Edvo-Kit # 150

Edvo-Kit #150

Survey of Protein Diversity

Experiment Objective:

The objective of the experiment is to use SDS-polyacrylamide gel electrophoresis to develop an understanding of protein structure, function and diversity.

See page 3 for storage instructions.

Version 150.220222

EDVOTEK®

Table of Contents

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

Components		Storage	Check (✓)
A	Standard Protein Markers	-20°C Freezer with desiccant	❑
B	Milk Whey Proteins	-20°C Freezer with desiccant	❑
C	Serum Proteins	-20°C Freezer with desiccant	❑
D	Egg White Proteins	-20°C Freezer with desiccant	❑
E	Spinach Leaf Proteins	-20°C Freezer with desiccant	❑

This kit is designed for six (6) groups sharing three polyacrylamide gels.

REAGENTS & SUPPLIES
Store all components below at room temperature.

- Tris-glycine-SDS buffer (10x) ❑
- FlashBlue™ Protein Stain Powder ❑
- Practice gel loading solution ❑
- Transfer pipets ❑

Experiment Requirements *(NOT included with this experiment)*

- Vertical electrophoresis apparatus (EDVOTEK® Cat. #581 highly recommended)
- D.C. power supply
- Precast 12% SDS polyacrylamide gels (3)
- Micropipette and tips (Cat #638 Fine Tip Micropipette Tips recommended)
- Microwave or Hot plate
- Distilled or deionized water
- Beakers
- Aluminum foil or foam water bath float
- White Vinegar
- Ethanol (95% or higher)
- 750 mL or 1 L flask or beaker
- Small plastic tray or large weigh boat
- Plastic wrap
- White light box (recommended)
- Rocking platform (recommended)

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Background Information

Proteins are a highly diversified class of biomolecules. Differences in their chemical properties, such as charge, shape, size and solubility enable them to perform many biological functions. These functions include enzyme catalysis, metabolic regulation, binding and transport of small molecules, gene regulation, immunological defense and cell structure.

Cellular differentiation within an organism is determined by selective expression of genes. Consequently, the kinds of proteins present and their concentrations vary between different tissues. The protein profile within the same tissue can also vary with time and by specific induction of gene transcription with hormones and other chemicals. The majority of cellular proteins remain the same between various mammalian tissue types. The greatest variance in the types of proteins expressed between two different tissues is about 100 fold. However, the concentration of proteins common to different tissues may vary considerably.

The eukaryotic cells of mammals can contain 5,000 to 10,000 different proteins and a total of 5×10^9 protein molecules per cell. The average length of a gene is about 1200 base pairs and the number of base pairs in the haploid human genome is 3×10^9. Theoretically, 2.4×10^6 different proteins can be encoded. However, less than 1% of the maximum is actually used for this purpose. Many DNA sequences in eukaryotic cells do not code for proteins, such as introns and repetitious sequences, rRNA and tRNA genes, transcriptional regulatory units and pseudo genes. By contrast, *E. coli* contains approximately 2000 different proteins and a total of 3 x 106 protein molecules per cell. There are 4 x 106 base pairs in the *E. coli* genome, theoretically coding for 3,300 different proteins. Over 60% of the genome actually encodes protein. The control of gene expression in prokaryotes is in response to immediate nutritional requirements and adaptations to changes in the physical environment. These features are in accordance with their short life cycle and rapid growth kinetics. The majority of cells in multicellular organisms remain in a stringently regulated constant environment. Gene control in this case deals mainly with differentiation and development, and is generally irreversible. Reversibly inducible genes do occur in higher organisms such as liver cells.

NOTES:
"!" is used to represent factorial. For example, n! is equal to n (n-1) (n-2) (n-3)...(1).

Sequence isomerism is the major reason for the great structural and functional diversity of proteins. A tetrapeptide having four different amino acids has 4!=24 sequence isomers. A small polypeptide consisting of 20 each of the standard amino acids has $20! = 2 \times 10^{18}$ different sequence isomers. A moderately sized protein of molecular weight 34,000, consisting of only 12 of the standard amino acids, has 10^{300} possible isomers. The sequence variations provide a virtually unlimited set of polypeptides. A protein can have a net negative or a net positive charge, depending on its amino acid composition and the pH. At certain values of pH, the molecule can be electrically neutral overall, i.e. negative and positive charges are balanced. In this case, the protein is isoelectric. In the presence of an electrical field, a protein with a net charge will migrate towards the electrode of opposite charge.

Proteins exhibit many different three-dimensional shapes and folding patterns which are determined by their amino acid sequence and intracellular processing. The precise three-dimensional configuration of a protein is critical to its function. Proteins have spherical, elliptical or rodlike shapes. The molecular weight is a function of the number and type of amino acids in the polypeptide chain. Proteins can consist of a single polypeptide or several polypeptides specifically associated with each other. Proteins that are in their normal, biologically active forms are called native.

POLYACRYLAMIDE GEL COMPOSITION

The properties of proteins affect the way they migrate during gel electrophoresis. Gels used in electrophoresis (e.g. polyacrylamide) consist of microscopic pores of a defined size range that act as a molecular sieve. Only molecules with net charge will migrate through the gel when it is in an electric field. Small molecules pass through the pores more easily than large ones. Molecules having more charge than others of the same shape and size will migrate faster. Molecules of the

same mass and charge can have different shapes. In this case, those with a more compact shape, like a sphere, will migrate through the gel more rapidly than those with an elongated shape, like a rod. In summary, the charge density and charge, the size and shape of a native protein all affect its electrophoretic migration rates. Electrophoresis of native proteins is useful in the clinical and immunological analysis of complex biological fluids, such as serum.

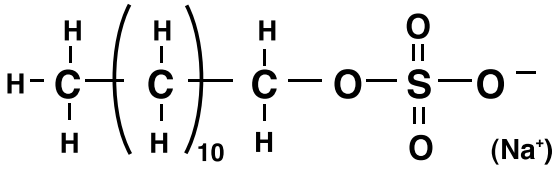


Figure 1:
The chemical structure of sodium dodecylsulfate (SDS).

Sodium dodecylsulfate (SDS) is a detergent which consists of a hydrocarbon chain bonded to a highly negatively charged sulfate group as shown in Figure 1. SDS binds strongly to most proteins and causes them to unfold to a random, rodlike chain. No covalent bonds are broken in this process. Therefore, the amino acid composition and sequence remains the same. Since its specific three-dimensional shape is abolished, the protein no longer possesses biological activity. Proteins that have lost their specific folding patterns and biological activity but have their polypeptide chains remaining intact are called denatured. Proteins which contain several polypeptide chains that are associated only by non-covalent forces will be dissociated by SDS into separate, denatured polypeptide chains. Proteins can contain covalent crosslinks known as disulfide bonds. These bonds are formed between two cysteine amino acid residues that can be located in the same or different polypeptide chains. High concentrations of reducing agents, such as β-mercaptoethanol, can break disulfide bonds. This allows the SDS to completely dissociate and denature the protein. Proteins that retain their disulfide links bind less SDS, causing anomalous migration. Figure 2 illustrates these ideas with a protein containing two differently sized polypeptide chains that are cross-linked by a disulfide bond. The chains are also associated by non-covalent forces. The circles represent the native structure.

