Laboratory 12
Analysis of Proteins in Different Animal Organs, Part B

I. Introduction

In the first part of this experiment, you set up and ran a SDS-polyacrylamide gel to determine the mobilities of a series of standard proteins, made a tissue homogenate or an acetone powder suspension of a certain animal organ, and measured the protein concentrations of the suspension using the Coomassie Plus protein reagent.

In the second part of this experiment, you will

- use SDS-PAGE to separate and compare the proteins in the acetone powders suspensions that were made last week

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this experiment. After preparing for the lab, you should be able to answer the following questions. **Record the answers to these questions in your lab notebook before the lab.**

A. How does polyacrylamide gel electrophoresis differ from agarose gel electrophoresis?

B. What is the difference between a denatured and a nondenatured protein?

C. What chemicals are used to denature proteins?

D. Why is 2-mercaptoethanol often added to denaturation solutions?

E. What determines the rate of movement of a protein during gel electrophoresis under denaturing conditions?

F. What is the relationship between protein size and mobility in an SDS-polyacrylamide gel?

G. Why was the dye bromophenol blue included in the sample buffer?

H. Why do all of the proteins in an SDS-polyacrylamide gel move towards the positive electrode?

I. How will the gels be stained to reveal the proteins after electrophoresis?

J. What is meant by semi-log graph paper?

K. What are the possible advantages of using a gradient polyacrylamide gel rather than a gel with a single gel concentration?

L. Why will molecular mass markers be included in the new gels of the proteins from the tissue extracts?

M. Which tissues would you expect to contain the most complex mixtures of proteins? Which should contain simpler combinations of proteins?

N. Many proteins in a cell have similar sizes. If you find a band with a molecular weight of 35,000, how do you know if it represents one protein or several different proteins?

O. If you find a protein with a molecular mass of 35,000 in both the calf liver acetone powder and the porcine brain acetone powder, how could you determine if they are the same protein?

III. Background Information

A. Gel Electrophoresis of Proteins

Electrophoresis is a powerful preparative and analytical technique that is widely used to separate molecules such as proteins and nucleic acids. There are many forms of electrophoresis, but all of them are based on the migration of charged molecules in an electric field. While electrophoresis can be done in a liquid solution, most currently-used protocols use a solid support medium such as a gel, a sheet of paper, or a synthetic membrane. This type of electrophoresis is referred to as zone electrophoresis.

The movement of a particular molecule in an electric field can be described by the equation:

$$\mathbf{v} = \frac{\mathbf{E\,q}}{\mathbf{f}}$$

where **v** is the velocity or rate of movement, **E** is the strength of the electric field in volts/cm, **q** is the net charge on the molecule, and **f** is the frictional coefficient. The frictional coefficient depends on the size and shape of the molecule of interest and on the type of medium through which it must pass. From this equation, it is clear that the velocity of a molecule increases with its net charge and the strength of the electric field and decreases with the frictional coefficient. Since the strength of the electric field is usually constant of all of the molecules in a mixture, these molecules can be separated if they differ in size, shape, or net charge. Because the charge on any particular molecule may vary with pH, the composition and pH of the surrounding buffer are often important factors in determining the direction or rate of movement.

1. Nondenaturing and denaturing gel electrophoresis.

Proteins can be separated under either nondenaturing or denaturing conditions. Nondenaturing electrophoresis is used to separate proteins in their natural or native state while denaturing electrophoresis is used to separate proteins after their native conformations have been destroyed. For electrophoresis under nondenaturing or native conditions, the proteins are suspended in a buffer that will not result in loss of three-dimensional structure or in separation of oligomeric proteins with subunits into their individual polypeptide chains. Depending on the pH of the solution, some of the proteins in a nondenatured mixture may migrate toward the positive electrode but others may migrate toward the negative electrode depending on their overall net charge. Nondenaturing gel electrophoresis is often used to determine the native molecular mass of a particular protein (that is, its total size) or to detect a particular protein within the gel by its catalytic or

binding activity.

For electrophoresis under denaturing conditions, the proteins are suspended in a buffer designed to cause denaturation and loss of conformation. Such buffers commonly contain high concentrations of 2-mercaptoethanol, which will break disulfide bonds between cysteine residues, and the anionic detergent sodium dodecyl sulfate (SDS), which is also called sodium lauryl sulfate. The detergent facilitates denaturation of proteins by interacting with hydrophobic residues and coats the proteins with a large negative charge. During electrophoresis, all of the proteins migrate toward the positive electrode. Denaturing gel electrophoresis is often used to determine the number of proteins in a complex mixture. It is also used to determine the subunit molecular mass of a particular protein. That is, it is used to determine the sizes of the different polypeptide chains or subunits that make up a natural or native protein. Gel electrophoresis of denatured proteins will be done in this experiment.

