EXERCISE 8C - Lab Procedures

SAFETY WARNING: Acrylamide in the unpolymerized form is a skin irritant and a potential neurotoxin. Fortunately, the acrylamide in your gels is polymerized, so it should not present a safety hazard. However, as a precaution you are required to wear gloves when handling gels, buffers, stains, and destaining solutions.

SAFETY WARNING: Do NOT touch the electrophoresis chamber or wires when the voltage is turned on. Voltages may be as high as 300 V and shocks can be fatal. Before handling the electrophoresis apparatus, always make sure it is disconnected from the power supply.

I. Prepare the electrophoresis apparatus for SDS-PAGE

1. Carefully study the diagram below. It shows what your electrophoresis apparatus should look like when it is fully set-up and ready to run:

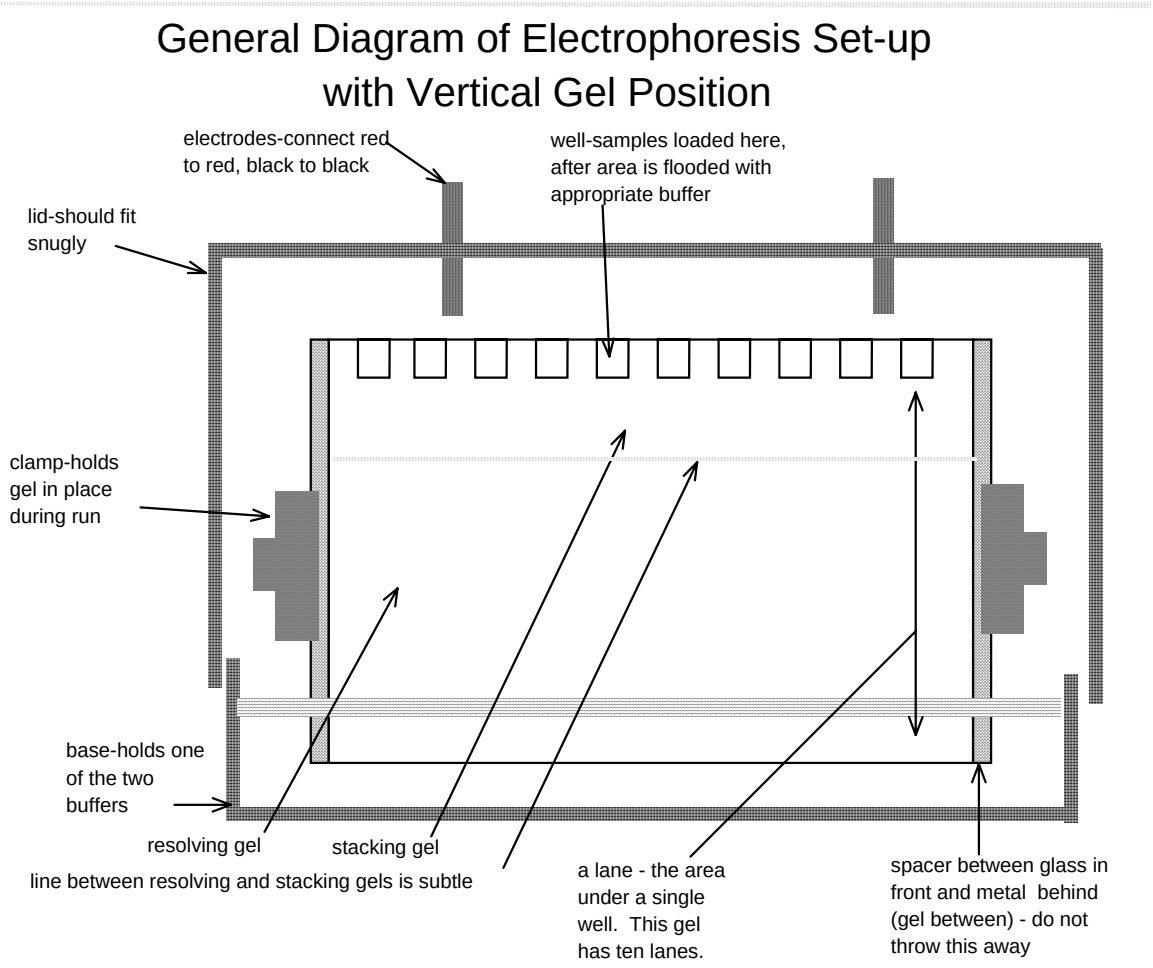


Diagram courtesy of Steve Bostic, ACC faculty

2. With gloved hands, carefully unwrap the cellophane from your electrophoresis gel. Notice that the actual gel is sandwiched between a white metal plate and a glass plate. Two gray plastic spacers are located on either side of the gel, and a white plastic comb is sticking out from the top of the gel. This comb was used to form the wells (indentations) in the top of the gel.

