Edvo-Kit # 110

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Molecular Weight Determination of Proteins

Experiment Objective:

The objective of this experiment is to determine the molecular weight of a protein using SDS horizontal gel electrophoresis. Students will develop a basic understanding of protein structure and denaturation.

See page 3 for storage instructions.

Version 110.190812

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	Pre-stained LyphoProtein™ Gel Marker (Molecular Weight Standard Protein Markers)	-20°C	❑
B	Unknown Pre-stained LyphoProtein™	-20°C	❑
C	Unknown Pre-stained LyphoProtein™	-20°C	❑
D	Unknown Pre-stained LyphoProtein™	-20°C	❑
•	Practice Gel Loading Solution	Room Temp.	❑
•	Protein Agarose™ Powder	Room Temp.	❑
•	Tris-Glycine-SDS Powdered Buffer	Room Temp.	❑
•	FlashBlue™ Protein Stain Powder	Room Temp.	❑

Supplies

- Transfer Pipets ❑
- 1 mL Pipet ❑

Quick Reference:

There is enough sample for 6 gels if you are using an automatic micropipet for sample delivery. Use of transfer pipets will yield fewer gels.

Although the proteins in this experiment are pre-stained and can easily be visualized directly during and after electrophoresis, staining with FlashBlue™ Protein Stain may enhance the visibility of the bands.

Requirements

- Horizontal gel electrophoresis apparatus (EDVOTEK® Cat. #502-504 highly recommended)
- D.C. power supply (EDVOTEK® Cat. #509 or Cat. #5010-Q highly recommended)
- Micropipets with tips
- Microwave
- 250 mL or 1 L flask
- Large beaker
- Lab tape or markers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Distilled or deionized water

For Staining with FlashBlue™ Protein Stain (optional):

- 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 diverse group of large molecules, or macromolecules, that perform many of the essential functions in our cells. Scientists first observed proteins in the 18th century and quickly determined that these molecules were critical for maintaining body structure. Since then, it has become clear that proteins also have a role in many cellular processes, including motility, transport, and communication. It is estimated that about 2-4 million proteins are found in every cubic micron of most mammalian cells.

Proteins are polymers, composed of hundreds to thousands of smaller organic compounds known as amino acids. Amino acids are simple molecules consisting of a central carbon atom bonded to four different groups: an amine group, a carboxyl group, a hydrogen atom, and a unique side chain (Figure 1). The simplest amino acid, glycine, has a single hydrogen atom as a side chain, while other amino acids feature more complex side chains. The chemical properties of side chains determine the polarity of each amino acid and whether the amino acid is acidic, basic, or neutral.

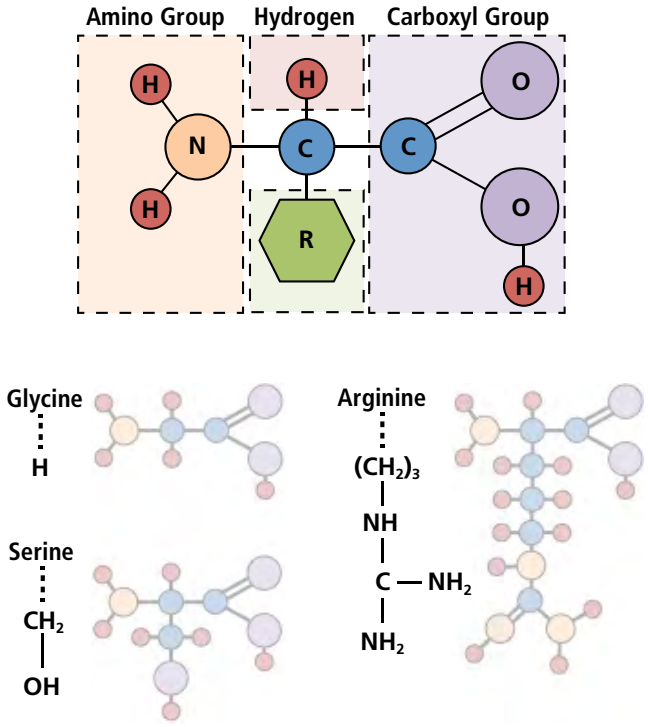


Figure 1: The Structure of Amino Acids.

During protein synthesis, a specific sequence of amino acids is connected together to form a continuous chain. Adjacent amino acids in the chain are linked to each other by peptide bonds. These strong covalent bonds link the carboxyl group of one amino acid and the amine group of a second amino acid (Figure 2). A chain of linked amino acids is known as a polypeptide, and one or more polypeptides combine to make a protein. The amino acid sequence gives each protein specific properties. For example, the molecular weight and charge of a protein is based on the number and type of amino acids, while the shape is determined by the order of amino