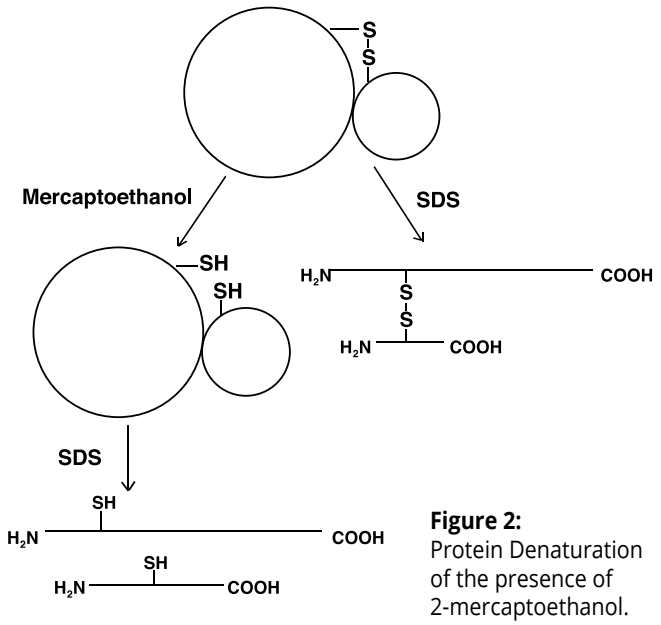


Figure 2:
Protein Denaturation of the presence of 2-mercaptoethanol.

In most cases, SDS binds to proteins in a constant ratio of 1.4 grams of SDS per gram of protein. On average, the number of bound SDS molecules is half the number of amino acid residues in the polypeptide. The negative charge due to SDS is much more than the negative and positive charges of the amino acid residues. The large quantity of bound SDS efficiently masks the intrinsic charges in the protein. Consequently, SDS denatured proteins are net negative and since the binding of the detergent is proportional to the mass of the protein, the charge to mass ratio is constant. In addition, the shapes of SDS denatured proteins are the same (rodlike). The size of the rodlike chains is the only gross physical difference between SDS denatured proteins. The larger the molecular weight of the protein the longer the rodlike chain. The pores in the gel distinguish these size differences. During SDS electrophoresis, protein migrates through the gel towards the positive electrode at a rate that is inversely proportional to their molecular weight. In other words, the smaller the protein, the faster it migrates. The molecular weight of the "unknown protein" is obtained by the comparison of its position after electrophoresis to the positions of standard SDS denatured proteins electrophoresed in parallel. The molecular weights of the standard proteins have been previously determined. After the proteins are visualized by staining, their migration distance is measured. The $\log_{10}$ of the molecular weights of the standard proteins are plotted versus their migration distance. Taking the logarithm or the R_f allows some of the data to be plotted as a straight line. The molecular weight of an unknown protein is then calculated from the standard curve.

The electrophoretic mobility of the proteins is also affected by the gel concentration. Higher percentage gels are more suitable for the separation of smaller proteins and peptides. Polyacrylamide gels are formed by mixing the monomer, acrylamide; the cross-linking agent, methylenebisacrylamide; and a free radical generator, ammonium persulfate in aqueous buffer (Figure 3). Free radical polymerization of the acrylamide occurs. At various points the acrylamide polymers are bridged to each other by methylenebisacrylamide.

It should be noted that acrylamide is a neurotoxin and can be absorbed through the skin. However, in the polymerized polyacrylamide form it is non-toxic. The polymerization process is inhibited by oxygen. Consequently, polyacrylamide gels are usually prepared between two glass plates separated by strips called spacers. As the liquid acrylamide polymerization mixture is poured between the plates, air is displaced and polymerization proceeds more rapidly.

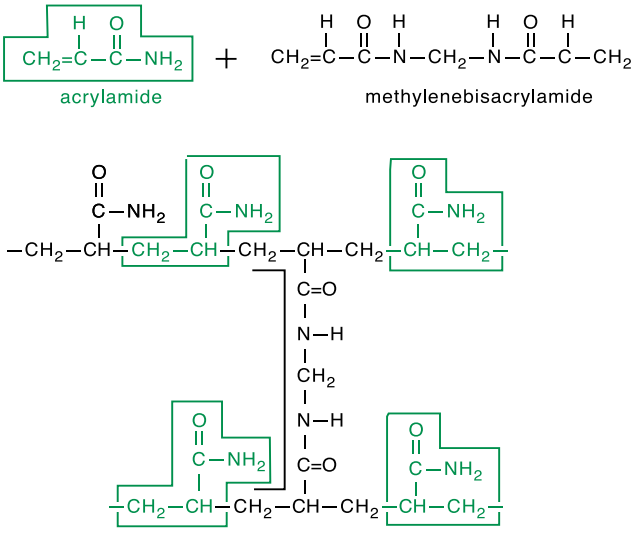


Figure 3:
Polymerization of polyacrylamide.

SURVEY OF PROTEIN SAMPLES

Standard Protein Markers are a mixture of proteins that give the following denatured molecular weights: 94,000; 67,000; 38,000; 30,000; 20,000; and 14,000 Da. The denatured values have been rounded off for convenience in graphical analysis.

Milk Proteins

The major milk proteins are caseins which, with emulsified lipids, gives the liquid its color. In milk the caseins are complexed with calcium which causes them to form aggregates and micelles. The casein fraction can be precipitated from skimmed (defatted) milk by acid titration to pH 4.7. The fat and caseins can also be removed by salting out with ammonium sulfate. The resulting green-yellow supernatant is whey, which contains 20% of the total milk protein. The whey fraction is a filtered derivative of serum. It contains small amounts of albumin, transferrin and lactoferrin. These proteins may be visualized as faintly staining bands between the 67,000 and 94,000 markers. Transferrin binds and transports iron to the various tissues from the blood plasma and is presumably a source of iron for the nursing young. In the presence of CO_2 transferrin binds 2 Fe^{+3} atoms per molecule. As are most excreted proteins, transferrin is glycosylated and consists of a single polypeptide chain of approximately 80,000 daltons. There are at least 20 different variants of this protein in humans.

Immunoglobulins comprise 10% of the whey proteins. The secretory immunoglobulin IgA predominates. The basic configuration of the immunoglobulins are 2 "light" polypeptide chains of molecular weight 26,000 and 2 "heavy" chains of molecular weight 54,000 to 75,000 depending on the class. Together these chains form a flexible Y-shaped molecule containing numerous intra and interchain disulfide bonds. One disulfide link occurs between each pair of light and heavy chains. Two disulfide links connect the heavy chains with each other. The N-terminal region of both chains are of variable sequence from one molecule to another. Consequently, the immunoglobulin fraction of normal plasma and milk is heterogeneous. The variable regions of both types of chains form the antigen binding site; there are two per molecule. The remaining portions of the light and heavy chains are of constant sequence (within their respective subclasses) from one immunoglobulin molecule to another.

The major whey protein in ruminants is β-lactoglobulin. The native protein has a molecular weight of 35,000 and consists of two identical polypeptide chains. The protein appears as a single band of molecular weight 17,500, just under the 20,000 dalton marker. β-lactoglobulin binds folic acid which is the precursor of tetrahydrafolate, an important coenzyme in metabolic carbon transfer.

