

Bio 6 – SDS-PAGE Lab

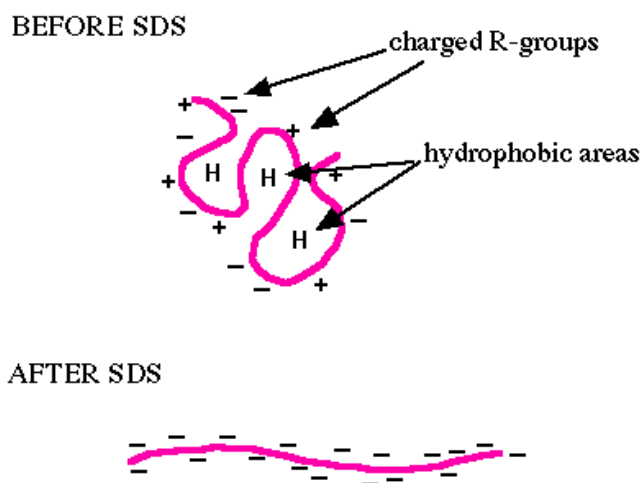
Objectives

Upon completion of this laboratory you will understand how to load and run protein samples on an SDS-polyacrylamide gel, stain the gel, and analyze the resulting bands of protein on the gel to estimate the molecular weight of each protein.

Introduction

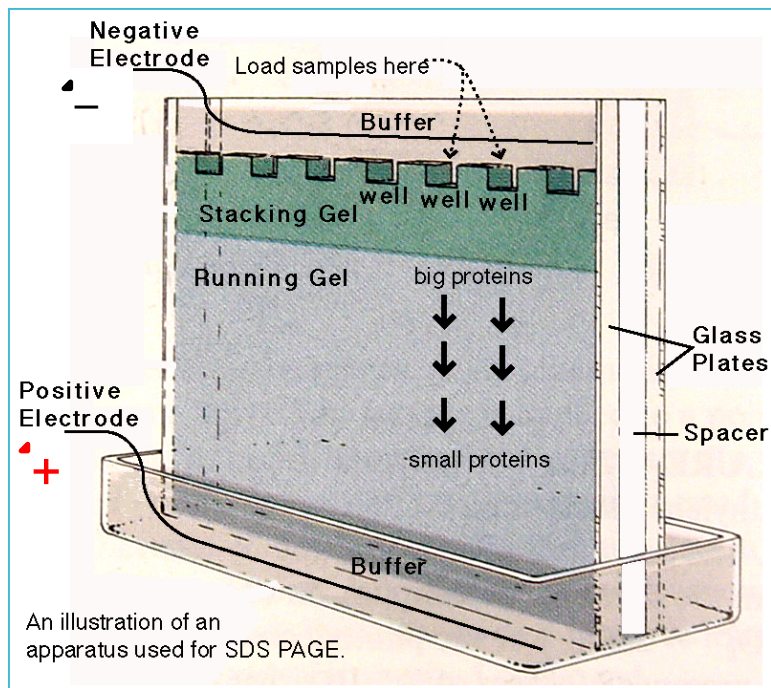
SDS-PAGE is a very common laboratory technique used to analyze proteins. The acronym SDS-PAGE stands for sodium dodecyl sulfate – polyacrylamide gel electrophoresis. **Sodium dodecyl sulfate** or **SDS** is a detergent commonly used in biology laboratories to denature proteins, i.e., disrupt the 3-dimensional structure of proteins without breaking the polypeptide backbone. **Polyacrylamide** is a polymer prepared as a gelatinous medium or “gel” through which proteins can be resolved based on molecular weight (MW). **Electrophoresis** refers to the movement of charged soluble particles such as proteins through a medium during exposure to an electric field. SDS-PAGE is therefore a technique by which proteins move through a polyacrylamide gel that is subjected to electric current.