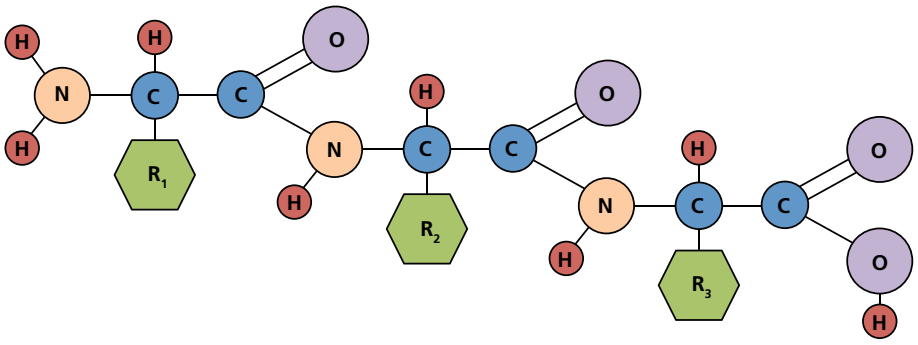


Figure 2: Polypeptide of three linked amino acids.

acids. This three-dimensional configuration, which is critical to protein function, includes twists, folds, and interactions between multiple polypeptides.

To analyze proteins, researchers will first determine a protein's molecular weight. Protein weight is most often described in unified atomic mass units or Daltons (Da). A mass of 1 DA is approximately the mass of one nucleon. If the protein's amino acid composition or DNA sequence is known then the molecular weight can be calculated. (In some cases this calculation must also factor in non-amino molecules like heme, zinc, and covalently bonded carbohydrates.) However, when the molecular composition of the protein is unknown, the molecular weight must be determined experimentally. This can be done using ultracentrifugation and light scattering or by running an SDS gel electrophoresis experiment.

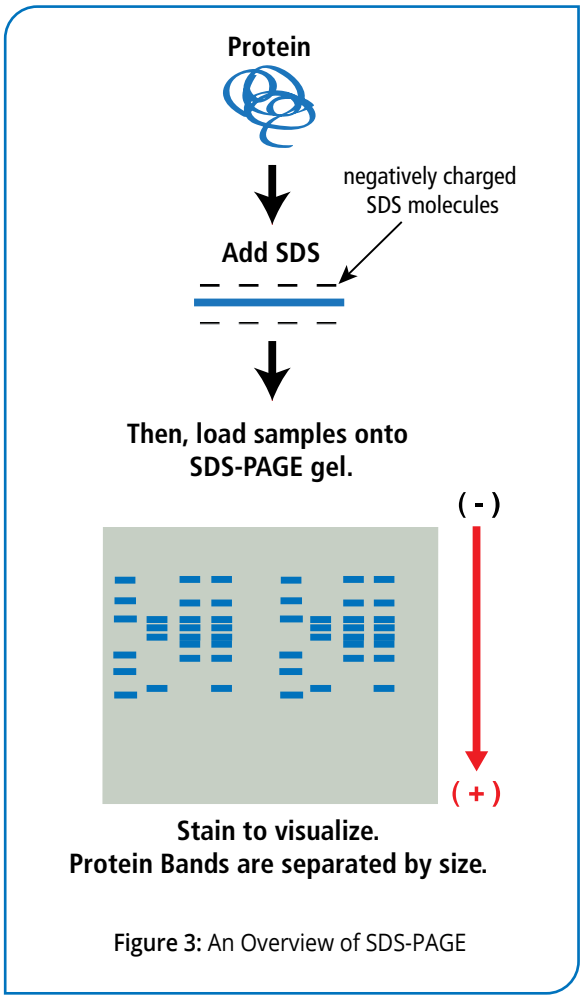


Figure 3: An Overview of SDS-PAGE

SDS gel electrophoresis is a simple but powerful method that provides information about the expression and purity of a molecule, along with its molecular weight. Often proteins are run on Polyacrylamide (PAGE) gels. However, acrylamide can be difficult and dangerous to work with when in liquid form. In addition, PAGE gels require specialized equipment. Alternatively, a high-density agarose gel can be used.
Both PAGE gels and high-density agarose gels contain a tight network of microscopic pores and channels. During electrophoresis, the proteins moved through the gel using these pores and channels.

To perform protein electrophoresis, a gel is prepared, placed in an electrophoresis chamber and flooded with buffer. Next, the protein samples are loaded into small indentations, or wells, in the gel. Finally, an electrical current is applied to the gel box, pushing the charged proteins through the gel towards the positive electrode (Figure 3). As the proteins migrate they move through the gel's pores and channels at different speeds. Smaller proteins have an easier time fitting than larger proteins and will migrate further in the same amount of time. By comparing the travel distance of unknown proteins to the travel distances of several known proteins the molecular weight of most protein can be determined.

PROTEIN DENATURATION FOR ELECTROPHORESIS

Proteins produce a unique challenge for electrophoresis because they have complex shapes and different charges, which affect how they migrate through the gel. Structural differences can cause two proteins with similar molecular weights to migrate at different rates - a complicated, spread-out protein will move slower through the gel than one with a compact shape. Similarly, positively and negatively charged proteins will migrate in different directions through the electric field in a gel. Scientists solve both these problems by using chemicals that denature the proteins. This eliminates the complex structure and neutralize the charge of the native protein.