α-lactalbumin is a single polypeptide having a molecular weight of approximately 15,000 daltons. It appears as a faint

band near the lowest molecular weight marker. This whey protein forms a one-to-one complex with mammary galactosyl transferase. The free enzyme (which occurs in many other tissues) catalyzes the transfer of the galactose unit of UDP-galactose to terminal N-acetylglucosamine residues on the oligosaccharide chains of glycoproteins. When complexed with α-lactalbumin the enzyme substrate specificity is changed to accept glucose as the galactosyl acceptor, resulting in the production of the milk sugar lactose. The α-lactalbumin has no catalytic activity itself but is a specific protein modifier. The galactosyltransferase-α-lactalbumin complex is called lactose synthase.

Blood Serum Proteins

Blood plasma is thought to contain over 100 different proteins. SDS polyacrylamide gel electrophoresis are useful methods for the fractionation and analysis of these proteins, particularly in clinical tests. The SDS electrophoretic profile of the plasma proteins will reveal bands ranging from approximately 200,000 to 15,000 daltons. The largest (top) band in the plasma sample has a denatured molecular weight of 190,000 and corresponds to α2-macroglobulin. The native molecular weight of the protein is approximately 800,000. It consists of two dimer subunits associated with each other through non-covalent forces. However, the dimer subunits consist of two polypeptides (190,000) that are associated by non-covalent forces and disulfide bonds. Macroglobulin is a protease inhibitor and may be involved with the control of proteolytic processes such as blood coagulation and complement cascades. Transferrin is a major plasma protein, comprising 3% of the total protein. Transferrin is a major band in the SDS electrophoretic profile, migrating with or just beneath the 94,000 molecular weight marker.

The major band of the plasma proteins is due to albumin, with a molecular weight of approximately 68,000. It is directly under the transferrin band. Albumin is the most abundant plasma protein and is one of the few that is not a glycoprotein. It consists of a single polypeptide chain with 17 interchain disulfide bonds. As in the immunoglobulins, the disulfide links help the albumin fold into three structural domains, each consisting of three sub-domains. These domains form the wide variety of ligand binding sites found in the protein. Albumin functions in the binding and transport of fatty acids, Cu^{+2}, Ni^{+2}, bilirubin, tryptophan, steroid hormones, and many drugs such as sulfonamides, penicillin and aspirin. A major physiological role of albumin is in osmotic regulation. The protein is responsible for 80% of the osmotic properties of plasma since it is more than half the plasma proteins by weight, has the lowest molecular weight of the major proteins and contains 18 negative charges at physiological pH. The charge effects the distribution of sodium and chloride ions in the extracellular fluids and consequently, plasma osmolarity. Below the albumin, there are several partially resolved bands that have mobilities between 67,000 and 43,000, as judged by the markers. The heavy chain family of the immunoglobulins (mostly IgG subclasses) migrate in this region in addition to α-antitrypsin (53,000) which is a relatively abundant protein involved with proteolytic inhibition. It consists of 12% carbohydrate by weight. Abnormally low levels of antitrypsin may cause a predisposition to emphysema since lung cells are damaged by proteolysis.

The haptoglobins are multisubunit proteins with native molecular weights ranging from 100,000 to 400,000. The haptoglobins consist of two pairs of different polypeptide chains, α and β, having molecular weight of 9,000 and 43,000 respectively. The polypeptide chains are joined to each other by disulfide bonds and the protein has the subunit stoichiometry a_2b_2.

The haptoglobins form a 1:1 complex with hemoglobin. The bound hemoglobin cannot be excreted by the kidneys or cause damage to them. The complexes are destroyed by the reticuloendothelial system and the iron of the heme group is salvaged and reused in heme biosynthesis. Plasma hemoglobin arises from the intravascular hemolysis of red blood cells.

Egg White Proteins

The SDS electrophoretic profile of egg white proteins also reveals a high degree of specialization. Egg white proteins are secreted by the cells of the oviduct under hormonal stimulation. The majority of egg white protein consists of ovalbumin. The protein consists of a single globular polypeptide chain having a molecular weight of 45,000. Ovalbumin contains a short oligosaccharide covalently linked to an asparagine residue. The oligosaccharide consists of N-acetylglucosamine and mannose. Ovalbumin functions as a storage form of amino acids for the developing embryo. Egg white also contains globulins which are represented by a prominent band corresponding to a molecular weight of approximately 65,000 to 68,000. Very

faint bands may be observable above the globulin band at a molecular weight of near 80,000 corresponding to conalbumin. This protein is the functional equivalent of transferrin. Lysozyme is an enzyme that degrades the polysaccharide chains in bacterial cell walls, predisposing the cell to lysis. The enzyme is a single polypeptide with a molecular weight of approximately 14,500. Egg white contains substantial amounts of this protein which can be observed as a band co-migrating with the lowest molecular weight marker. There are several functional similarities between egg white and milk protein. Both contain iron transport proteins. They have large amounts of "nutritional" proteins (caseins in milk and ovalbumin in egg) and contain antibacterial surveillance (immunoglobulins in milk and lysozyme in egg). These features satisfy several requirements for the developing young.

Plant Proteins

Spinach leaf proteins reveal a complex pattern of bands after electrophoresis, particularly in the lower molecular weight range. Many of these proteins are from the numerous chloroplasts that are found in the leaf tissue of higher plants. A prominent feature is a major band at a molecular weight of approximately 56,000. This band is due to the enzyme ribulose-1, 5-biphosphate carboxylase. This enzyme catalyzes the addition of CO_2 to the phosphopentose ribulose biphosphate producing two molecules of the phosphotriose 3-phosphoglycerate. This reaction is responsible for the fixation of carbon dioxide in the dark reactions of photosynthetic organisms. The enzyme is the most abundant protein in plants and the biosphere. The enzyme is responsible for the annual incorporation of 5×10^{14} kilograms of carbon dioxide into organic linkage. The carboxylase is present in the stroma of chloroplasts and contains multiple subunits. The 56,000 polypeptide is termed L, and it is encoded by chloroplast DNA. There is also a small subunit with a molecular weight of 14,000, termed S, which is coded in the nucleus. The band containing the S polypeptide co-migrates with the smallest molecular weight marker in the SDS gel. The subunit stoichiometry is L_8S_8, giving a native molecular weight of 560,000. The active sites are located on the L subunits. The S subunit is thought to regulate the enzymes activity but this has not been clearly demonstrated. Magnesium ion is required for catalytic activity. The carboxylase's activity is regulated by a chloroplast enzyme which catalyzes the addition of carbon dioxide to specific lysine residues in the L subunits. The modification activates the carboxylase.

In this experiment, the protein samples have been denatured by incubation with the anionic detergent sodium dodecyl sulfate (SDS) and will be submitted to polyacrylamide gel electrophoresis. Under the experimental conditions, the proteins will have a mobility in the gel that is inversely proportional to the logarithm of their molecular weights.

Proteins of known molecular weights will be electrophoresed in parallel. The protein samples contain buffer, SDS, β-mercaptoethanol as a reducing agent for disulfide bonds, glycerol to create density greater than that of the electrode buffer and the negatively charged tracking dye bromophenol blue. The tracking dye will migrate ahead of the smallest proteins in these samples toward the positive electrode (bottom).

The molecular weight estimates obtained from SDS polyacrylamide gel electrophoresis are of denatured proteins. Since proteins often consist of multiple subunits (polypeptide chains) the method can give minimum subunit molecular weights. Incubation of SDS and β-mercaptoethanol causes disruption of cell membranes and lysis. Clarification of the sample is done by centrifugation and the supernatant is used for electrophoresis.

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of the experiment is to use SDS-polyacrylamide gel electrophoresis to develop an understanding of protein structure, function and diversity.