The rate at which a protein moves through the microscopic pores of a polyacrylamide gel during electrophoresis is dependent on three physical properties – **molecular weight**, **3-dimensional shape**, and **net charge**. Separation of proteins based solely on the property of MW is possible only if the variables of 3-dimensional shape and net charge are eliminated. This is accomplished by the detergent SDS, which due to its **amphiphilic** properties of being hydrophobic on one end and charged on the other is able to disrupt all non-covalent interactions in a protein – i.e., **denature** the protein. This results in unfolded or linear polypeptides that have a net negatively charge as shown below:

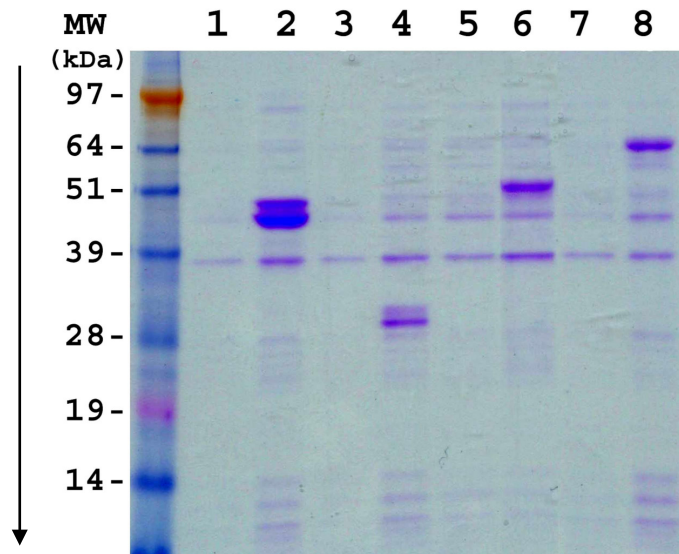


SDS thus eliminates any differences in shape and overall charge among proteins, leaving one variable physical property – molecular weight – to influence the rate at which a protein moves through the gel.

As shown below, SDS-PAGE is run vertically and the gel consists of a “stacking gel” on top of a “resolving gel” (aka “running gel”). Both ends of the gel are in contact with an electrolyte buffer solution containing the positive and negative electrodes which generate electric current through the gel.



The stacking gel contains cavities called **wells** into which you will load protein samples. When electric current is applied, negatively charged solutes such as SDS-treated proteins will experience electromotive force toward the oppositely charged positive electrode and thus move into the stacking gel. The stacking gel has an acrylamide percentage and pH that ensures all proteins enter the resolving gel at the same time. Once in the resolving gel, proteins will migrate at a rate inversely proportional to MW. When the gel is finished running, it is stained to reveal the position of each protein on the gel as shown below:



Each stained band on the gel corresponds to a one or more types of protein with the same molecular weight (keep in mind that each band contains *billions* of proteins of that MW). The larger the protein, the slower it moves through the gel and thus the higher its position. Smaller proteins move at faster rate and are seen as bands further down the gel. A protein MW standard (a collection of proteins of known size) is always run on the gel and used to estimate the sizes of proteins in the other lanes.

Part 1: Running an SDS-PAGE gel

To begin this lab you will denature and load several different protein samples on a polyacrylamide gel after which you will run the gel as described. Preparing an acrylamide gel for SDS-PAGE is a bit tricky, so the polyacrylamide gels have been prepared for you. To denature your proteins it is essential that you add **sample loading buffer** and then boil the sample. The sample loading buffer contains several key components needed to fully denature the proteins and have them run through the gel properly:

- **SDS** to denature the polypeptides
- **Dithiothreitol (DTT)** to break disulfide bonds (covalent bonds between cysteines)
- **Glycerol** to make the sample dense enough to sink into the well
- **Bromophenol Blue**, a negatively charged dye to monitor gel progress

The loading dye is prepared at a 2X concentration so that it can be diluted to 1X when mixed with the protein sample. Once loading dye has been added, the heat from boiling facilitates denaturation of the proteins and breaking of disulfide bonds. Disulfide bonds (refer to chapter 5 of your textbook) are the only covalent interactions formed between amino acid R groups and are **not** disrupted by SDS, thus the need for DTT. Only in this fully denatured state will each protein sample move through the gel at a rate dependent on molecular weight only.