Sodium Dodecyl Sulfate (SDS) is a common detergent used to disrupt interactions between amino acids. The SDS molecule consists of a hydrocarbon chain bonded to a negatively charged sulfate group. When incubated with proteins and heated, SDS will unfold the protein's three-dimensional structure. To break the stronger disulfide bonds in proteins researchers also

use reducing agents such as β-mercaptoethanol (β-ME) or Dithiothreitol (DTT). Although the amino acid composition and sequence stay the same, a protein treated with SDS, β-ME, and DTT will no longer have biological activity because the specific three-dimensional shape has changed. Such proteins are said to be denatured. Proteins can also be denatured by briefly heating them.

In this experiment, you will be testing three unknown proteins to confirm their presence and purity and to estimate their molecular weight. For convenience, the proteins in this experiment have already been denatured and are pre-stained. You will run these proteins in a specialized protein agarose SDS gel along with several standard proteins of known molecular weight. Following electrophoresis you will compare the migration distances of the unknown proteins to the migration distances of the standard proteins in order to estimate their approximate size.

Experiment Overview

EXPERIMENT OBJECTIVE:

The objective of this experiment is to determine the molecular weight of a protein using SDS horizontal gel electrophoresis. Students will develop a basic understanding of protein structure and denaturation.

LABORATORY SAFETY

- 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.
- Always wash hands thoroughly with soap and water after working in the laboratory.


Wear gloves and safety goggles

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:

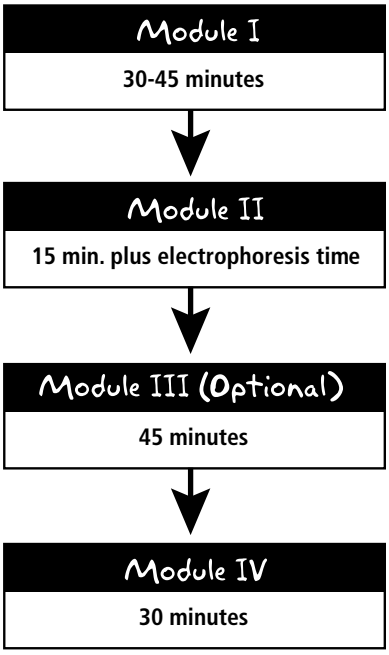
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

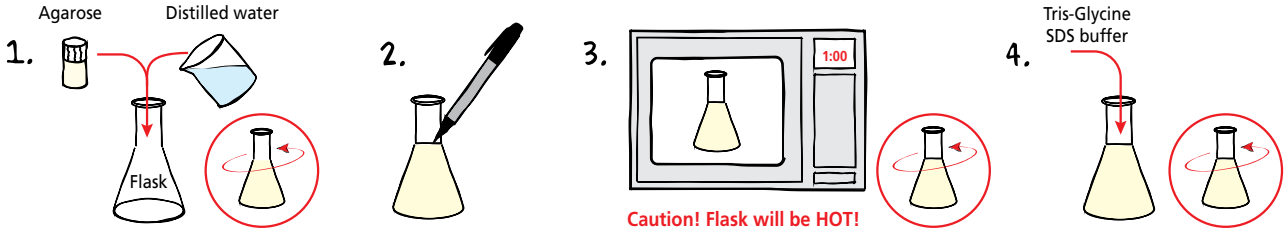
- Record your observations.

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: Preparing Agarose Gels For Protein Electrophoresis



1. **COMBINE** distilled water and agarose in a large flask (at least 250 mL). Use Table A to determine the correct volume and weight for your gel size. **MIX** by swirling the flask. *DO NOT add the SDS buffer until step 4 (after the solution has been heated).*

NOTE: High percentage agarose can easily bubble over during heating. To prevent this prepare each gel in a large flask, carefully monitor the solution as it is being heated, and add the Tris-Glycine-SDS buffer only after the solution has been heated. If you do observe a head of fine bubbles beginning to form in step 3 quickly remove the flask from the microwave and the let the bubbles subside. Swirl briefly before heating again.

2. **MARK** the height of the solution in the flask using lab tape or a nonpermanent marker.

3. **DISSOLVE** the agarose powder by boiling the solution.
   a. **MICROWAVE** the solution on high for 1 minute.
   b. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask.
   c. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved. (The solution should be clear like water.)
   d. **REMOVE** your solution from the microwave.
   e. **OBSERVE** if any evaporation has occurred. If it has, **ADD** distilled water until the solution is back to its original volume as marked in step 2.
   f. **MIX** by swirling.

4. **ADD** the concentrated Tris-Glycine-SDS buffer. Use Table A to determine the correct volume for your gel size. **MIX** by gently swirling the flask.

