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A Geno Technology, Inc. (USA) brand name

Protein Electrophoresis

Teacher's Guidebook

(Cat. # BE-406)

MATERIALS INCLUDED

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 bottle PAGE: PAGE Mix (19:1 Acrylamide/Bisacrylamide)
- 1 vial PAGE: APS (Amm. Persulfate)
- 1 bottle Detergent Solution (10% SDS)
- 1 bottle PAGE: PAGE Stacking Buffer (1M Tris-HCl, pH 6.8)
- 1 bottle PAGE: PAGE Separating Buffer (1M Tris-HCl, pH 8.8)
- 1 vial PAGE: TEMED
- 1 vial PAGE: Sample Loading Buffer (2X)
- 1 bottle PAGE: Electrophoresis Running Buffer (10X)
- 8 vials PAGEmark™ Blue PLUS Protein Marker
- 1 vial Protein: Tissue Extract & Pestle
- 1 bottle PAGE: LabSafe GelBlue

SPECIAL HANDLING INSTRUCTIONS

- Store the Protein Marker and Tissue Extract & Pestles frozen at -20°C.
- All other reagents can be stored at room temperature

The majority of reagents and components supplied in the *BioScience Excellence*™ kits are non toxic and are safe to handle, however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.

For further details on reagents please review the Material Safety Data Sheets (MSDS).

The following items need to be used with particular caution.

Part #	Name	Hazard
P151	PAGE: TEMED	Toxic
PP011	PAGE: APS (Amm. Persulfate)	Oxidizing
P061	PAGE: PAGE Mix (19:1 Acrylamide/Bisacrylamide)	Very Toxic

ADDITIONAL EQUIPMENT REQUIRED

- Protein Electrophoresis Equipment
- 70% isopropanol
- Waterbath or beaker and thermometer
- Light box

TIME REQUIRED

- 3-4 hours

OBJECTIVES

- Learn the principle of protein electrophoresis.
- Prepare protein samples and electrophoresis gels, and run electrophoresis with test samples.
- Use electrophoresis gels for visualization of resolved protein bands.
- Use of protein standard for determination of protein mass.
- Understand non-denaturing and denaturing protein electrophoresis.

BACKGROUND

Protein electrophoresis is a relatively simple, rapid and highly sensitive tool to study the properties of proteins. It is the principle tool in analytical chemistry, biochemistry, and molecular biology. The separation of proteins by electrophoresis is based on the fact that charged molecules will migrate through a matrix upon application of an electrical field. The matrix for protein electrophoresis separation is polyacrylamide.

The chemical agents used to form polyacrylamide are monomeric acrylamide and N, N'-methylene-bis-acrylamide (bis-acrylamide). The most popular method for polymerizing acrylamide and bis-acrylamide is using TEMED (tetramethylethylenediamine) and ammonium persulfate.

The size of pores in the polyacrylamide gel matrix is determined by the amount of total acrylamide used per unit volume and relative percentage of bis-acrylamide used. The effective range of polyacrylamide gel is between 3-30%.

Two fundamentally different types of gel system exist, non-dissociating (non-denaturing) and dissociating (denaturing). Non-dissociating (non-denaturing) system is designed to separate native protein under conditions that preserve protein function and activity. In contrast, a dissociating system is designed to denature protein into their constituent's polypeptides and hence examines the polypeptide composition of samples.

Sodium dodecyl sulfate (SDS) is commonly used for denaturing proteins into their constituents and the method is known as sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis is the most commonly used system and this separates proteins strictly by their size.

SDS-PAGE uses two types of buffer systems: the continuous buffer system and the discontinuous buffer system. In the continuous buffer system the pH of the gel matrix remains constant throughout the separation. In contrast, the discontinuous buffer system consists of a narrow layer of stacking gel (of large pore size and acidic pH) above the main separating or resolving gel matrix of alkaline pH (pH 8.8). The stacking gel concentrates the protein sample before entering the separating gel and hence

enhancing resolution. SDS-PAGE with a discontinuous buffer system is the most popular electrophoresis technique used to analyze polypeptides.

In SDS-PAGE, the protein mixture is denatured by heating at 100°C in the presence of excess SDS and a reducing reagent is employed to break disulfide bonds. Under these conditions, all reduced polypeptide bind the same amount of SDS on a weight basis (1.4g SDS/g polypeptide) independent of the amino acid composition and sequence of the protein. The SDS-protein complex forms a rod with its length proportional to the molecular weight of the protein. All proteins are now negatively charged with similar charge density and thus can be separated on the basis of their size only.

SDS-PAGE is used mainly for the following purpose:

a. Estimation of protein size.
b. Determination of protein subunits or aggregation structures.
c. Estimation of protein purity.
d. Protein quantitation.
e. Monitoring protein integrity.
f. Comparison of the polypeptide composition of different samples.
g. Analysis of the number and size of polypeptide subunits.
h. Post-electrophoresis applications, such as Western blotting.

This lab activity is provided with all of the reagents, buffers, and supplies needed for SDS-PAGE with a discontinuous buffer system, including a mixture of Acrylamide/Bis-acrylamide (19:1) and two acrylamide polymerization catalysts (ammonium persulfate and TEMED).

Other buffer reagents included with this kit are buffers for casting polyacrylamide gels, both stacking and resolving (separating) gel and buffers for preparing protein samples, (10X) ten fold concentrated running electrophoresis, and gel staining solution for visualization of protein bands. Test protein samples and protein standards are also provided with this kit.