Loading samples into the wells of the gel can be challenging since the gel is very thin, too thin to insert the pipet tip directly into the well. As shown in the picture, you will need to *gently* rest the pipet tip on top of the inner plate at an angle so that the pipet tip is resting just above the well you want to load. It is especially important that you **do not press the pipet tip down between the plates**, otherwise you may pry them apart. If this happens, even just a little bit, your samples will leak down between the gel and the plate and be lost. Before you load your protein samples you will load a practice sample so you will have some experience before loading your real samples.



Exercise 1 – Loading and running your gel

All of the materials you will need can be found on carts at the side of the lab where you will obtain an ice bucket and a tray of materials. Once you have all your materials, be sure to half fill the beaker with hot tap water and put it on a hot plate set to the maximum so boiling water will be ready when you need it:

1. Each protein sample is 5 μ l. Add 2X sample loading buffer to each protein sample so the final concentration is 1X (calculate this as you did in the “Metric System” lab).
2. Tap each tube gently to mix and boil the samples along with the molecular weight marker for 5 minutes (the protein molecular weight marker is 5 μ l and already contains 1X sample loading buffer).
3. Identify the wells you plan to use for your protein samples (avoid using deformed wells), and indicate in your lab notebook how you will load your gel. Each person should practice loading 10 μ l of 1X sample loading buffer in the unused lanes. Your instructor will demonstrate how to do this.
4. Load all of the protein molecular weight marker and each protein into their designated lanes.
5. Place the cover on the gel apparatus and connect the red and black plugs to the corresponding colors in the power supply. *NOTE: If the buffer level in the upper chamber is not above the inner plate of the gel, there will be no flow of electric current.*
6. Be sure the power supply is plugged in and set the toggle switch below the digital display to “Volts”, the Range Select switch to “Low” and the Voltage Select knob to “Min.”



7. Turn on the power (toggle switch on upper left) and adjust the Voltage Select knob until the digital display reads between 110 and 120 volts.

NOTE: If the gel is running properly you will see a curtain of tiny bubbles rising from the wire in the upper buffer chamber. You should also see a much lower amount of “bubbling” from the wire in the lower buffer chamber. If you don’t see this, be sure to consult your instructor.

8. Let the gel run until the bromophenol blue dye is ~1 cm from the bottom of the gel (~90 minutes).
9. When the run is complete, turn the Voltage Select knob to “Min.” and turn off the power.

Part 2: STAINING PROTEINS IN A POLYACRYLAMIDE GEL

To visualize the proteins in your polyacrylamide gel you will use a stain called **Coomassie Brilliant Blue**. This is not the only protein stain one could use, however it is a very commonly used stain to view proteins on polyacrylamide gels. The staining process may require an overnight incubation so you may not see the protein bands on your gel until the next laboratory session.

