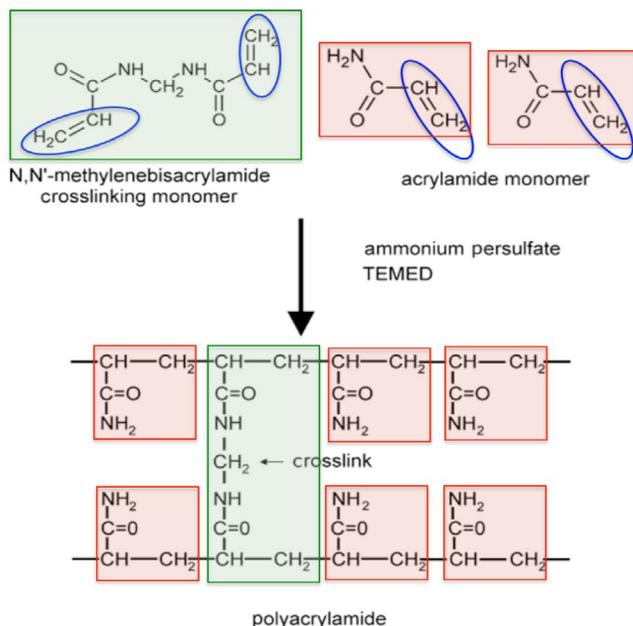
In gel electrophoresis, an electric field is used to move charged molecules through a matrix of a polymerized substance such as agarose or polyacrylamide. The rates at which individual molecules move through the gel depend on the properties of both the separation system and the molecules themselves. Gel matrices are permeated with networks of pores through which the molecules move. The amount of resistance that the matrix presents to the movement of a molecule depends on the diameter of the pore as well as the size and geometry of the molecule. Researchers can control the size of the pore by adjusting the concentration of gel monomer within a certain range. In general, smaller, more highly charged molecules migrate more rapidly through gels than larger or less charged molecules. The mobility of a molecule is also affected by the buffer system and the strength of the electrophoretic field used for the separation.

You have already used agarose gel electrophoresis to separate DNA molecules. Recall that the size of a linear DNA molecule can be estimated from the rate at which it moves through an agarose gel, because DNA molecules have a uniform charge to mass ratio. Protein electrophoresis is somewhat more complicated than DNA electrophoresis. Proteins are much smaller than DNA molecules, so polyacrylamide gels are used for their separation. In addition, proteins are much more structurally diverse than DNA, so chemical treatments (see below) are used to impart a uniform geometry and charge/mass ratio to the proteins.

### **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).



### Acrylamide gel polymerization.

Ammonium persulfate and TEMED catalyze the polymerization of acrylamide and bis-acrylamide monomers into a crosslinked network.

### Proteins are denatured prior to electrophoresis

Compared to DNA molecules, proteins are structurally very diverse. Proteins show tremendous variation in their amino acid compositions and in the distribution of amino acids in their folded structures, features with important implications for electrophoresis. Recall that proteins are mixtures of hydrophobic and hydrophilic amino acids and that the primary sequence of the protein determines its final folded form. Because of the hydrophobic effect, the surfaces of proteins have a higher frequency of polar and charged amino acids than the interiors, where hydrophobic residues predominate. Folded proteins assume many different geometries and their surfaces are mosaics with respect to the distribution of R groups with different chemistries. 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. Positively and negatively charged proteins would migrate in different directions!

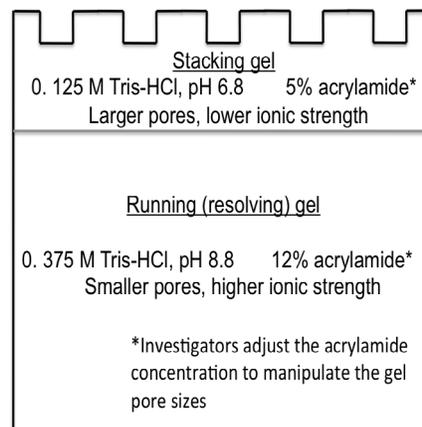
To resolve the proteins in a sample according to their size, investigators must convert the proteins to a uniform geometry and impart a uniform charge/mass ratio to the proteins. In SDS-PAGE, the solution is to denature the proteins by boiling them with the anionic detergent, sodium dodecyl sulfate (SDS) 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. Like other detergents, SDS is an amphipathic molecule, consisting of a hydrophobic 12-carbon chain and a hydrophilic sulfate group. The SDS

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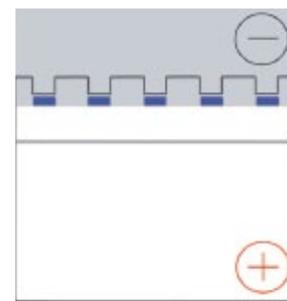
hydrocarbon chain permeates the protein interior and binds to hydrophobic groups, reducing the protein to a random coil, coated with negatively charged detergent molecules all along its length. Denatured proteins bind quite a lot of SDS, amounting to ~1.4 g SDS/g protein, or ~one SDS molecule for every two amino acids.