LABORATORY SAFETY:

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.

Wear gloves and safety goggles

Acrylamide is a known neurotoxin and carcinogen and should be handled with extreme caution. Liquid acrylamide, used in the manufacture of SDS-PAGE gels, should only be handled in a chemical fume hood while wearing gloves and goggles. Polymerized acrylamide, including precast acrylamide gels, is safe but should still be handled with caution at all times.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

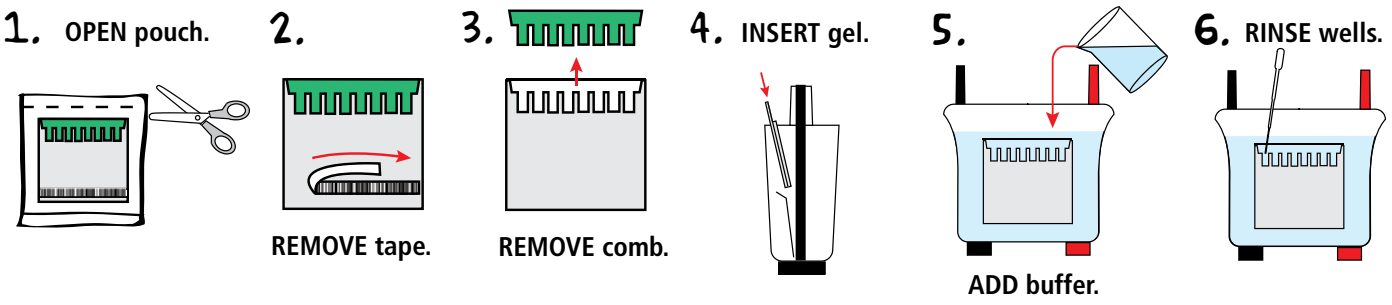
During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I-A: Preparing Precast Polyacrylamide Gels For Electrophoresis

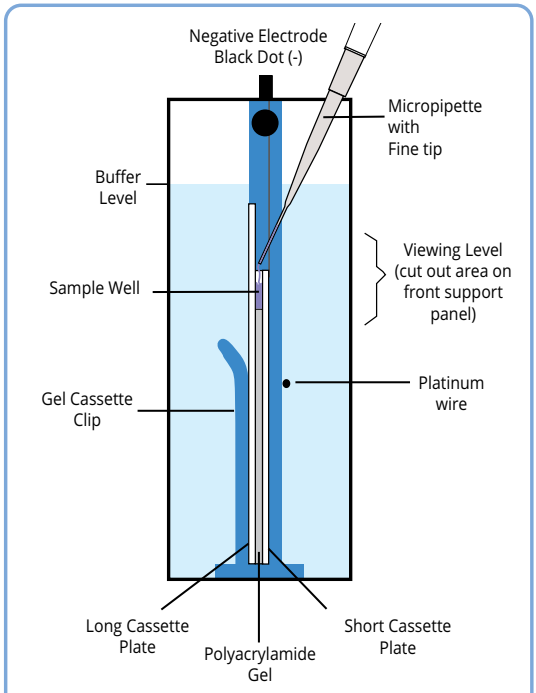


PREPARING THE POLYACRYLAMIDE GEL AND CHAMBER

NOTE: Although precast polyacrylamide gels and protein chambers will vary slightly in design, the procedure for their use will be similar.

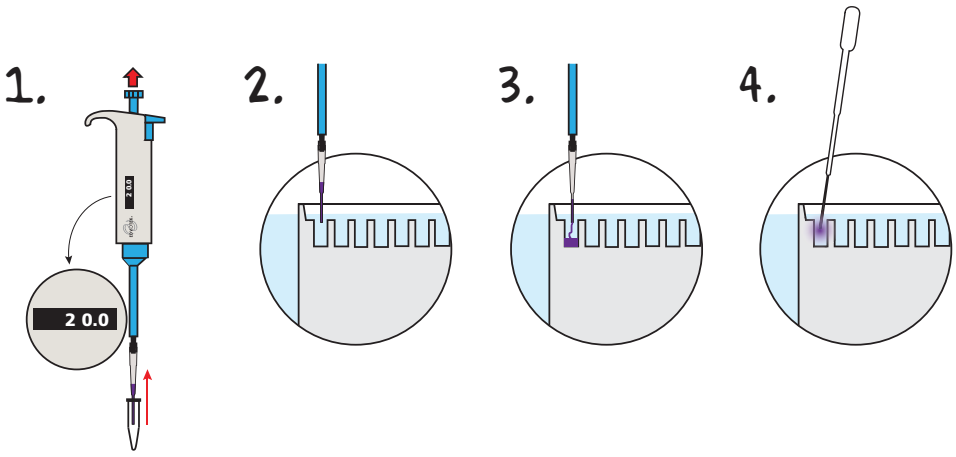
1. **OPEN** the pouch containing the gel cassette. Remove the cassette and place on bench with the shorter front plate facing up.
2. Gels may feature a sticker or tape at the bottom of the front plate. **REMOVE** the tape (if present) to expose the bottom of the gel.
3. Carefully **REMOVE** the comb by gently pulling upwards. Pull the comb straight up to prevent damage to the wells of the gel.
4. **INSERT** the gel into the electrophoresis chamber. Orient the gel according to the manufacturer's instructions. ***NOTE: For EDVOTEK® vertical electrophoresis chambers, the short plate should face the middle of the apparatus.***
5. **ADD** diluted electrophoresis buffer to the chamber. The buffer should cover the top of the shorter plate.
6. **RINSE** each well by squirting electrophoresis buffer into the wells using a transfer pipet. Using the transfer pipet, carefully straighten any wells which may have been distorted during comb removal or rinsing.

The gel is now ready for practice gel loading.



A polyacrylamide gel cassette in the EDVOTEK® Vertical Electrophoresis Apparatus, Model #MV10.

Module I-B: Practice Gel Loading



NOTE: EDVOTEK® Cat. #638, Fine Tip Micropipette Tips are recommended for loading samples into polyacrylamide gels. A regular microtip may damage the cassette and result in the loss of protein samples.

Wear gloves and safety goggles

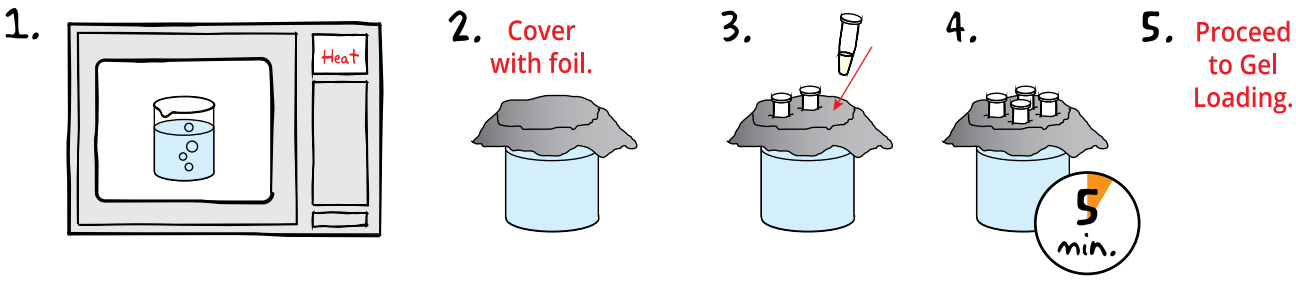
1. **PLACE** a fresh tip on the micropipette. **REMOVE** 20 µL of practice gel loading solution.