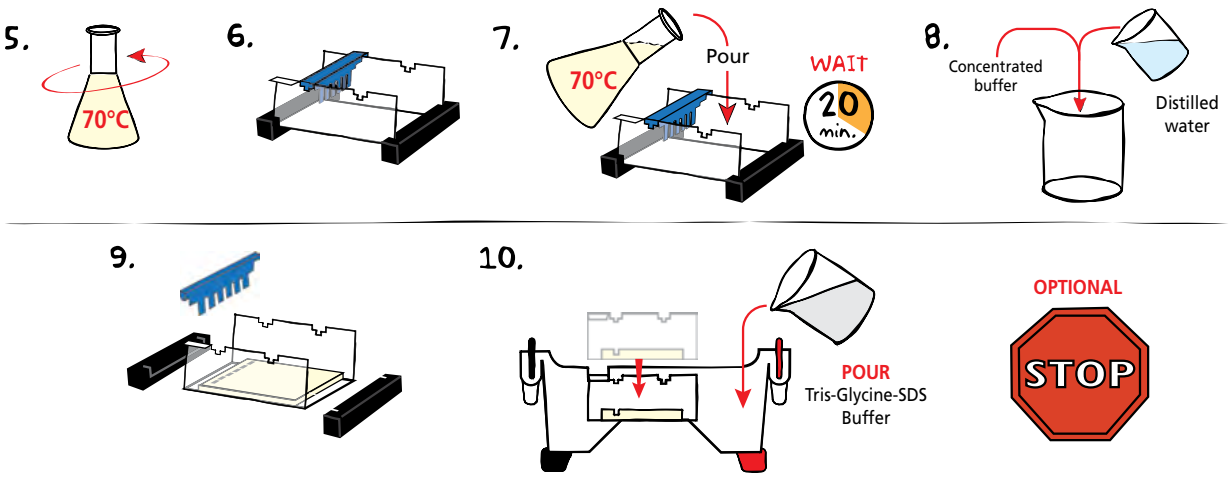
Table A — Preparing Individual 3.2% Protein Agarose Gels

Size of Gel Casting tray	Amt. of Protein Agarose	+	Distilled Water	+	Tris-Glycine SDS Buffer (STEP 4)	=	TOTAL Volume
7 x 7 cm	0.96 g		27 mL		3 mL*		30 mL

*Do not add the SDS buffer until step 4 (after the solution has been heated).

NOTE: Tris-Glycine-SDS contains a detergent that exacerbates bubbling. Gently handle the agarose solution after this buffer has been added. Even with careful pouring some small bubbles may form in the gel. These will not interfere with the experiment and may disappear as the gel solidifies. Bubbles can also be nudged to the side of the gel using a toothpick or plastic pipette tip during step 7.

Module I: Preparing Agarose Gels For Protein Electrophoresis, continued



5. **COOL** the agarose solution to 70°C. Periodically **MIX** to promote even dissipation of heat.

6. While the agarose is cooling, **SEAL** the ends of a gel casting tray with the rubber end caps. **PLACE** the well template (comb) in the first set of notches.

7. Slowly, **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes.

8. While the agarose solidifies, **PREPARE** the electrophoresis buffer by diluting the remaining Tris-Glycine-SDS buffer in distilled water according to Table B.

9. **REMOVE** both end caps and then remove the comb by pulling straight up. Work slowly and carefully to prevent damage to the wells.

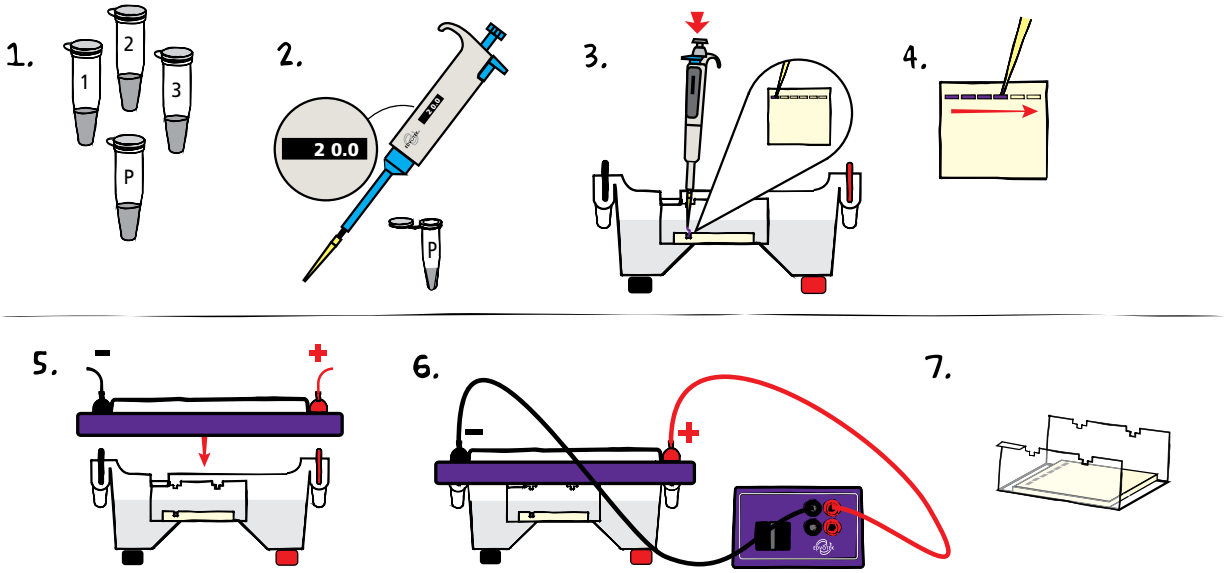
10. **PLACE** the gel (still on the tray) into the electrophoresis chamber. **COVER** the gel with 1X Electrophoresis Buffer using the recommended volume in Table B. The gel should be completely submerged.