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

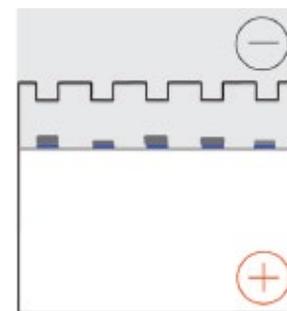
The Laemmli (1970) SDS-PAGE system can be considered a 3-component system. The stacking and running (resolving) gels have different pore sizes, ionic strengths and pHs. The third component is the electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH ~8.3), which contains large amounts of glycine. 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. The  $pK_a$  of the amino group is 9.6, considerably higher than the pH of the chamber buffer. 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. Let's follow the progress of protein samples during SDS-PAGE to see how differences in the composition of these three components generate the high resolving power of SDS-PAGE gels.



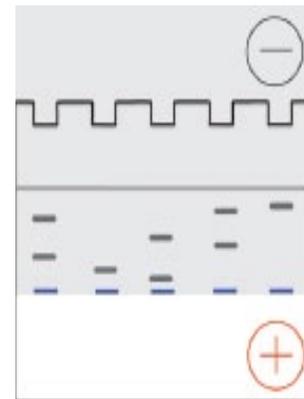
The sample buffer used for SDS-PAGE contains a tracking dye, bromophenol blue (BPB), which will migrate with the leading edge of the proteins being separated on the gel. The sample buffer also contains glycerol, which allows the protein samples to settle into the bottom of the gel wells. The gel is vertically positioned in the electrophoresis apparatus and covered with chamber buffer containing glycine (right, shaded).



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 closer to the  $pK_a$  of the glycine amino groups, 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, thereby eliminating the voltage difference that controlled protein mobility through the stacking gel. 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.

With few exceptions, naturally-occurring proteins are invisible on SDS-PAGE gels. Consequently, researchers often use pre-stained protein standards to monitor the approximate positions of proteins during electrophoresis. The pre-stained standards are produced by covalently attaching a large number of chromophores to a protein. The addition of the chromophores increases the MW of the protein and also produces more diffuse bands on the gel. The diffuseness of the bands reflects variation in the number of dye molecules attached to individual protein molecules. We will use prestained standard protein ladders in our gels, so you will be able to visualize the protein separation that is occurring. Yeast proteins will not be visible, however, because they have not been modified with chromophores.

To visualize the positions of proteins after electrophoresis is complete, investigators stain the gels with various dyes that bind noncovalently and with very little specificity to proteins. During the staining process, proteins are also “fixed” in the gel, meaning that proteins become insoluble and unable to diffuse out of the gel. In our experiments, we will use Simply Blue, a colloidal suspension of Coomassie Brilliant Blue G-250. Brilliant Blue G-250 binds proteins nonspecifically through a large number of ionic and Van der Waals interactions. In this procedure, gels are rinsed with water to remove the buffer salts used for electrophoresis and then treated with the colloidal G-250 suspension. 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.

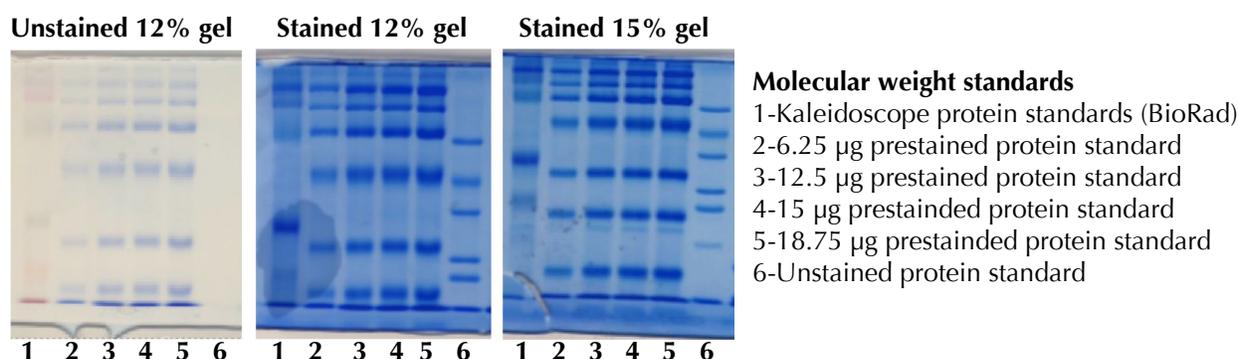
### Protein molecular weights can be calculated from their migration on gels