2. **PLACE** the lower portion of the pipette tip below the surface of the electrode buffer, directly over a sample well. The tip should be at an angle pointed towards the well. The tip should be partially against the back plate of the gel cassette, but the tip opening should be over the sample well. **Do not try to jam the pipette tip in between the plates of the gel cassette.**

3. **EJECT** all the sample by steadily pressing down on the plunger of the automatic pipette. Do not release the plunger before all the sample is ejected. Premature release of the plunger will cause buffer to mix with sample in the micropipette tip. Release the pipette plunger after the sample has been delivered and the pipette tip is out of the buffer.

4. **REMOVE** the practice gel loading solution from the sample wells. **FILL** a transfer pipet with buffer and **SQUIRT** a stream into the sample wells. This will displace the practice gel loading solution, which will be diluted into the buffer and will not interfere with the experiment. ***NOTE: Practice gel loading solution must be removed from the sample wells prior to sample loading.***

Module II: Performing SDS-PAGE with Protein Samples



PROTEIN DENATURATION:

NOTE: PROCEED to gel loading if your lab instructor has already heated the protein samples.

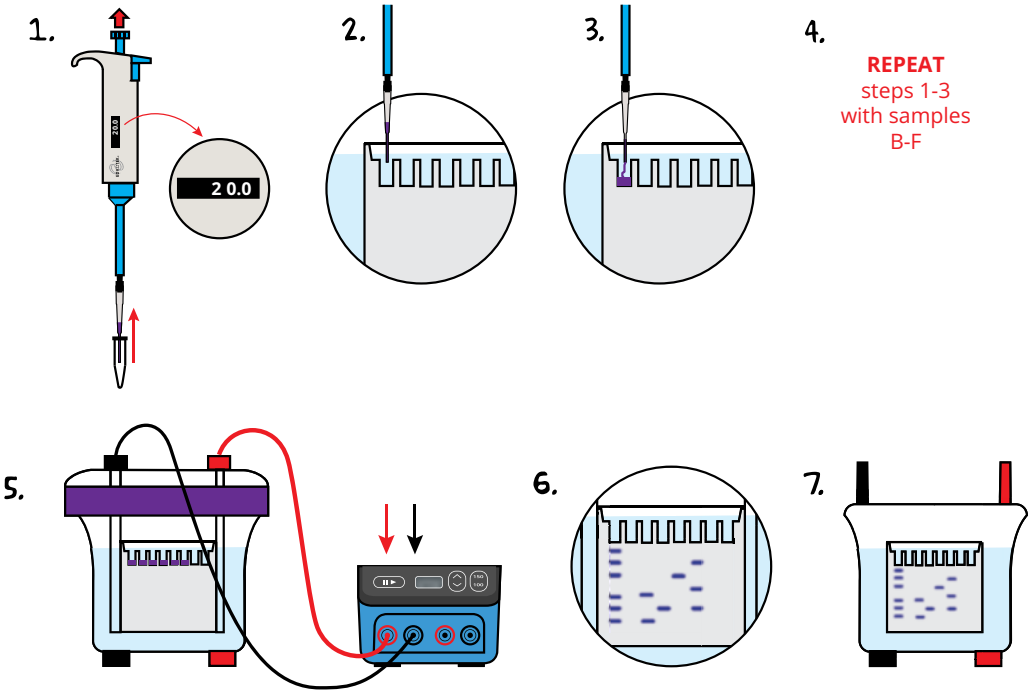
1. Using a hot plate or microwave, **HEAT** a beaker of water until it boils.
2. **COVER** with aluminum foil and carefully remove from heat.
3. Tightly **CAP** sample tubes. **PUSH** tubes through foil to suspend in the boiling water.
4. **INCUBATE** the samples for 5 minutes.
5. Immediately **PROCEED** to loading the gel while the samples are still warm. (For loading, the samples can be aliquoted into individual microcentrifuge tubes or placed at a classroom pipetting station for students to share.)

Wear gloves and safety goggles

FREEZING PROTEINS:
Unused portions of the protein samples can be frozen for later use. When needed, repeat steps 1-4 and proceed to gel loading.

Samples must be boiled in screw top microcentrifuge tubes!

Module II: Performing SDS-PAGE on Protein Samples, continued



LOADING THE PROTEIN SAMPLES:

1. Using a fresh pipette tip, **MEASURE** 20 µL of the Standard Protein Marker (A).
2. **PLACE** the pipette tip under the buffer and directly above the sample well, resting gently against the back plate of the gel cassette.
3. Slowly **DISPENSE** the sample by depressing the plunger.
4. **REPEAT** steps 1-3 with protein samples B-E, changing the tip between each new sample.
5. Once all samples have been loaded, carefully **PLACE** the cover onto the electrode terminals and **CONNECT** the electrical leads to the power supply.
6. **SET** the voltage of the power supply and **PERFORM** electrophoresis (See Table A for time and voltage guidelines). Allow the proteins to separate on the gel for the recommended length of time, or until the tracking dye reaches the bottom of the gel.
7. **TURN OFF** the power supply and carefully **REMOVE** the lid. The gel can now be removed from the chamber and stained.

Table 1: Gel Loading

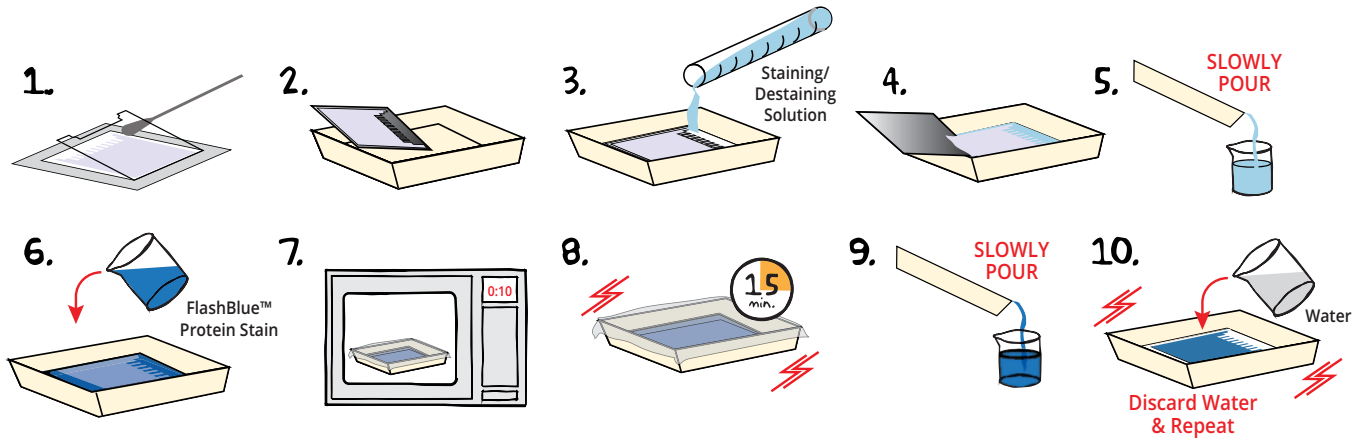
Lane	Tube	Sample
1	A	Standard Protein Markers (Group A)
2	B	Milk whey proteins (Group A)
3	C	Serum proteins (Group A)
4	D	Egg white proteins (Group A)
5	E	Spinach leaf proteins (Group A)
6	A	Standard Protein Markers (Group B)
7	B	Milk whey proteins (Group B)
8	C	Serum proteins (Group B)
9	D	Egg white proteins (Group B)
10	E	Spinach leaf proteins (Group B)

Table A Time and Voltage Guidelines

	Recommended Time	
Volts	Minimum	Optimal
100	80 min.	95 min.
125	60 min.	75 min.
150	50 min.	60 min.