2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The most commonly-used support medium for separating denatured proteins is a polyacrylamide gel. Polyacrylamide gels are formed by the polymerization of acrylamide and N, N'-methylene-bis-acrylamide. While solutions of acrylamide and bis-acrylamide are stable by themselves, these molecules form a crosslinked matrix in the presence of free radicals. Free radicals can be generated either photochemically, by irradiating a dye like riboflavin, or chemically, by dissolving a salt such as ammonium persulfate in water. The presence of unpaired electrons oxidizes the acrylamide and promotes polymerization. To facilitate rapid propagation of free radicals and electrons through the solution, a catalyst such as N, N, N, N-tetramethylethylenediamine (TEMED) is usually added to the gel mixture along with the other chemicals that form a buffer.

Polyacrylamide gel electrophoresis is most often done in vertical gel systems. Acrylamide gels can be cast as either tube gels or slab gels. In the first case, the liquid acrylamide solutions are mixed with a source of free radicals and poured into a glass tube. In the second case, the liquid acrylamide solutions are mixed with a source of free radicals and poured into the space between a pair of glass or plastic plates. The plates are separated by small spacers of known thickness and held together by clamps. A comb is inserted into the top of the plate system while the gel solution is still liquid. As the gel sets, small wells are formed. These wells can then be filled with samples once the comb is removed. Most protein separations by SDS-PAGE today are done in slab gels because they allow better comparison of proteins samples and molecular mass markers.

While proteins can be separated in a slab gel containing a single layer of polyacrylamide, they can be analyzed more effectively using two gels that are stacked on top of one another. The separating or running gel, which comprises most of the gel matrix, usually has a relatively high acrylamide concentration (in the range of 5%-20%) and is made in a high ionic strength buffer at pH 8.8. Sometimes a separating gel with a single acrylamide concentration is used, but sometimes a separating gel with a gradient of acrylamide in used. In this case, the gel concentration gradually increases towards the bottom of the gel. This slows down the movement of the smaller proteins and results in the separation of a wider range of proteins in the sample. The stacking or spacer gel, which comprises the upper part of the matrix, has a lower acrylamide concentration (in the range of 3%-5%) and is made in a low ionic strength buffer at pH 6.8. The sharp increase in acrylamide concentration between the two parts of the gel and the sudden change in pH causes the proteins in the sample to form much tighter bands.

Once the stacking and separating gels have been cast, the slab system is placed between two buffer compartments, one containing the positive electrode and one containing the negative electrode. Buffer is

added to the compartments and the wells are filled with samples. The system then is connected to a power supply and a voltage is applied. This causes the proteins to move towards the bottom of the gel which is immersed in buffer containing the positive electrode. Because most proteins have no intrinsic color, a negatively charged tracking dye such as bromophenol blue is added to the sample mixture so that the movement of the molecules can be followed (Figure 12.1)

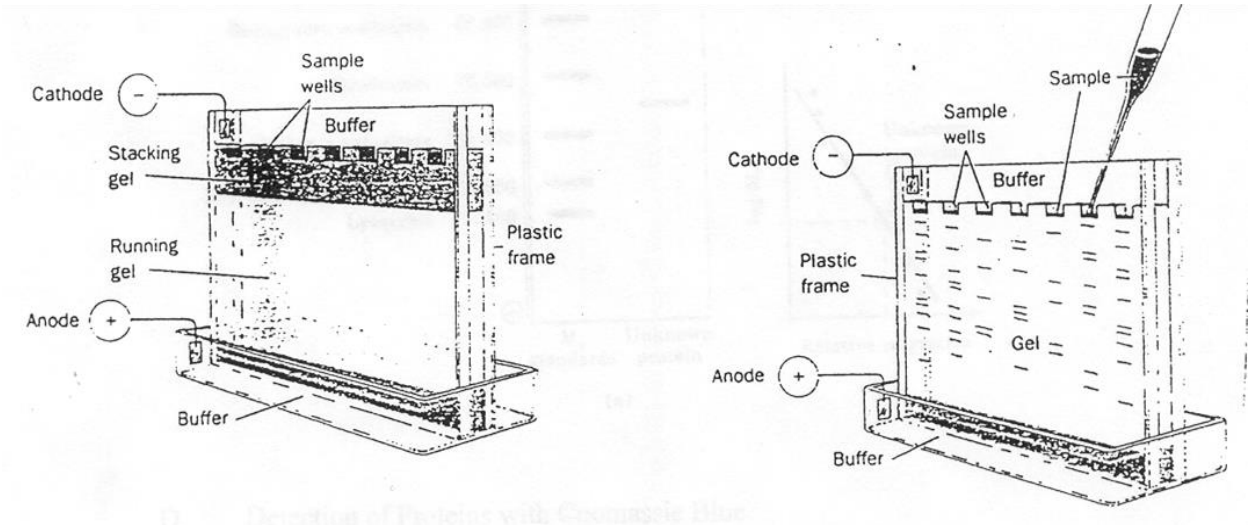

Figure 12.1 Example of polyacrylamide gel electrophoresis.