The sizes of proteins in an extract can be calculated by comparing their migration to a set of standard proteins run on the same gel. Researchers select standard proteins that will be well-resolved on the particular gel that they are running. For example, an investigator using a 7.5% gel will select standards with higher molecular weights (MWs) than an investigator using a 15% gel,

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which is better suited to the analysis of small proteins. The principles used to estimate MWs are the same used for agarose gel electrophoresis. A plot of the  $\log_{10}$  MW of the standard proteins against the distance that each protein migrated on the gel will give a straight line in the region where the gel has good resolving power. (Note: MW is not the same as the mass of a protein. MW is a dimensionless term. For example, myoglobin has a mass of 16.7 kDa and a MW of 16,700.) The sizes of unknown proteins can be estimated by interpolating experimental values on a graph of standard proteins. Proteins whose molecular weights fall outside this range will not be well-resolved on the gel.

The figure below illustrates several of the points discussed above. The same sets of unstained and pre-stained protein standards were separated on either 12% or 15% SDS-PAGE gels. The prestained standards in lanes 1-5 are visible without staining, but they become much more pronounced after staining. The unstained standard in lane 6 requires staining to become visible, but the bands are much more discrete and will give more reliable values when calculating MWs of unknown proteins, because chromophores have not been attached to the proteins. The data in lanes 2-5 also demonstrate that Brilliant Blue staining is a quantitative procedure, because the intensity of bands in each lane increases in direct proportion to the amount of protein in the lane.



When analyzing your experimental data, remember to consider the additional amino acids that have been added during the cloning procedure. The Met proteins that you are working with are fusion proteins with additional amino acids at the C-termini the Met proteins. The BG1805 plasmid encodes HA and His6 epitopes, as well as the ZZ immunoglobulin binding domain. Together these sequences add a walloping ~19 kDa to the expected mass of *S. cerevisiae* Met proteins (Gelperin et al., 2005). The pYES2.1 plasmid encodes 33 amino acids that are added to cloned ORFs. The additional sequences include a V5 epitope tag and a (His)<sub>6</sub> purification tag at the C-termini of overexpressed proteins. Together, these amino acids add ~5 kDa to the size of the protein.

NOTE: The MW of the LacZ control protein without the V5 epitope is ~120 kDa. Because this is such a large protein, it will be very difficult to get an accurate estimate of its MW.

## Casting SDS-PAGE gels

These instructions are designed for constructing two 12% SDS-PAGE gels with the BioRad Mini Protean system. One gel will be used for Simply Blue staining in the next lab. The second gel will be used for western blotting.

### Assemble the gel casting apparatus

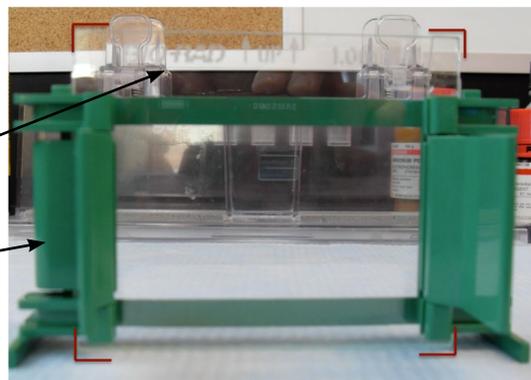
1. Assemble the components that you will need for casting the gel: a tall glass plate with attached 1 mm spacers, a small glass plate, a green casting frame and a casting stand.
2. Place the green casting frame on the bench with the green “feet” resting firmly against the bench and the clamps open (perpendicular 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. ***To 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.

#### Assembling plates in the casting frame.

Large spacer plate with spacer goes in rear, oriented so the arrows point up and the logo is readable. Small plate is placed in front. (Corners of the plates are marked with bracket marks.)

Green gates of casting frame are open.

Casting frame “feet” and bottom edges of plates are flush against the benchtop.



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## Prepare two resolving gels.

**SAFETY NOTE: Acrylamide and bisacrylamide monomers are weak neurotoxins. Gloves and goggles should be used when working with acrylamide.**

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

**NOTE: catalysts should NOT be included into the mixture until you are ready to pour the gels!!**

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 $\mu$ L	30 $\mu$ L
TEMED (catalyst)	10 $\mu$ L	7.5 $\mu$ L