Module III: Gel Staining with FlashBlue™ Protein Stain

Although the protein samples are provided in a pre-stained format it is possible to increase the intensity of the bands by using FlashBlue™ Protein Stain. Staining is rapid and sensitive. Student groups that shared a polyacrylamide gel during electrophoresis should also stain this gel together.



1. After electrophoresis, **LAY** the cassette down and **REMOVE** the front plate by placing a thin spatula or screwdriver at the side edge and gently lift it away from the larger back plate. In most cases, the gel will stay on the back plate. If it partially pulls away with the front plate, let it fall onto the back plate. ***Handle very carefully as the thin gels are extremely fragile.***

2. **TRANSFER** the gel on the back plate to a clean tray.

3. **ADD** a sufficient volume (approximately 50-75 mL) of the staining/destaining solution into the tray to **COVER** the gel and back plate.

4. Carefully **REMOVE** the back plate from the tray, leaving just the gel in the tray containing the staining/destaining solution. Bands may be easier to see once the cassette is removed. **OBSERVE** the gel and take a photo/sketch the banding pattern in your notebook before continuing. ***NOTE: If the gel sticks to the plate, gently nudge the gel off the plate using two GLOVED fingers.***

WEAR GLOVES AND SAFETY GOGGLES

Gloves must be worn during this procedure. Avoid touching the gel without gloves.

Polyacrylamide gels are very thin and fragile. Use care in handling to avoid tearing the gel.

5. **DISCARD** the staining/destaining solution. ***Pour slowly to keep the gel in the container.***

6. **ADD** 30 mL of prepared FlashBlue™ Protein Stain.

7. (OPTIONAL) **COVER** the container with plastic wrap and **MICROWAVE** for 10 seconds to gently heat the solution.

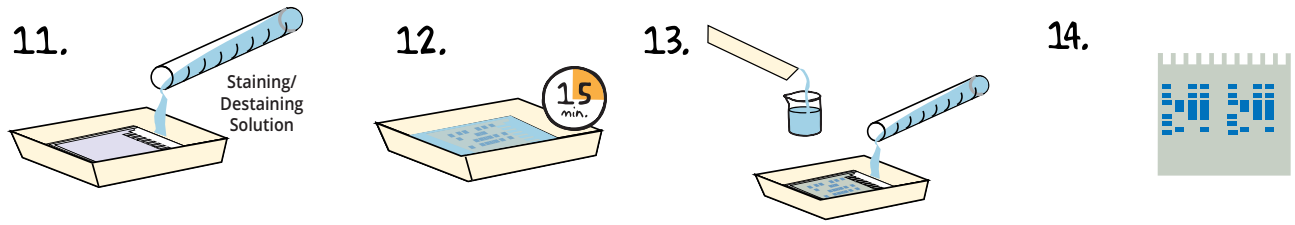
8. **INCUBATE** for 15 minutes at room temperature, **SHAKING** occasionally.

9. **DISCARD** the FlashBlue™ Protein Stain solution. ***Pour slowly to keep the gel in the container.***

10. **WASH** the gel by partially filling container with water and gently rocking back and forth several times. **DISCARD** the used water and **REPEAT** with fresh water.

continued

Module III: Gel Staining with FlashBlue™ Protein Stain, continued



11. **ADD** 30 mL of staining/destaining solution to the gel.

12. **INCUBATE** for 15 minutes at room temperature. **EXAMINE** the gel.

13. (OPTIONAL) **DISCARD** the used staining/destaining solution and **ADD** an additional 30 mL of staining/destaining solution. **INCUBATE** for 15-60 minutes at room temperature until the appearance and contrast of the protein bands against the background improves.

14. After staining, protein bands will appear medium to dark blue against a light background. A white light box can be used to better visualize the protein bands. **OBSERVE** and/or **PHOTOGRAPH**.

STORING THE GEL

- Gel may be left in deionized water for several hours with no loss in sensitivity and band intensity. This step should be performed once a desired background and stained protein bands are obtained. Pour off the destaining solution from Step 12 (or 13) and add a sufficient amount of deionized water to cover the gel.

- For permanent storage, the gel can be dried between two sheets of cellophane (saran wrap) stretched in an embroidery hoop. Air dry the gel for several days until the gel is paper thin. Cut the "extra" saran wrap surrounding the dried gel. Place the dried gel overnight between two heavy books to avoid curling. Tape it into a laboratory book.

Study Questions

1. Would changes occur in the SDS electrophoretic profiles of serum proteins treated in the absence or presence of high concentrations of β-mercaptoethanol? If so, would proteins show differences in migration rates?

2. The α chain of haptoglobin exhibits genetic polymorphism since glutamic acid can be replaced by lysine (a^{1S} and a^{1F}). Which method could potentially distinguish the polymorphs, SDS or native polyacrylamide gel electrophoresis?

3. An SDS lysate of red blood cells was submitted to SDS polyacrylamide gel electrophoresis. The sample revealed a major band with a molecular weight of approximately 17,000. What protein do you think is responsible for this band?

Instructor's Guide

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Prior to starting this experiment, carefully check the list of components and requirements on page 3 to ensure you have all the necessary components and equipment.

This experiment requires three 12% polyacrylamide gels to be shared by the 6 student groups. Each group requires 5 sample wells.

Preparation For:	What to do:	When:	Time Required:
Module I: Preparing Precast Polyacrylamide Gels for Electrophoresis	Prepare diluted electrophoresis buffer	Up to one day before performing the experiment.	15 min.
	Rehydrate and aliquot protein samples	Up to one day before performing the experiment and stored at -20˚ C.	15 min.
Module II: Performing SDS-PAGE on Protein Samples	Prepare waterbaths for denaturing proteins	Up to one day before performing the experiment.	15 min.
	Denature proteins (optional)	No more than 10 min. before performing the experiment.	10 min.
Module III: Gel Staining with FlashBlue™ Protein Stain	Prepare staining solutions	Anytime before performing the experiment.	10 min.

Pre-Lab Preparations

PREPARING PROTEIN SAMPLES

1. Add 130 µL of distilled or deionized water to each tube (A-E) and allow the samples to hydrate for several minutes. Vortex or flick tube vigorously to mix. Resuspended proteins may be kept at room temperature for immediate use or frozen until needed.
2. The protein samples must be heated in their original 1.5 ml screw-top microcentrifuge tubes before use. This step can be completed by laboratory instructors immediately before the lab period or it can be performed by the students during the lab period. For instructions on denaturing the protein samples please refer to Module II, page 12.
3. Samples can be aliquoted for each of the 6 student groups, or students can share the rehydrated sample stock tubes. Have students load samples onto the polyacrylamide gel while the samples are still warm to avoid aggregation. The volume of sample to load per well is 20 µL.
4. Store any unused portion of reconstituted sample at -20° C and repeat step 2 when using samples at a later time.

PREPARING ELECTROPHORESIS BUFFER

Prepare the electrophoresis buffer by adding and mixing 1 part Tris-Glycine-SDS 10x buffer concentrate to 9 parts distilled water.