3. Mobility of Proteins under Denaturing Conditions

When denatured proteins are subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), they move through the gel towards the positive electrode in order of molecular size. That is, the smallest proteins move most rapidly while the largest proteins move most slowly. There is a direct relationship between the mobility of a protein and the log of its molecular mass. The mobility of a protein is defined as:

$$\text{mobility} = \frac{\text{distance travelled by protein}}{\text{distance travelled by tracking dye}}$$

Since the distance traveled by the tracking dye is the same for all of the proteins, most scientists just use the **actual distance** from the bottom of the sample well to the center of a particular protein band as a measure of mobility. The mass of a protein is expressed in daltons (Da) or atomic mass units. Because most cellular proteins are relatively large, size is often express in kilodaltons (1 kDa, = 1000 daltons).

In most SDS-PAGE experiments, a mixture of proteins of known molecular mass is separated in the gel along with the unknown samples. In this way, the actual relationship between mobility and molecular mass for a particular gel system can be determined. A standard curve is then made by plotting the **log** of the mass of each standard protein on the Y axis as a function of its mobility on the X axis (Figure 12.2)

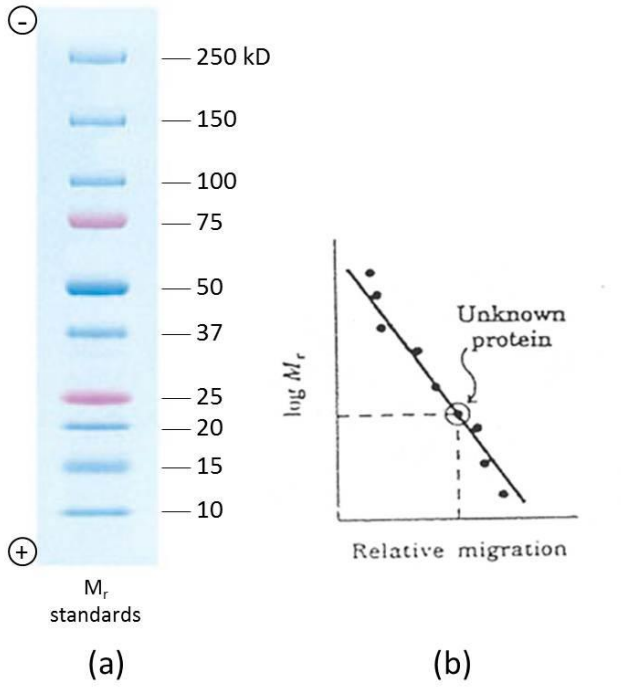

Figure 12.2 Picture of (a) the Precision Plus Protein Dual Color Standard (Bio-Rad) and (b) a standard curve created based on the log of the size of the protein standards and the relative migration in the gel.

There are two ways to generate the log size vs. distance plot. One is to actually calculate the log of the fragment size using a calculator and then to plot the data on graph paper. You can also do this using Excel. The other is to use **semi-log graph paper**. This type of paper has a linear scale on the X axis and a log scale on the Y axis. Depending on the type of graph paper, there may be 1 to 5 log cycles along the Y axis. When you plot numbers on the paper, it is as if you were plotting the log of a certain number because the intervals get smaller as you go along each cycle.

For example, suppose you collect the following set of data:

X variable:	1	3	5	6	7	9	10	12
Y variable:	5000	3000	1000	500	100	60	50	20

Since the Y variable changes over a very wide range of values, it is easier to plot log Y as a function of X rather than Y itself. Figure 12.3 shows a plot of these data on three-cycle semi-log paper.