Table B — Electrophoresis Buffer (Chamber Buffer)

EDVOTEK Model #	Total Volume Required	Dilution: Tris-Glycine SDS Buffer	+ Distilled Water
M6+ & M12 (new)	300 mL	30 mL	270 mL
M12 (classic)	400 mL	40 mL	360 mL
M36	1000 mL	100 mL	900 mL

OPTIONAL STOPPING POINT:
Gels can be stored overnight submerged in the electrophoresis chamber. Gels can also be stored for several days in the refrigerator. Keep refrigerated gels hydrated by storing each gel in a watertight plastic bag with a small amount of electrophoresis buffer.

Module II: Performing Electrophoresis



1. **COLLECT** the three unknown protein samples and standard protein marker from your instructor.
2. Using a fresh pipet tip, **MEASURE** 20 µL of the standard protein marker.
3. **PLACE** the pipet tip under the buffer and directly above the sample well. Slowly **DISPENSE** the sample by depressing the plunger.
4. **REPEAT** steps 2-4 with the three unknown protein samples.
5. **PLACE** safety cover on the chamber. **CHECK** that the gel is properly oriented. Remember that the samples will migrate toward the positive (red) electrode.
6. **CONNECT** leads to the power supply and **PERFORM** electrophoresis (See Table C for time and voltage guidelines.)
7. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

Table 1: Gel Loading

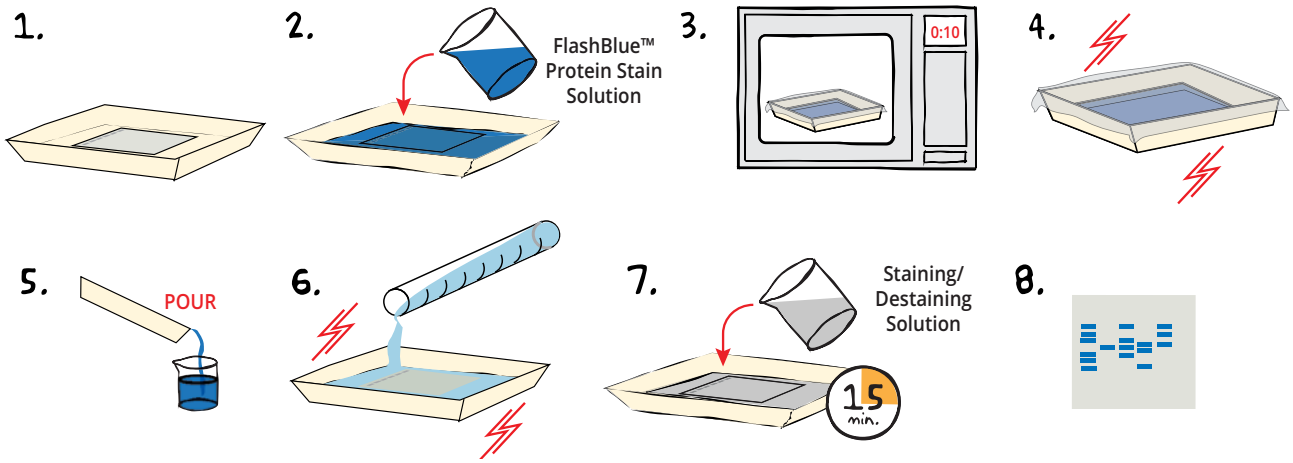
Lane	
1	Standard Protein Marker
2	Unknown Protein Sample 1
3	Unknown Protein Sample 2
4	Unknown Protein Sample 3

OPTIONAL STOPPING POINT:
Gels can be stored overnight. For long term storage the gel should be submerged in a fixative solution.

Table C: Time & Voltage

Volts	Recommended Time Minimum	Maximum
125	30 min.	45 min.
70	40 min.	1.5 hours
50	60 min.	2 hours

Module III: Supplemental Gel Staining (Optional)



1. **PLACE** the gel into a shallow container.
2. **ADD** 30 mL of prepared FlashBlue™ Protein Stain.
3. (OPTIONAL) **COVER** the container with plastic wrap and **MICROWAVE** for 10 seconds to gently heat the solution.
4. **INCUBATE** for 15 minutes at room temperature, **SHAKING** occasionally.
5. **DISCARD** the FlashBlue™ Protein Stain solution. ***NOTE: Pour slowly to keep the gel in the container.***
6. **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.
7. **ADD** 30 mL of Protein Staining/Destaining solution to the gel and **INCUBATE** for 15 minutes at room temperature.
8. **EXAMINE** the gel.
9. (OPTIONAL) **DISCARD** the used Protein Staining/Destaining solution and **ADD** an additional 30 mL of Protein Staining/ Destaining solution. **INCUBATE** for 15-60 minutes at room temperature until the appearance and contrast of the protein bands against the background improves.