3. Handle the gel sandwich gently to avoid distorting or breaking the gel. Carefully remove the white plastic comb from the gel and drain any excess water out of the wells. Wash and save the gel comb - do NOT throw it away. Now clamp the entire gel sandwich onto the electrophoresis apparatus, with the metal plate pushed firmly against the rubber gasket.

4. Place a clear plastic template on the glass plate and align the black lines with the wells in the gel. This will allow you to locate the wells once they have been filled with cathode buffer.

5. Use a Pasteur pipette to fill the wells with "**cathode buffer**." Force the liquid into the wells with some velocity in order to sweep any non-polymerized gel components out of the wells. No harm is done if some Cathode Buffer splashes or overflows into the "top" reservoir (the narrow space behind the gel support chamber) since this reservoir will be filled with the same solution.

6. Pour cathode buffer into the "top" reservoir until the liquid level is 3 – 4 mm below the top of the glass plate. This is necessary in order to make electrical contact between the electrode and the gel.

7. Check to make sure that the cathode buffer is not leaking down from the upper buffer chamber. If it is leaking, add vacuum grease to the rubber seal and adjust your clamps to stop the leak before continuing with the experiment.

II. Prepare your samples and load them into the wells of your gel

1. Last week you saved 8 Eppendorf tubes containing samples of your milk fractions with 2X sample treatment buffer added. These tubes have been placed in an ice bucket along with an additional Eppendorf tube containing a mixture of Molecular Weight Standards ("MW").

2. If you need to dilute any of your samples before loading them into your gel, make your dilutions now. Make your dilutions according to the table below, which you completed as part of the Prelab:

Dilution of Milk Fractions to Make 40 μL of Diluted Sample with a Protein Concentration of 1.25 μg/μL

Milk fraction	Amount of milk fraction with 2X sample treatment buffer needed (μL)	Amount of 1X sample treatment buffer needed (μL)
Skim milk		
Pellet		
Whey		
Column fraction ___		
Column fraction ___		
Column fraction ___		
Column fraction ___		
Column fraction ___		

3. Insert all **9** Eppendorf tubes (milk fractions plus MW standards) into a holder and place them in the 60° C water bath. Make sure you use the diluted samples for those fractions that required dilution, and the undiluted samples for those fractions that did not require dilution. Heat the samples for 5 minutes, removing them briefly at 1 minute intervals to mix by tapping the sides of the tubes. After 5 minutes, dry the Eppendorf tubes and place them in the microcentrifuge in balanced positions. Centrifuge for 5 sec to force the solutions to the bottoms.

4. Return the Eppendorf tubes to the ice bucket and leave them undisturbed to allow any aggregated proteins and insoluble particulate matter to settle.

5. Your gel contains 10 wells. The wells are identified as #1 through #10, starting with #1 on your left as you face the front of the gel. Following the instructions below, load 16 μL of sample into each well.

   Important: Load your samples carefully, to make sure that each sample stays in its proper well.