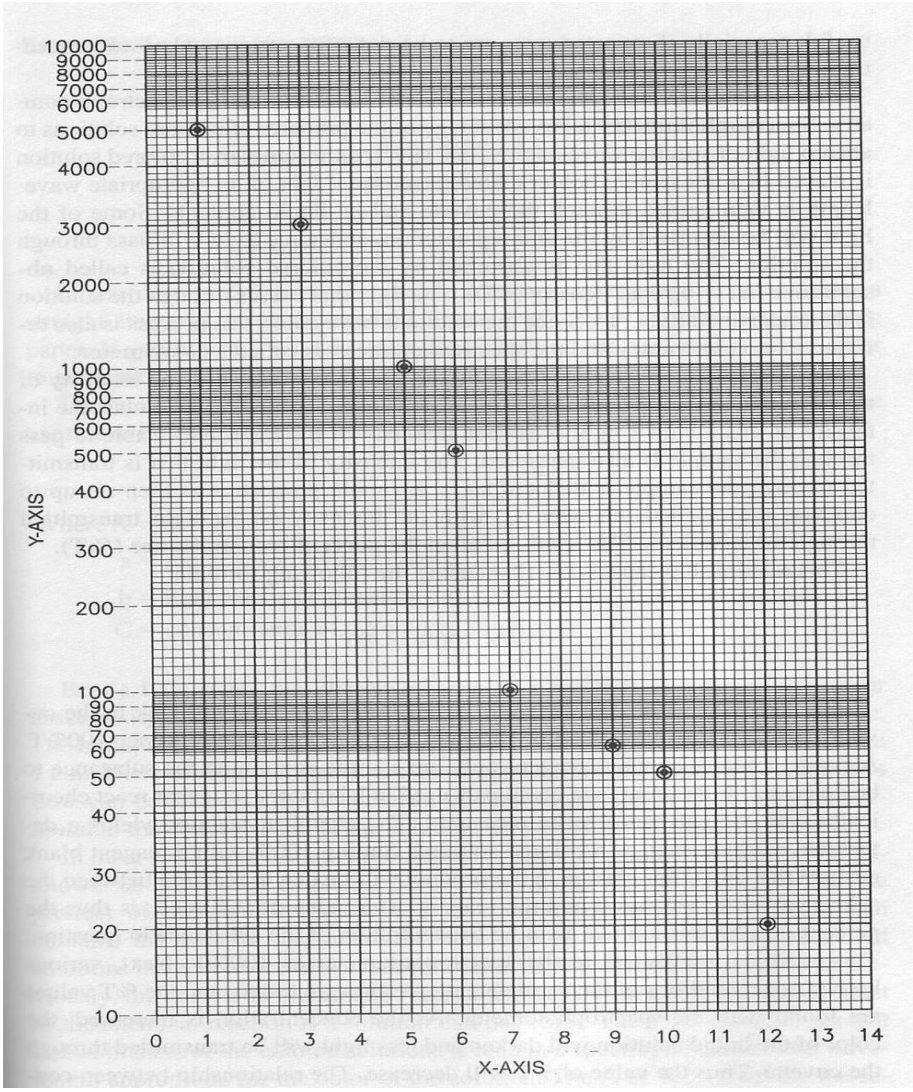

Figure 12.3. Shows an example of numbers plotted on semi log paper.

Note that in the semi log paper shown beginning at the bottom, the first log cycle goes log cycle goes from 10 to 100, the second from 1000 to 10000, and the third second from 10000 to 100000. So when the data are plotted, the X value samples for 1, 3, and 5 fall in the upper-most cycle, the X value samples for 4, 5, and 7 fall in the middle cycle, and the X values samples for 9, 10, and 12 fall in the bottom cycle. When this data set is plotted, the points fall on what might be a straight line. The best-fit straight line to these points can be drawn with a ruler. Note: the paper you will use may be two cycle semi log paper in which case you would start at 1000, rather than 10.

The molecular mass of an unknown protein can then be determined from a standard curve such as the one noted on the previous page. In our example if a value on the X axis was known to be 7, the corresponding value on the y axis would be ~300 if you draw a straight line through the points given. Or in the case of the figure on the previous page the size of the unknown protein may be ~37,000

4. Detection of Proteins with Coomassie Blue

Because most proteins do not have an intrinsic color that can be seen by humans, it is necessary to stain gels after electrophoresis in order to locate the proteins. The most commonly-used stain is called Coomassie Blue R-250 (also called Brilliant Blue R) (Figure 4.5). Coomassie Blue binds tightly to proteins, giving them a blue color. However, not all proteins bind the dye to the same extent and some proteins do not bind it at all. As a result, while the intensity of a protein band is generally related to the amount of the protein in the gel, it is not a simple relationship).

To prevent the migration of proteins within the gel or out of the gel into the solution during the staining process, the gel is often fixed and stained at the same time. This usually involves placing the gel in a solution containing Coomassie Blue, methanol, and acetic acid. This results in uniform staining of the gel. The gel is then "destained" by shaking it gently in a solution of methanol and acetic acid. Free dye gradually is washed from the gel while dye molecules that are bound tightly to proteins remain in the gel and produce a series of blue bands.

In this experiment, a simpler and more rapid staining procedure developed by the Pierce Chemical Company will be used. The gels will be washed first with several large volumes of water to remove the SDS. The gel then will be stained with a colloidal suspension of Coomassie Blue R-250, which binds almost exclusively to the protein bands. Staining is usually complete within several hours and the background staining of the gel can be eliminated by washing with water. Once the gel bands are visible, the gel can be stored in water indefinitely.

IV. Experimental Procedures

This experiment involves only three steps. Because it takes some time for the electrophoresis to run, you will analyze a practice gel while your gels are running. The actual analysis of the new gels will be done as part of next week's lab.

The following is a flow chart for this laboratory session:

Preparation of Protein Samples (Section IVA)
↓
↓
SDS-PAGE of Proteins in Acetone Powder Suspensions (Section IVB)
↓
↓
Analysis of Standard Protein Gels (Section IVC)

A. Preparation of Protein Samples

The objective of this part of the experiment is to prepare the samples to be analyzed by SDS-polyacrylamide gel electrophoresis. You will use the acetone powder suspensions prepared during the last lab.