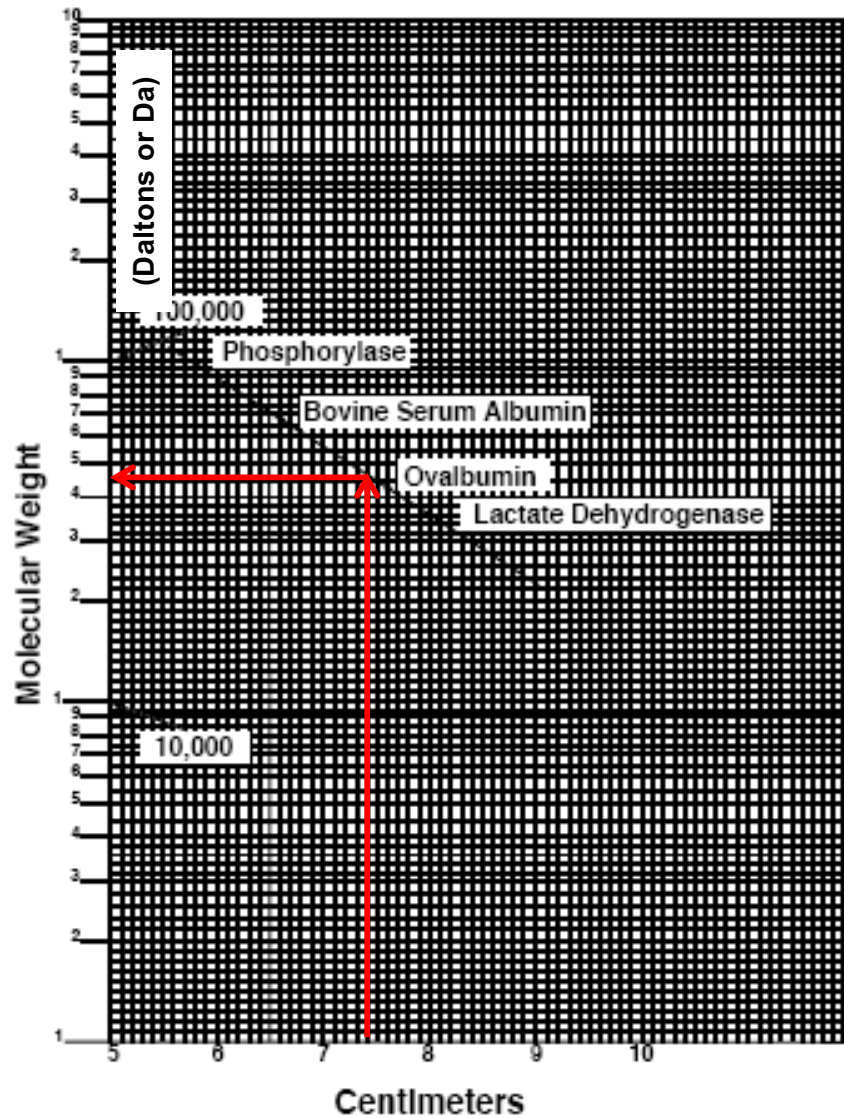
Exercise 2 – Staining a polyacrylamide gel

Be sure to wear gloves when you handle the gel and staining solutions, and to clean up as indicated by your instructor:

1. When the gel run is complete be sure the Voltage Select knob is set to “Min.” and the power is off, then remove the cover of the gel apparatus.
2. Carry the gel apparatus to the sink and discard the running buffer from both chambers into the sink.
3. Loosen the clamps holding the gel in place, carefully remove the plates containing your gel and lay it on a flat surface with the taller outer glass plate facing up.
4. Use the plastic spatula supplied as a wedge to **carefully** pry apart the 2 plates. The gel should stick to one plate or the other as you do so. If it seems to stick to both, ask for assistance from your instructor.
5. Place ~100 ml of fixative solution in the staining tray provided.
6. Gently separate a bottom corner of the gel from the glass plate using the spatula, **carefully** peel the gel off the plate (it’s OK if the stacking gel tears off, you won’t need it) and place it in the fixative solution. If the gel curls up, carefully unravel it with your gloved hand until it flattens out.
7. Place a Protein InstaStain card (which contains Coomassie Brilliant Blue stain) face down in the fixative solution over the gel, place the lid on the container (be sure to label the lid with your group number) and place the container on the slow rocker at the side of the lab. Leave for 1 to 3 hours or overnight.
8. When the protein bands are clearly visible, photograph your gel as indicated by your instructor being sure to include a ruler aligned with the top of the resolving gel. Store an image of the gel in your lab notebook.

Part 3: ESTIMATING THE MOLECULAR WEIGHT OF PROTEINS ON A POLYACRYLAMIDE GEL

You will use the protein molecular weight standard you ran on your gel to estimate the molecular weights of the other proteins on your gel. As mentioned earlier, the protein molecular weight standard is a set of proteins of known molecular weight. Since the rate at which a denatured protein moves through a polyacrylamide gel during electrophoresis depends only in its molecular weight, you can estimate the molecular weight of a protein based on its position on the gel in relation to the molecular weight standard proteins. In theory, the most accurate way to do this is to create a standard curve on semi-log graph paper by plotting molecular weight in daltons (Da) or kilodaltons (kDa) vs distance traveled on the gel in cm. The distance traveled refers to the distance from the middle of the protein band to the top of the resolving gel (**not** the bottom of the well in the stacking gel).