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.

Module IV: Size Determination of Unknown Proteins

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.

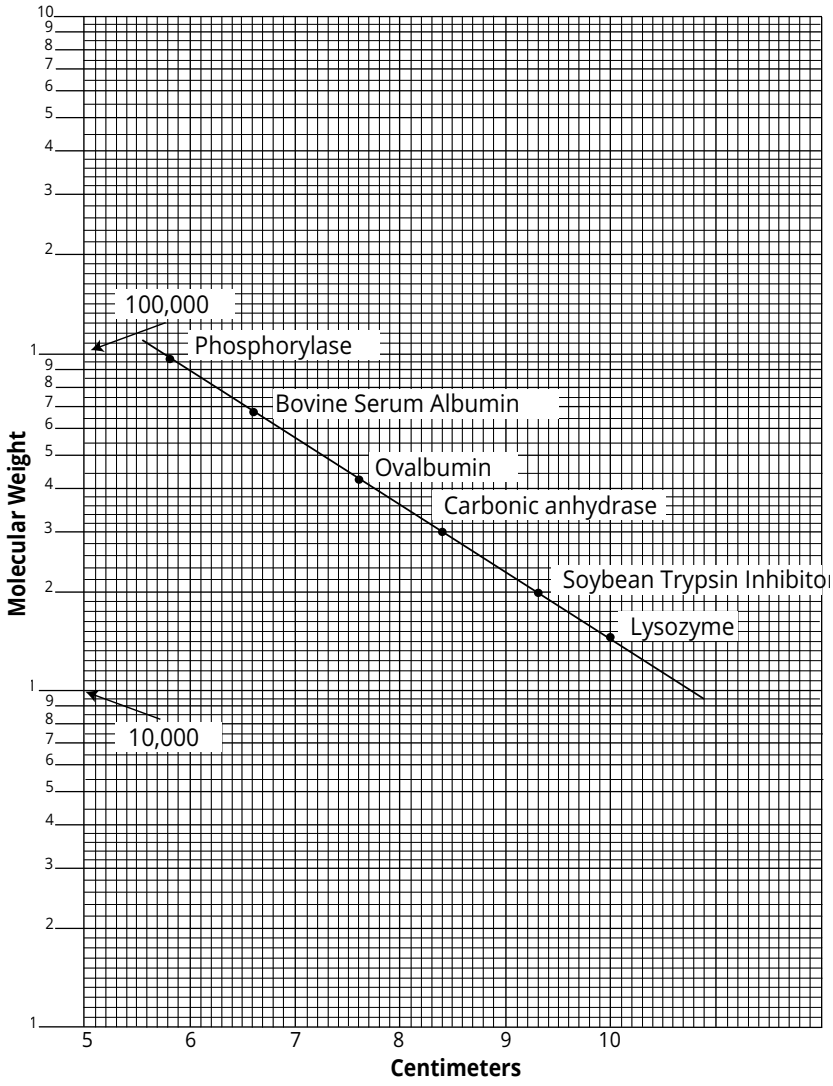
2. Using semilog graph paper, **PLOT** the migration distance or relative mobility (Rf) of each standard protein on the non-logarithmic x-axis versus its molecular weight on the logarithmic y-axis. **CHOOSE** your scales so that the data points are well spread out.