1. Each group will be provided with a microcentrifuge tubes containing 5-10 µl of the Precision Plus Dual Color Protein Standard (Figure 12.2a)

   The Precision Plus Protein Dual Color standard is a mixture of 10 proteins (10-250kD), including eight

blue-stained bands and two pink reference bands (25 and 75 kD). Each tube of standard contains sample buffer (30% (w/v) glycerol, 2% SDS, 62.5 mM Tris, pH 6.8, 50mM DTT, 5 mM EDTA, 0.02% NaN_3, 0.01% bromophenol blue).

2. The samples of acetone powder suspensions prepared in lab last week will be available. Thaw one of your samples for use in the gel analysis; save the LDH tube in the freezer until next week's lab. You should use your own sample from last week to prepare a protein solution at an appropriate concentration in sample buffer. You will then share your solution with the other lab groups and they will share their solutions with you.

   The six samples will be labeled as shown below.

   PK: porcine kidney
   CB: chicken breast muscle
   BP: bovine pancreas
   CL: calf liver
   PH: porcine heart
   PB: porcine brain

3. Based on the protein assay that was done last week, prepare a small amount (300 µl) of your own sample at a concentration of 2 mg/ml in a clean 1.5 ml microcentrifuge tube. The dilution factor for this working solution can be calculated as follows:

$$\frac{\text{actual concentration}}{\text{desired concentration}} = \text{dilution factor}$$

   For example, if the actual protein concentration of the calf liver suspension is 5.32 mg/ml and you want to make a 2 mg/ml solution, it must be diluted:

$$\frac{5.32 \text{ mg/ml}}{2 \text{ mg/ml}} = 2.66$$

   To make 300 µl of solution, you will need to add:

$$300 \text{ µl} \times \frac{1}{2.66} = 113 \text{ µl of original solution}$$

   The 113 µl of the tissue sample should be combined with (300 µl - 113 µl) = 187 µl of water.

4. Then add 300 µl of 2X SDS sample buffer to your diluted protein solution. The final protein concentration in the sample thus will be 1 mg/ml.

5. Transfer 100 µl portions of your diluted sample in sample buffer to each of 5 more 1.5 ml microcentrifuge tubes. Label the tubes with the correct letter for your sample.

6. Trade these samples with the other groups in the lab so that each group has 1 tube with 100 µl of each of the six samples.

7. Heat the molecular mass standards and the six samples of the organ proteins in sample buffer at 100 ^{o}C for five minutes. Allow the solutions to cool to room temperature and centrifuge the samples briefly in the microcentrifuge to collect the liquid at the bottom of each tube.

B. SDS-Polyacrylamide Gel Electrophoresis of Proteins in Acetone Powder Suspensions

The objective of this part of the experiment is to use SDS-polyacrylamide gel electrophoresis to separate and analyze the proteins in the different organ acetone powder suspensions. We will use Bio-Rad mini-PROTEAN Tetra vertical gel electrophoresis systems, which hold two 8.6 cm x 6.8 cm mini slab gels that have 10 sample wells each. To save time, you will use pre-cast gels that contain a 4-15% gradient polyacrylamide separation gel and a 4% polyacrylamide stacking gel. The proteins in the acetone powder suspensions will be compared to the molecular mass marker proteins in the Precision Plus Protein Dual Color Standard (PPS). Because acrylamide is toxic and Coomassie Blue will stain proteins in fingers, students should wear gloves whenever they are working with the gels.

Figure 12.4 shows a diagram of the Mini-PROTEAN Tetra system we will be using. The system allows two plate cassettes to be clamped tightly to a central core, which is then held in a clamping frame. This forms an inner buffer compartment. It contains the negative electrode and is continuous with the top of each gel. The entire system is then placed in a large mini-tank or buffer box, which will also contain buffer. This lower buffer compartment contains the positive electrode and is continuous with bottom of each gel. When voltage is applied to the system from a power supply, ions flow from the negative electrode in the inner buffer compartment to the positive electrode in the lower buffer compartment, and proteins move from the top of each gel towards the bottom.