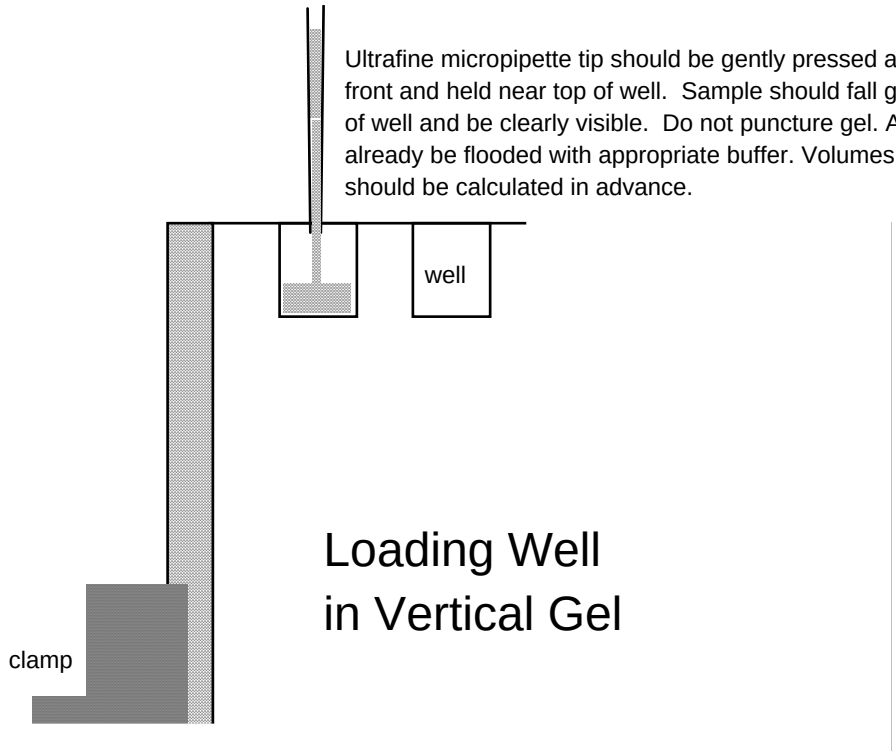


Diagram courtesy of Steve Bostic, ACC biology faculty

To load your first sample, adjust an automatic pipettor to deliver 16 μL and attach an ultra-thin disposable capillary tip. Withdraw the correct amount of your milk sample from your Eppendorf tube labeled "Milk" and insert the tip of the pipettor into the top of well #1. Make sure that the pipettor tip is between the glass and metal plates of the gel sandwich. Keep the tip at least 4mm above the bottom of the well. Take care not to puncture the sides or the bottom of the well with your pipettor tip. This takes a steady hand – it may help to support the micropipettor with your other hand and to support your elbows on the lab bench top.

Very slowly and gently expel the solution from the pipettor tip into the well while holding the pipettor steady. The blue solution should fall to the bottom of the well, gradually filling it. **Do not press the pipettor to the second stop – it is important to avoid blowing air bubbles into the well. Do not release your thumb from the pipettor until you have completely withdrawn the tip from the well.**

If the sample overflows into an adjacent well, you may be trying to load too much sample (do not exceed 16 μL). Alternatively, you may be expelling the sample with too much force, or you may be inserting the pipettor tip too far into the well so there is not enough room for your sample as it fills the well.

6. Place a new tip on the automatic pipettor, and then follow the procedure just described to load 16 μL of "MW standards" into wells 2 and 9.

7. Use the same procedure to load the other milk samples into the remaining wells on the gel. Be sure to use a clean tip for each protein sample, and keep a record of which sample was loaded into each well.

III. Run your gel

1. Remove the template from the front of the glass plate, and then pour anode buffer into the bottom reservoir. Wipe up any spills with paper towels so that your work area is dry. Place the protective plastic cover over the electrophoresis apparatus. Make sure the cover fits snugly.

Electric shock hazard! Be sure to follow instructions exactly!

2. **First make sure that the power supply is NOT connected to your electrophoresis apparatus.**

3. Plug in the power supply, turn it on, and adjust the voltage to 150V. It may be hard to get it to stay at exactly 150V, but get as close to that setting as possible. Once the voltage has been adjusted, **turn off the power supply and unplug it.**

4. As demonstrated by your instructor, connect the electrophoresis apparatus to the power supply and then plug in the power supply. Have your instructor check your set-up and connections. When given the OK by your instructor, turn on the power supply. Record the time when the power was turned on.

5. Running time is generally between 50 minutes and one hour. As current flows through the gel, proteins that are negatively charged will be pulled towards the bottom of the gel. You will not be able to see the proteins since they are colorless, but the blue tracking dye should remain visible. A few minutes after the power is applied, the blue bands should concentrate as a thin line below each well at the interface between the stacking gel and the resolving gel. The blue bands should then move slowly down through the resolving gel gradually becoming thicker as they move downward.

6. When the blue dye is within 2-3 millimeters of the bottom of the gel, turn off the power supply and unplug it. Finally, disconnect the power supply from the electrophoresis apparatus.

IV. Remove your gel and stain it with Coomassie Blue Solution

1. With the apparatus disconnected from the power supply, remove the cover from the electrophoresis apparatus. With gloved hands, carry the apparatus to the sink, secure the central section with your thumbs, and invert the apparatus to discard the buffer from the two reservoirs.

2. Return the electrophoresis apparatus to your work area. Place a piece of plastic wrap on your table. Remove the clamps holding the "gel sandwich" in place, and lay the gel sandwich on the plastic wrap with the glass plate on top.

3. Slide the two gray Teflon® spacers out from between the plates of the gel sandwich.

Wash and save the Teflon® spacers - do NOT throw them away!