The approximate volume of 1x electrophoresis buffer required for EDVOTEK Protein Vertical Electrophoresis units are listed in Table B. The buffer should just cover the back plate of the gel cassette.

Table B — Tris-Glycine-SDS Electrophoresis (Chamber) Buffer

EDVOTEK Model #	Total Volume Required	Concentrated Buffer (10x)	+ Distilled Water
MV10	580 mL	58 mL	522 mL
MV20	950 mL	95 mL	855 mL

ELECTROPHORESIS TIME AND VOLTAGE

Your time requirements will dictate the voltage and the length of time it will take for your samples to separate by electrophoresis. Approximate recommended times are listed in Table A.

Run the gel until the bromophenol blue tracking dye is near the bottom edge of the gel.

Table A — Time and Voltage Guidelines

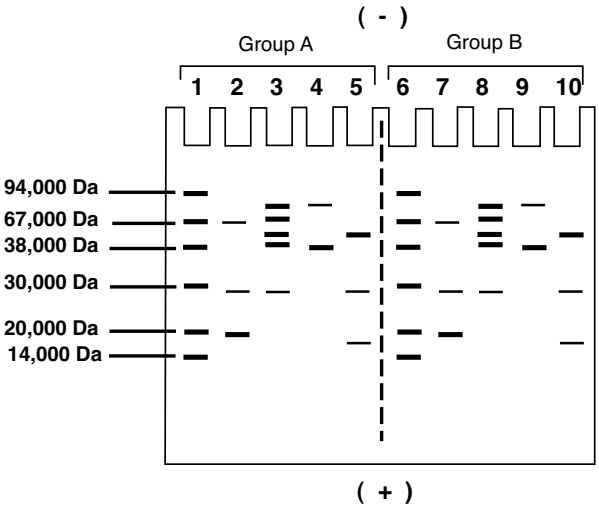
	Recommended Time	
Volts	Minimum	Optimal
100	80 min.	95 min.
125	60 min.	75 min.
150	50 min.	60 min.

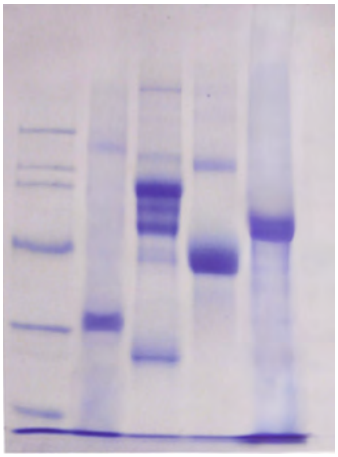
PREPARATION FOR STAINING GELS

1. Prepare a stock solution of white vinegar and ethanol* by combining 400 mL white vinegar with 200 mL ethanol. Gently mix. Label as "Staining/Destaining Solution".
2. Add 125 mL of the Staining/Destaining Solution to a 250 mL flask or beaker. Add the entire contents of the FlashBlue™ Protein Stain powder and briefly stir or shake to mix. Residual powder can be rinsed from the tube using an additional 1 mL of Staining/Destaining Solution.
3. Store both solutions at room temperature until needed.
4. TWO student groups will share: 30 mL FlashBlue™ Protein Stain, 140 mL Staining/Destaining Solution, water, a staining tray, and plastic wrap.

**White vinegar, sometimes called distilled or spirit vinegar, is an easy to find cooking and cleaning vinegar with an acetic acid concentration between 5-8% and a pH ~2.6. Ethanol is a common lab supply which is available at various concentrations. Our FlashBlue™ Protein Stain has been designed to work with a wide range of white vinegars. However, we do recommend using 95% Ethanol or higher.*

Experiment Results and Analysis





The figure on the left is an idealized schematic showing relative positions of the protein bands. Actual results will yield broader bands of varying intensities. The idealized schematic shows the relative positions of the bands, but are not depicted to scale.

Lanes	Tube	
1, 6	A	Standard Protein Markers
2, 7	B	Milk Whey Proteins
3, 8	C	Serum Proteins
4, 9	D	Egg White Proteins
5, 10	E	Spinach Leaf Proteins

Please refer to the kit insert for the Answers to Study Questions

Appendices

A Size Determination of Unknown Proteins

B EDVOTEK® Troubleshooting Guide

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Appendix A

Size Determination of Unknown Proteins

	Migration Distance (cm)	Molecular Weight (daltons)
Marker Protein 1 (Phosphorylase)		94,000
Marker Protein 2 (Bovine Serum Albumin)		67,000
Marker Protein 3 (Ovalbumin)		38,000
Marker Protein 4 (Carbonic anhydrase)		30,000
Marker Protein 5 (Soybean Trypsin Inhibitor)		20,000
Marker Protein 6 (Lysozyme)		14,000
Protein Sample 1		
Protein Sample 2		
Protein Sample 3		
Protein Sample 4		

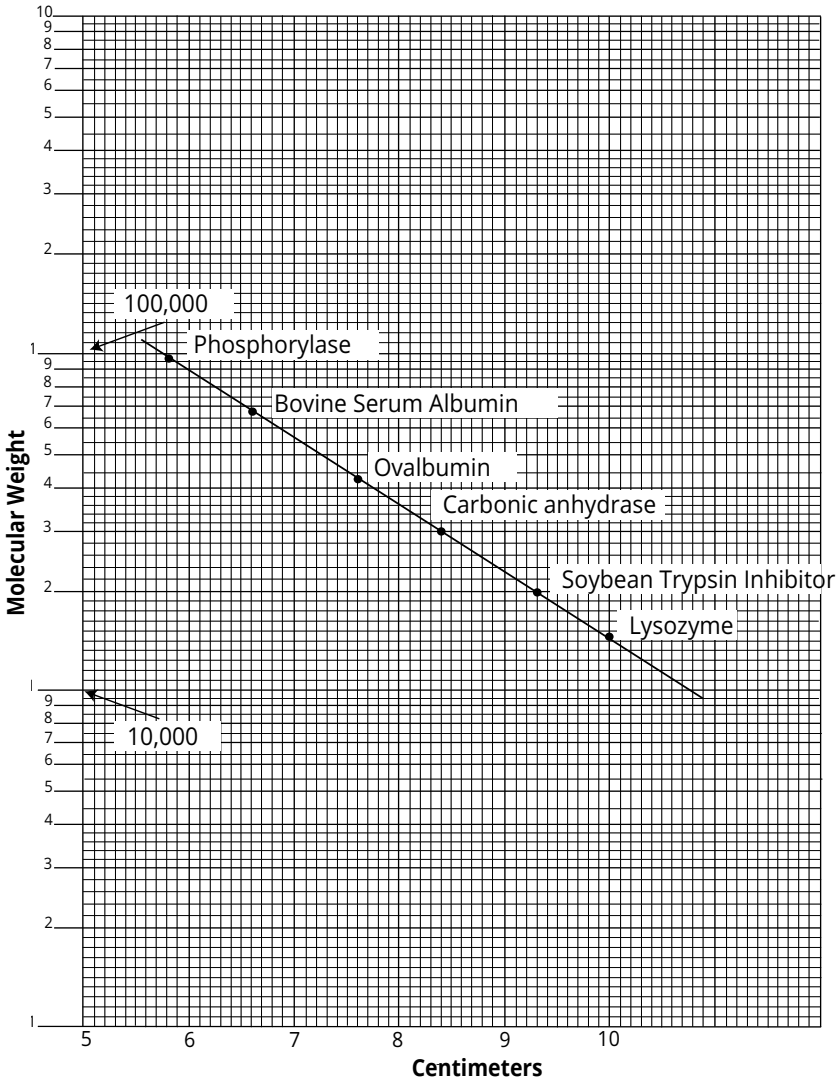
1. **MEASURE** the migration distance, in centimeters (to the nearest millimeter) of every major band in the gel. All measurements should be from the bottom of the sample well to the bottom of the protein band.