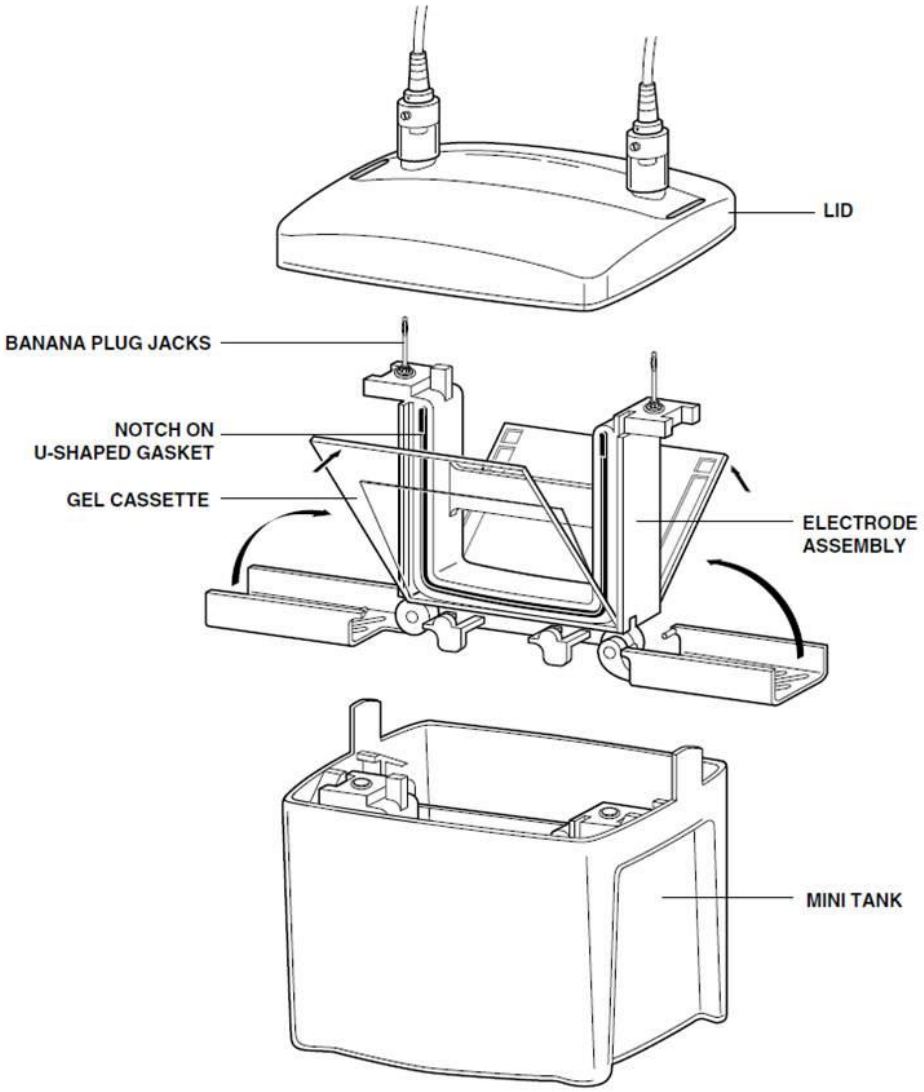

Figure 12.4 (Assembling the Mini-PROTEAN Tetra Cell (Bio-Rad))

1. Each group will be provided with a microcentrifuge tubes containing 5-10 µl of the Precision Plus Dual Color Protein Standard (Figure 12.2a)

   The Precision Plus Protein Dual Color standard is a mixture of 10 proteins (10-250kD), including eight blue-stained bands and two pink reference bands (25 and 75 kD). Each tube of standard contains sample buffer (30% (w/v) glycerol, 2% SDS, 62.5 mM Tris, pH 6.8, 50mM DTT, 5 mM EDTA, 0.02% NaN_3, 0.01% bromophenol blue).

2. Wearing gloves, remove two pre-cast Bio-Rad Mini-PROTEAN® TGX™ 4-15% gradient polyacrylamide gels from the sealed pouches. Remove the combs by pulling upward in one smooth motion. **Remove the green tape** from the bottom of each cassette. Mount each gel cassette into the electrode assembly as demonstrated by the instructor and shown in Figure 12.5. The shorter plate should face in towards the inner buffer compartment and fit up against the flat green gasket on the central core.

3. Gently push the gels toward each other making sure that they rest firmly and squarely against the green gasket that is built into the electrode assembly. Align the short plates to ensure the edge sites just below the notch at the top of the green gasket.

4. While gently squeezing the gel cassette and buffer dam against the green gaskets (maintaining constant pressure) slide the green arms of the clamping frame one at a time over the gels, locking them into place.

5. Place the electrophoresis module in the back position of the Mini-PROTEAN Tetra Tank (Front of the tank is the face that has the 2-Gels and 4-Gels line markings). Make sure that the red (+) electrode jack matches the red marking on the top right inside edge of the tank.

6. Add Tris-Glycine-SDS electrophoresis buffer to the inner chamber and check for leaks then add it to the lower buffer compartment. The buffer in the inner chamber should come up over the lower plates in the central core. The buffer in the mini-tank should come up to the bottom of the green gasket.