The graph above shows a sample standard curve consisting of 4 known proteins (NOTE – the standard proteins on your gel are different, this is an example). Once the standard curve is drawn, the molecular weight of any protein on the gel can be estimated using the distance it traveled on gel. For example, if a protein on the gel pertaining to this standard curve traveled 7.4 cm, identify 7.4 cm on the X-axis and move vertically to find the corresponding position on the standard curve. The molecular weight on the Y-axis that corresponds to this point on the standard curve will be your estimate. In this example the molecular weight estimate would be ~45,000 daltons or 45 kDa. While this method can be very precise, its accuracy depends on the quality of the distance measurements and the precision of the best fit curve.

A simpler method to estimate molecular weight is to compare the protein band of interest to the protein molecular weight standard and *visually* estimate its molecular weight. For example, if your protein of interest is positioned approximately halfway between standard proteins that are 40 kDa and 50 kDa, then you would estimate 45 kDa. If the band is just above and very close to the 40 kDa standard protein then you might estimate 41 or 42 kDa. While this may not seem very precise, it can give you a value very close to that obtained using a standard curve and for most purposes this should be sufficient. In the next exercise you will estimate the MW of proteins using both methods described above.

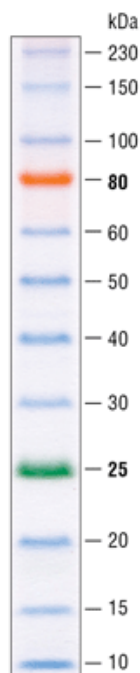
Exercise 3 – Estimating protein molecular weight

*NOTE: A 12% polyacrylamide gel can effectively resolve or separate proteins between 20 kDa and 100 kDa, so be sure **NOT** to include molecular weight standard proteins outside this range in your standard curve.*

1. Place your stained gel on a white transilluminator and use a small ruler to measure in **cm** the distance from the **middle of the band to the top of the resolving gel** for each molecular weight standard protein* between 20 kDa and 100 kDa. Record these values in your notebook.
2. Plot these values on the semi-log graph paper found at the end of this lab and draw the best fit line through the points. A copy of this standard curve should be placed in your lab notebook.
3. For each protein sample loaded on your gel, identify the most prominent band (this will be the protein of interest, the other bands are contaminants), measure the distance from the middle of the band to the top of the resolving gel. Record these values in your notebook.
4. Estimate the molecular weight of each protein using the standard curve as shown on the previous page and record in your notebook.
5. Estimate the molecular weight of each protein by visual comparison with the protein molecular weight standards and record in your notebook.
6. Answer the study questions associated with this lab.

NOTE: For this laboratory you will turn in a formal lab report.

*The protein molecular weight standard most likely used on your gel is shown below. If a different protein molecular weight standard was used your instructor will provide the details.



SDS-PAGE Lab Study Questions

NAME:

1. SDS-PAGE is an acronym referring to 3 specific components (SDS, PA and GE). Indicate what SDS-PAGE stands for and briefly describe each of these 3 components.
2. What does it mean to “denature” a protein and why is this important for SDS-PAGE?
3. Describe how you denatured the proteins you used for SDS-PAGE.
4. What substance in sample loading buffer is responsible for breaking disulfide bonds?
5. Diagram a disulfide bond under “BEFORE” and also diagram what it should look like after boiling in the presence of the substance from the previous question (“AFTER”).

BEFORE

AFTER

6. What is the purpose of loading “protein molecular weight standards” on the gel?

7. List the molecular weights of the standard proteins run on your gel and check the ones that you used to make your standard curve.

8. Describe each of the two methods you used to estimate protein molecular weight.

9. Why did you measure the distance each protein traveled into the gel from the top of the resolving gel and not the bottom of the well?