4. Insert a spatula a short distance into the space where the Teflon spacers were and gently pry the top (glass) plate away from the gel. After you remove the glass plate, the gel should remain on the bottom (metal) plate.

5. Use the spatula to cut and discard a small triangular piece (approximately 5 mm on a side) from the lower right corner of the gel. Later, this will allow you to identify the lower right corner of the gel.

6. You should see a faint seam between the stacking gel (on top) and the resolving gel (on the bottom). Cut along this seam with a spatula and scrap away the stacking gel while leaving the resolving gel on the metal plate.

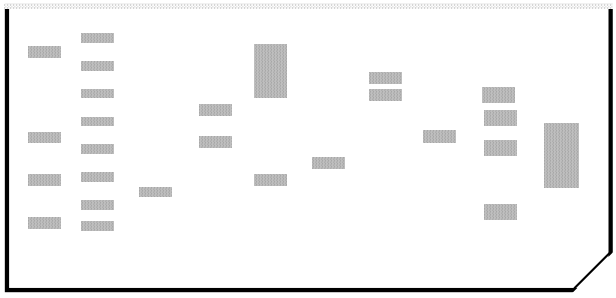
7. The resolving gel must be transferred to a Petri dish containing Coomassie Blue staining solution in order to stain the protein bands on the gel. The gel is very thin and fragile, so take care not to tear it. Use a dH_20 squeeze bottle to wet the gel. Hold the metal plate with the resolving gel upside down, just above the Petri dish containing Coomassie Blue solution. Using a spatula, gently peel the gel from the plate starting with the edge furthest away from the dye. Gently pull the corner of the gel away from the metal plate until it begins to peel away from the plate. Once the gel starts peeling from the plate it should fall into the Coomassie Blue solution without any help from you. When the gel is in the staining solution, cover the Petri dish and use a piece of tape to label it with your group name.

8. Place the dish on a rotary agitator and allow it to agitate slowly for 30 min.

   While the gel is staining, wash the Teflon® spacers, template, and metal and glass gel plates, being careful not to scratch them. Give them a final rinse with dH_2O, and leave them on absorbent toweling to dry.

9. Rinse out the electrophoresis apparatus with tap water and then with dH_2O. Turn it upside down on a paper towel at your work station to drain.

Gel as it is likely to apppear near end of run-- Fuzzy line near bottom is tracking dye; proteins are not yet visible.



Gel as it is likely to apppear after staining and de-staining; Note that resolving gel has been removed, a notch has been cut below lane ten, and numerous bands (each a single protein) have appeared. Lanes 5 and 10 contain smears. Lane 1 contains 4 proteins; lane 2 contains 8 proteins and gives the general appearance of "markers"--molecular weight standards.

Diagrams courtesy of Steve Bostic, ACC biology faculty

V. Remove background stain from the gel

1. The Coomassie Blue stain will be reused, so after the gel has been in the stain for 30 minutes, pour the stain through the filter-lined funnel into the discard bottle, while carefully holding the gel in place with your gloved hand. Pour out as much of the stain as possible.

2. Deliver several milliliters of dH_20 from a squeeze bottle to the Petri dish containing the gel, swirl briefly to rinse the gel, and again holding the gel in the dish with your gloved hand, discard the rinse water into the sink.

3. Pour fresh “Destaining Solution" into the Petri dish containing the gel until it is half full. This solution is 50% Methanol + 40% dH_20 + 10% Acetic acid. Return the Petri dish to the agitator and record the time when destaining was begun.

4. After 15 minutes, pour the destaining solution from the Petri dish into the bottle labeled "Used Destaining Solution”. Once again, pour fresh “Destaining Solution" into the Petri dish until it is half full.

5. After 15 minutes, pour the destaining solution from the Petri dish into the bottle labeled "Used Destaining Solution." Ask your instructor to examine the gel and determine if it requires additional destaining. . The proteins within the gel should appear as blue horizontal bands in otherwise colorless lanes.
   If it is sufficiently destained, fill the Petri dish containing the gel about half-full with dH_2O.

6. Tape the Petri dish closed, and make sure it is labeled with your instructor’s name, your lab day and time, and your group name. Give the dish to your instructor, who will store it until your next lab period.

Clean up

Be careful not to lose any parts of the gel apparatus, including the plates, the template, the spacers, and the gel comb. Wash them in soapy water, rinse with tap water and dH_2O, and leave on absorbent toweling to dry.

Dispose of your excess solutions as directed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all glassware, give it a final rinse with dH_2O, and leave it inverted at your work area in order to drain.

All disposable glassware goes into the special glass disposal receptacle.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.