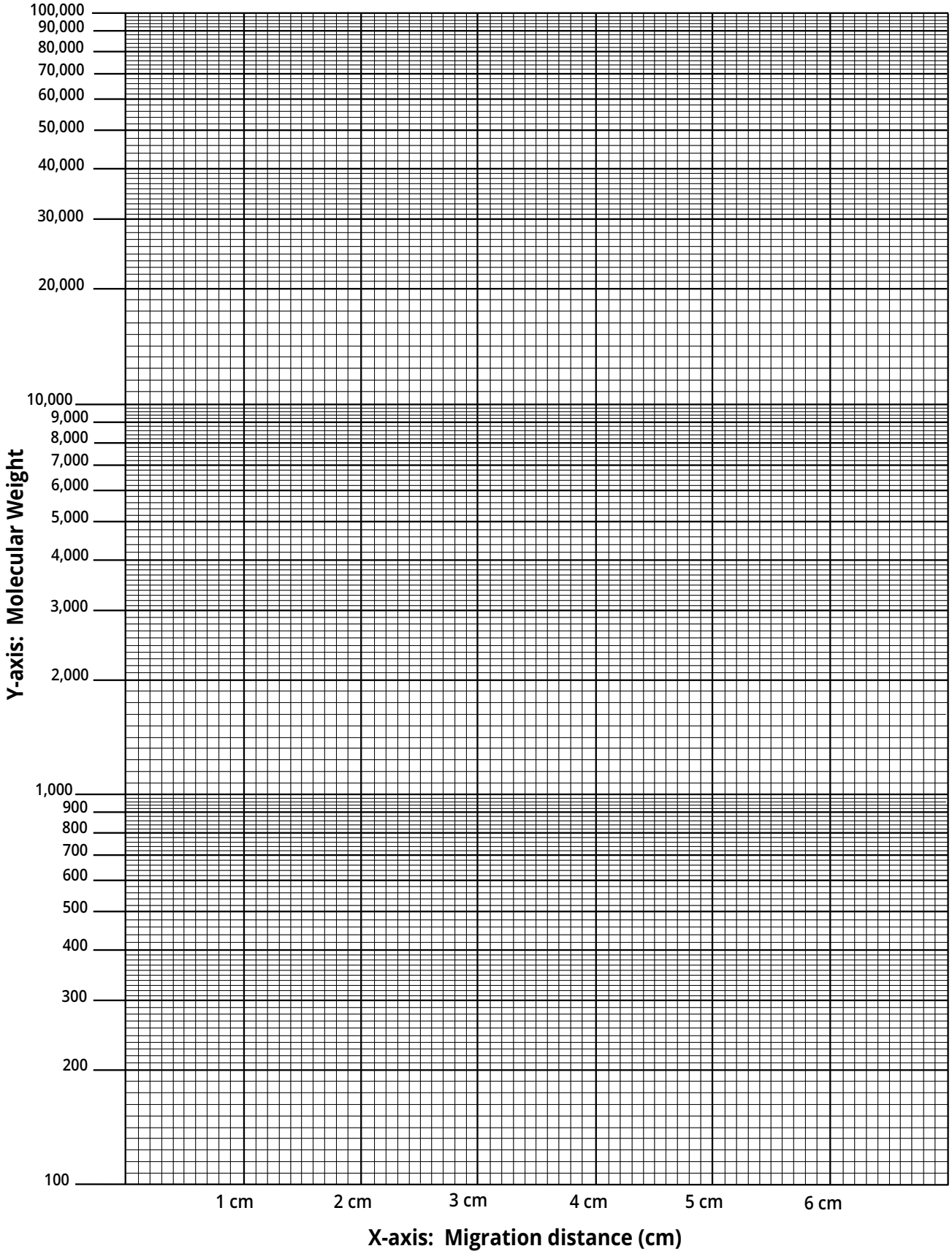
3. **DRAW** the best average straight line through all the points. This line should roughly have an equal number of points scattered on each side of the line. As an example, refer to the figure at left. This method is a linear approximation.

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.

	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
Unknown Protein Sample 1		
Unknown Protein Sample 2		
Unknown Protein Sample 3		





Study Questions

1. What is an amino acid? Draw or describe the four subparts of an amino acid.

2. What is Sodium Dodecyl Sulfate (SDS) and why is it so important to protein electrophoresis?

3. What would happen if a non-denatured (native) protein was run in a gel next to a denatured sample of the same protein?

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 is supplied with reagents for six groups to each run four samples. Each group can be provided with protein agarose gel or can create their own.

Preparation for:	What to do:	When?	Time Required:
MODULE I	Distribute student supplies OR	Anytime before Module I	10 minutes
	Batch prepare gels and electrophoresis buffer	Up to one week before Module I.	30 minutes
MODULE II	Rehydrate protein samples	Up to one week before Module II.	10 minutes
MODULE III	Prepare stain solutions (optional)	Up to one week before Module III.	10 minutes

Pre-Lab Preparations

MODULE I - PREPARING AGAROSE GELS FOR PROTEIN ELECTROPHORESIS

This experiment requires a total of six 3.2% protein agarose gels for the class. These gels can be prepared by each student group as described in Module I. Alternatively, to save class time, all six gels and the electrophoresis buffer can be prepared beforehand by the teacher (see Appendix B).

FOR MODULE I
Each group will need:

- Protein Agarose
- Distilled Water
- 10X Tris-Glycine-SDS Buffer
- Gel casting tray, seals, and comb
- Two large flasks (for gel preparation and electrophoresis buffer preparation)

Prepare Tris-Glycine-SDS Buffer

1. Measure 200 mL of distilled or deionized water and place in a large vessel.
2. Add the entire amount of Tris-Glycine-SDS Powdered Buffer powder to the vessel and mix well.
3. Label the vessel as "10X Tris-Glycine-SDS Buffer".
4. Use within 60 days of preparation.

MODULE II - PERFORMING ELECTROPHORESIS

FOR MODULE II
Each group will need:

- Protein agarose Gel
- Electrophoresis apparatus
- Power supply
- Micropipette and tips
- Standard Protein Marker
- Three Protein Samples

Reconstitute Proteins and Standard Protein Marker

1. Add 160 µL of distilled or deionized water to each tube (A-D) and allow the samples to hydrate for several minutes.
2. Vortex or flick tube vigorously to mix.
3. Either place re-hydrated proteins in their original tubes in a central location to be shared by the class or aliquot 25 µL of each protein into six appropriately labeled new tubes. The latter will require 24 additional small tubes.