7. With a transfer pipet, rinse out the wells with electrophoresis buffer. **Watch the buffer level to see if there are any leaks.**

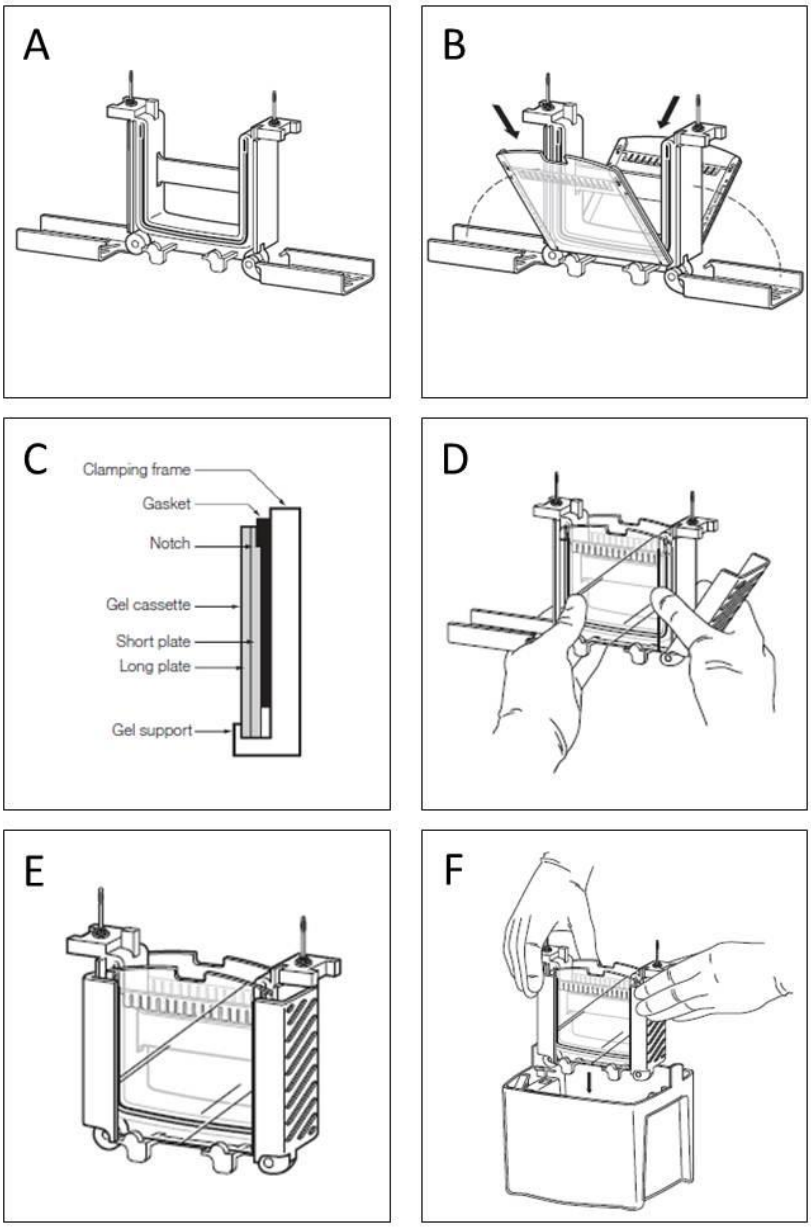

Figure 12.5 Assembly of Mini-PROTEAN® Tetra Cell for Mini Precast Gels

8. Using a P-10 or P-100 micropipetter and long plastic gel-loading tips, load different volumes of the standard protein mixtures and the proteins from the acetone powder suspensions into the wells in each system as shown in the following table. Note that you will load the samples through the buffer and between the plastic plates of the gel cassettes. You may find it helpful to place a yellow loading guide into the inner buffer compartment so that it lines up with the wells. It will take a little practice to see the wells and to load the samples, but once the first sample is loaded, the rest will be easier. In loading the samples into the wells, be careful to add the solution slowly so that it settles to the bottom of the well. Also, do not push the micropipetter down to the second stop, since this will force air into the well and may displace your sample. **Be sure to use a separate pipet tip for each sample.**

The following tables show the loading protocols for the two gels. All volumes are in μl.

Sample Loading Protocol for Gel A:

well:	1	2	3	4	5	6	7	8	9	10
volume:	5	10	20	30	10	20	30	10	20	30
sample:	PPS	PK	PK	PK	CB	CB	CB	BP	BP	BP

Sample Loading Protocol for Gel B:

well:	1	2	3	4	5	6	7	8	9	10
volume:	5	10	20	30	10	20	30	10	20	30
sample:	PPS	CL	CL	CL	PH	PH	PH	PB	PB	PB

PPS: Precision Plus Standard
PK: porcine kidney
CB: chicken breast muscle
BP: bovine pancreas
CL: calf liver
PH: porcine heart
PB: porcine brain

9. When both of the gels have been fully loaded, carefully place the lid on the system so that the electrodes are correctly aligned (red to red, black to black). Be sure to remove the yellow loading guide first. Then, insert the leads from the lid into the power supply. **The red lead should go into the red plug and the black lead should go into the black plug.** Be sure that the power supply is initially programmed to allow 150 V as the maximum voltage, 400 mA as the maximum current, and a time of 45 minutes.