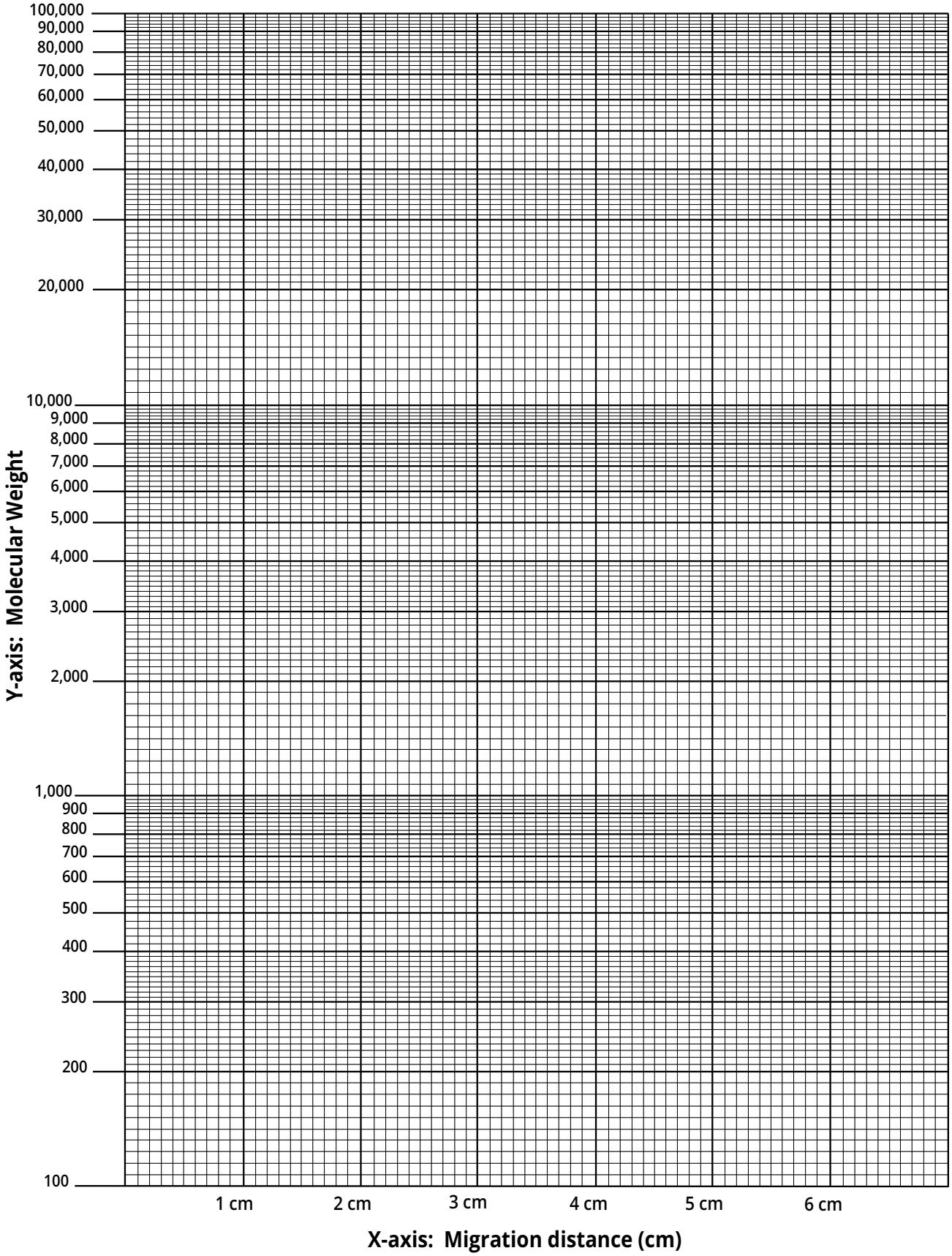
2. Using semilog graph paper, **PLOT** the migration distance or relative mobility (Rf) of each standard protein on the non-logarithmic x-axis versus its molecular weight on the logarithmic y-axis. **CHOOSE** your scales so that the data points are well spread out.

3. **DRAW** the best average straight line through all the points. This line should roughly have an equal number of points scattered on each side of the line. As an example, refer to the figure at left. This method is a linear approximation.

4. Using your standard graph, **DETERMINE** the molecular weight of the three unknown proteins. This can be done by finding the Rf (or migration distance) of the unknown band on the x-axis and drawing a straight vertical until the standard line is intersected.

5. A straight line is then made from the intersection across to the y-axis where the approximate molecular weight can be determined.





Appendix B
EDVOTEK® Troubleshooting Guide

PROBLEM:	CAUSE:	ANSWER:
Gel is not running properly.	Running buffer was not properly prepared.	Check buffer protocol, make fresh buffer.
	Wrong buffer used.	Check gel recipe, buffer must be compatible with the gel.
	Buffer volume is too low.	Buffer must fully cover the sample wells throughout the entire experiment.
	Gel is inserted in the wrong orientation.	Check with manufacturer for proper setup of the electrophoresis chamber.
	Malfunctioning electrophoresis chamber or power supply.	Consult with manufacturer of electrophoresis chamber or power supply.
	Tape at bottom of precast gel not removed.	Carefully remove tape before running the gel.
	Buffer volume is too low.	Buffer must fully cover the sample wells throughout the entire experiment.
	Electrodes not connected or polarity reversed.	Check electrode connections at the gel box and power supply.
Poor band resolution or separation.	Diffusion of samples before power was turned on.	Minimize time between loading samples and the start of electrophoresis.
	The gel is old or expired.	Make fresh gels or order new pre-cast gels.
	Wrong concentration of acrylamide gel.	The kit is designed for 12% acrylamide gels, other concentrations will affect results.
Smiling or frowing of bands.	Proteins have been overloaded.	EDVOTEK ® has optimized this kit to avoid overloading. Be sure to load the amount recommended by the protocol.
	Wrong buffer was used.	Check gel recipe, the buffer must be compatible with the gel.
	Incorrect voltage supplied to the gel.	Check the protocol for the recommended voltage (page 13).
No bands on gel/ smallest bands missing from gel.	Proteins ran off gel.	Use the appropriate length of time for the chosen voltage. Be sure to monitor the tracking dye while the gel is running. For best results, the tracking dye should run 8-9 cm and should not be allowed to run off the gel.
Proteins have accumulated in the wells of the gel.	Proteins have aggregated.	Ensure proteins have fully denatured; boil proteins for 5 min. and load while still warm.
Bands are smeary and distorted.	The gel has overheated.	Reduce voltage, check buffer concentration and dilute if necessary.
Bands are faint.	Proteins have diffused or faded.	Repeat staining with increased staining times and/or increased destaining times.
	Too little protein was loaded.	EDVOTEK ® has optimized this kit to avoid underloading. Be sure to load the amount recommended by the protocol.