NOTE: Rehydrated proteins may be kept at room temperature for immediate use or frozen until needed.

MODULE III - SUPPLEMENTAL GEL STAINING (OPTIONAL)

FOR MODULE III
Each group will need:

- 30 mL FlashBlue™ Protein Stain
- 30-60 mL Staining/Destaining Solution
- Water
- Staining Tray
- Plastic Wrap
- Rocking platform (recommended)
- White light (recommended)

Prepare Staining Solutions

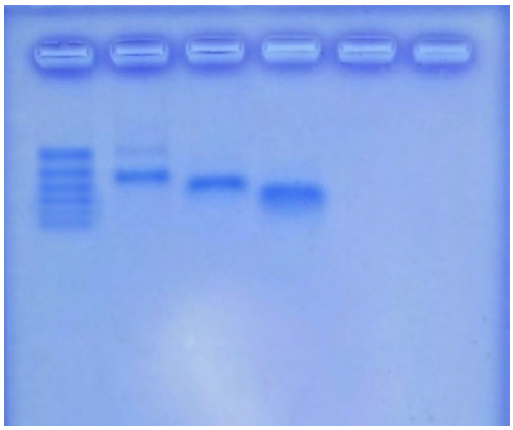
These solutions are only needed for long term gel storage or for supplemental staining.

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 180 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.

**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 expected banding patterns are shown below relative to the standard protein marker. The actual results may differ slightly due to variations in gel composition or thickness or due to fluctuations in how the samples run in the gel. Even in high percentage protein agarose gels, the last two proteins of the standard protein marker may be hard to observe.



Lane	Sample Name	Number/Size of bands
1	Standard Protein Marker (A)	----------
2	Unknown Protein 1 (B)	64,000 Da
3	Unknown Protein 2 (C)	40,000 Da
4	Unknown Protein 3 (D)	30,000 Da

Standard Protein Marker Molecular Weights
94,000 Da
67,000 Da
38,000 Da
30,000 Da
20,000 Da
14,000 Da

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

Appendices

A Practice Gel Loading

B Bulk Electrophoresis Preparation

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

Appendix A
Practice Gel Loading

If your students are unfamiliar with loading samples in agarose we suggest they practices the delivery techniques before performing this experiment. Below is one suggested activity for practice gel loading. Although the same gel can be used for both this activity and the actual experiment we suggest using a separate gel in case of damage.

1. Cast a separate practice gel with the maximum number of combs in it. For practice gel loading you can use any gel grade agar at any concentration or you can use Edvotek's reusable DuraGels™ (Cat. #S-43).

2. Place the gel under water or buffer either in the chamber or in a similarly deep tray.

3. Let students practice delivering the practice gel solution to the sample wells.

4. If the students need more practice, remove the practice gel loading solution by squirting buffer into the wells with a transfer pipet.

Appendix B
Bulk Electrophoresis Preparation

To save time, the electrophoresis buffer and agarose gels can be prepared ahead of time.

Bulk Electrophoresis Buffer

Bulk preparation for 2 liters of 1X Electrophoresis Buffer is outlined in Table D.

Table D Bulk Preparation of 1X Electrophoresis Buffer

Tris-Glycine-SDS	+	Distilled Water	Total Volume Required
200 mL		1800 mL	2000 mL (2L)

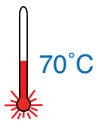
Batch Protein Agarose™ Gels (3.2%)

For quantity (batch) preparation of Protein Agarose™ gels, see Table E.

Table E Batch Preparation of 3.2% Protein Agarose™

Tray Size	Amt of Agarose (g)	+	Distilled Water (mL)	+	Tris-Glycine SDS Buffer (mL)	Total Volume (mL)
7 x 7 cm	5.76		162		18	180

1. In a 1 or 1.5 L flask, combine distilled water and agarose. Refer to Table E for volume and weight. Swirl to disperse clumps.
2. With a marking pen, indicate the level of solution volume on the outside of the flask.
3. Microwave the solution on high for 45 seconds. Remove the flask and mix by swirling. Continue to heat the solution in 15 second burst until the agarose is completely dissolved.
4. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 2.
5. Add the concentrated Tris-Glycine-SDS buffer and mix by gently swirling the flask.
6. Cool the agarose solution to 70°C with swirling to promote even dissipation of heat.



7. While the agarose is cooling, seal the ends of the gel casting trays with the rubber end caps. Place the well template (comb) in the first set of notches.
8. Dispense the required volume of cooled agarose solution into each assembled tray (30 mL for each 7 x 7 cm gel.)
9. Allow the gels to completely solidify. They will become firm and cool to the touch after approximately 20 minutes.
10. Proceed with electrophoresis. Alternatively, gels can be stored in the refrigerator for up to 1 week. Seal each gel in a water-tight plastic bag with 1-2 mL of 1X electrophoresis buffer.