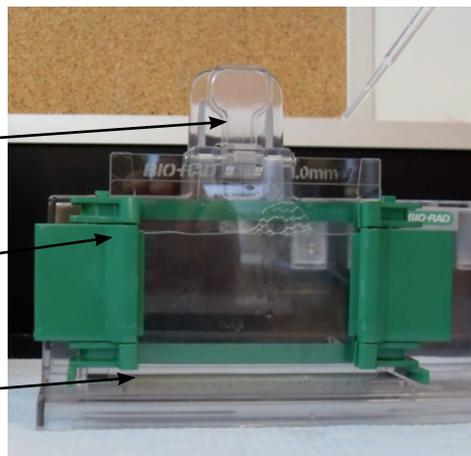
1. Label two 15 mL conical tubes “Resolving gel” and “Stacking gel”.
2. Prepare ONLY the resolving gels at this time. Mix the acrylamide solution, **pH 8.8** Tris buffer and water, as shown in the chart above. Mix the ingredients gently, trying not to introduce air. Oxygen inhibits polymerization of acrylamide gels.
3. To the resolving gel mixture, add 100  $\mu$ L of a 10% ammonium persulfate (APS) solution. Gently mix the solution, trying not to introduce air. Oxygen inhibits acrylamide polymerization.
4. Add 10  $\mu$ L of TEMED catalyst. Once again, gently mix in the catalyst trying not to introduce air bubbles. CAUTION: TEMED has an unpleasant odor. 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. Draw up any remaining acrylamide into the transfer pipet. (You will know that the acrylamide has polymerized when you can no longer push the acrylamide out of the pipet.)
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. (You can also check the remaining polyacrylamide in the transfer pipette to see if it has polymerized.)
8. **When polymerization is complete, remove the water from the top of the resolving gel by tilting the gel to the side and using a paper towel or Kimwipe to wick out the water.**

**Casting the SDS-PAGE gel.**

Casting stand and plates are clamped into the casting stand

Fill plates with running gel solution to the top of the closed green gates of the casting frame.

Bottom edges of plates are flush against the grey pad of the casting stand.

**Pour the stacking gels**

**SAFETY NOTE: Be sure you are wearing goggles when pouring 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  $\mu\text{L}$  10% APS and 7.5  $\mu\text{L}$  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. (The Bio Rad logo on the comb should be facing you.) Adding the comb will force some solution out of the gel, but this is fine. If air bubbles become trapped below the comb, remove the comb and reposition it.

**Save the SDS-PAGE gels**

1. Carefully remove the gels from the casting stand and then from their green frames.
2. Keeping the combs in the gel, wrap the gels in a wet paper towel. Then wrap the gels in plastic wrap to be used in later labs. ***The gels will be ruined if they are not kept wet and properly wrapped!***

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## *Running SDS-PAGE gels* **Set up the electrophoresis apparatus**

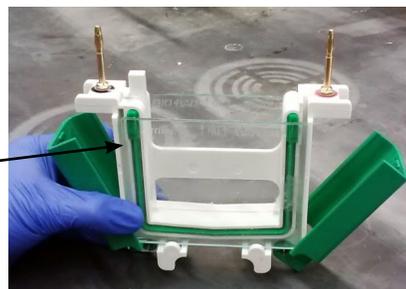
1. Retrieve one of the SDS-PAGE gels from the refrigerator.
2. Carefully remove the comb from the spacer gel.
3. Remove the casting frame from the gel cassette sandwich and place the sandwich against the gasket on one side of the electrode assembly, with the short plate facing inward. Place a second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

### **Electrode assembly**

One gel is positioned on each side of the electrode assembly

Top of short plate fits snugly into notch in the green gasket of the electrode assembly

Electrode assembly with two gels is lowered into the clamping frame



## **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. Allow the extracts to thaw and vortex vigorously for ~ 10 seconds to thoroughly mix the contents.
2. Using gel loading micropipette tips (tips have very long, thin points and fit P20s or P200s), load up to 15  $\mu\text{L}$  of sample into each well. Load 5  $\mu\text{L}$  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.

**NOTE: Be sure to record the order of samples loaded onto the gel.**

3. Connect the tank to the power supply. Fit the tank cover onto the electrodes protruding up from the electrode assembly. Insert the electrical leads into the power supply outlets (connect black to black and red to red).
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. Turn off the power supply.
2. Remove the gel apparatus from the tank. Open the clamping frame and remove the gel cassette sandwich. Carefully, pry the two plates apart with a spatula. With the spatula, remove the lower right or left corner of the gel to serve as an orientation marker. **Be sure to indicate in your lab notebook whether the notched corner corresponds to lane 1 or lane 10 of the gel.** You may also remove the stacking gel with the spatula, if you desire.
3. 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 ~2 minutes.
4. Drain the water from the gel and add enough Simply Blue to cover the gel, while allowing the gel to move freely when the tray is rocked. Cover the gel container with saran-wrap and rock overnight. Make sure that the gel does not stick to the bottom of the tray.
5. In the morning, drain the Simply Blue stain into an appropriately labeled waste container in the hood of the lab room.
6. 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.
7. When individual bands are detectable, record your data. You may photograph the gel with your cell phone camera against a white background. Alternatively, place the gel in a clear plastic page protector and scan the gel.
8. After recording the data, dispose of the gel in the Biohazard waste container.

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## References

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