10. Run the gel system at 150 Volts for about 30-40 minutes. Note the position of the bromophenol blue tracking dye throughout the run. You should see the dye move first towards the bottom of each well. The dye should eventually form a thin blue line within the gel.

11. When the tracking dye gets to within **1 cm** of the bottom of the gel cassette, disconnect the leads from the power supply and carefully remove the lid. Pour off the buffer from the upper and lower buffer chambers into the designated container and remove the cassettes with the gels from the clamping assembly.

12. Using a thin spatula or the plastic tool that comes with the gel system, carefully separate the two plates in one of the cassettes. The gel will usually stick to one of the plastic plates. Place the plate with the gel facing up in a plastic gel staining dish and add about 50 ml of deionized water.

13. Repeat the process with the other gel cassette. Use a separate staining dish and be sure to label the dishes (with tape) Gel A and Gel B.

14. Shake the staining dishes for about 5 min to begin to extract the SDS from the gels. The gels will probably float off of the plastic plates during this process, so you can remove the plates from the staining dishes. Decant the water and add 50 ml of fresh water. Be careful not to discard the gel as you decant the washing solution. Shake the gels again for about 5 min. Decant the water and add 50 ml of fresh water. Shake again for 5 min.

15. Add 20 ml of Pierce Gel-Code Blue Stain Reagent to each gel staining dish. Gently shake the gel in the stain and allow it to stain overnight.

16. The instructor will remove the staining solution the following day and wash the gels several times with water. The gels then will be stored in water until the next lab session when they will be scanned and analyzed.

C. Analysis of Standard Protein Gels

The objective of this part of the experiment is to learn how to analyze protein gels. You will do a similar analysis next week for the gels that you are running today.

1. Figure 12.6 shows a picture of an SDS-PAGE gel that has been stained with Gel-Code Blue stain.

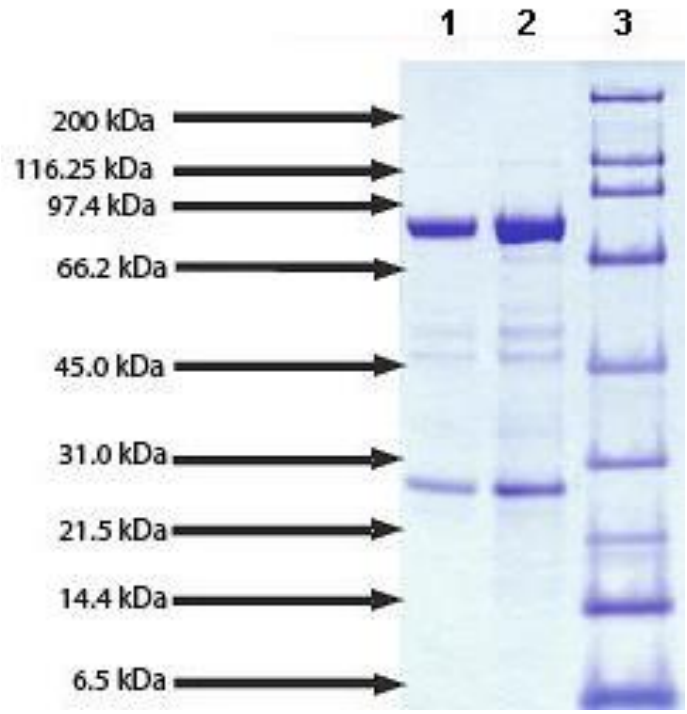

Figure 12.6 SDS-PAGE Practice Gel

Two unknown protein samples were subjected to SDS-PAGE and visualized by staining with Coomassie Blue Stain. Lane 1: Unknown protein sample A; Lane 2: Unknown protein sample B; Lane 3: Molecular Weight Standard. Sizes for proteins in molecular weight standard are shown in kDa. Wells are located at the top of the gel. (Image obtained from www.Abcam.com)

2. For each lane in each gel, measure the distance (in cm) from the bottom of the well to the center of each clearly visible protein band. Record the distances in the following table:

Table 12.1. Protein Migration Distance

Lane 1 Sample A	Lane 2 Sample B
Distance (cm)	

Lane 3 Standard	
Distance (cm)	Mass (kDa)
	116.25
	97.40
	66.20
	45.00
	31.00
	21.50
	14.40
	6.50

3. Use a piece of semi-log graph paper to construct a standard curve. Using the data from the table above, plot mobility (in cm) on the X axis and molecular mass on the Y axis. **Note: Only the molecular weight standard should be included as part of your standard curve. Do not include the unknown protein samples on this graph**.

4. Draw the best fit straight line through as many of the points as you can. You may find that the proteins with the lowest or highest molecular mass deviate from the straight line.

5. Now look at the lanes that were loaded with the unknown protein samples. Using the standard curve, determine the denatured molecular masses each of the proteins in the unknown samples.

Table 12.2. Denatured Molecular Masses in Unknown Samples

Lane 1 Sample A	Lane 2 Sample B
Protein Sizes (kDa)	