The sample loading buffer contains Tris-HCl, disulfide reducing agent TCEP (Tris[2-carboxyethyl]phosphine) and SDS (sodium dodecylsulfate), where as the 10X electrophoresis running buffer contains Tris base, glycine and SDS. TCEP is an odorless and powerful reducing agent. Other commonly used protein reducing agents such as β-mercaptoethanol and DTT (dithiothreitol) can also be used in the sample loading buffer and protein sample extraction buffer for electrophoresis.

The kit is also provided with a set of protein standards or proteins of known molecular weight and teaches the method for determination of molecular weight.

TEACHER'S PRE EXPERIMENT SET UP

Acrylamide/Bisacrylamide is toxic. Always wear gloves and protective clothing when handling the chemicals.

1. Dissolve 8g PAGE Mix (19:1 Acrylamide/Bisacrylamide) in distilled water to a final volume of 20ml. The concentration of Acrylamide/Bisacrylamide will be 40%. The solution can be stored in cold for two months.

2. Dissolve 0.15g APS (Amm. Persulfate) in 1.5ml distilled water to make a 10% solution. This solution is stable for one week.

    ***NOTE:*** *Protein Markers and Tissue Extracts are supplied with this kit. Teacher can prepare other protein samples for students to load on to the gel.*

3. Add 250µl distilled water and 250µl Sample Loading Buffer to the Tissue Extract vial. Leave at room temperature for 10 minutes. Use the Pestle to grind the tissue extract for 2 minutes. Centrifuge the tube for 5 minutes at maximum speed in a microcentrifuge. Transfer supernatant, without disturbing the pellet, to a 1.5ml tube.

4. Dilute 10X Electrophoresis Running Buffer to 1X with distilled water (mix 100ml buffer with 900ml distilled water). The amount of Electrophoresis Running Buffer needed depends on the device used. You may need to make more running buffer if necessary (0.025M Tris base, 0.194M Glycine, 0.1% SDS).

5. Aliquot reagents for each student group according to the next section.

MATERIALS FOR EACH GROUP

Supply each group with the following components. Several components will be shared by the whole class and should be kept on a communal table.

- 1 bottle 40% Acrylamide/Bisacrylamide solution (shared with whole class)
- 1 vial 10% APS (Amm. Persulfate) (shared with whole class)
- 1 bottle 10ml Detergent Solution (10% SDS) (shared with whole class)
- 1 bottle 10ml Stacking Buffer (shared with whole class)
- 1 bottle 10ml Separating Buffer (shared with whole class)
- 1 vial 100µl TEMED (shared with whole class)
- 1 vial PAGEmark™ Blue PLUS Protein Marker
- 10µl Sample Loading Buffer (2X)
- 50µl Tissue Extract in sample loading buffer
- 1X Electrophoresis Running Buffer (shared with whole class)
- 50ml LabSafe GelBlue

PROCEDURE

Acrylamide/Bisacrylamide is toxic. Always wear gloves and protective clothing when handling the chemicals.

I. Preparation of a Polyacrylamide Gel (Stacking and Resolving Gels).

1. The data provided in the following table is for making two 12% 8x10cm mini polyacrylamide gel. Different percentage resolving gels or multiple gels can be prepared. Calculate the reagents needed accordingly and fill in the blank columns.

Gel Concentration	**12% Separating Gel**		**4% Stacking Gel**	
Solution volume	5ml		5ml	
40% Acrylamide/Bis-acrylamide solution	1.5ml		0.5ml	
1M Separating Buffer	2ml		X	X
1M Stacking Buffer	X	X	0.6ml	
10% SDS	0.5ml		0.5ml	
10% APS	50µl		50µl	
Distilled water	To 5ml		To 5ml	
TEMED	5µl		5µl	

2. Clean the gel plates, spacers and stand as per manufacturer's instruction. Assembly the gel plates and spacers on the gel stand for casting the gel. Make sure the gel plates and spacers are aligned at the bottom.

3. Label two 15ml tubes for "Separating Gel" and "Stacking Gel". Add all the reagents, except TEMED, to the tube according to the table above. Mix the solution thoroughly. Do not add TEMED until you are ready to cast the gel.

 ***DO NOT ADD TEMED*** *until ready to cast gel or gel will polymerize in the tube.*

4. When ready to cast the gel, add TEMED to *only* the "Separating Gel" solution and mix well. Using a 1ml pipette swiftly add the running gel solution until the gel solution is 2.5-3.0cm from the top of the small plate.

5. Slowly add 1ml 70% isopropanol to prevent evaporation and create an even surface on top of the gel. Let the gel polymerize for 30-40 minutes.

6. Pour off the isopropanol and rinse the top layer of the resolving gel with 0.5ml distilled water.

7. Add TEMED to the "stacking gel" solution and mix well. Slowly fill up the top of the gel.

8. Carefully put the comb between the two glass plates without introducing air bubbles. Let the stacking gel polymerize for 30minutes. Now the gel is ready.

II. Running and Staining Electrophoresis Gel

1. Boil the protein samples for 5minutes in a 100°C water bath. Do NOT boil the protein marker

2. Vortex the sample briefly and centrifuge for 30 seconds to bring down the condensation.

3. Set up the electrophoresis gel to load your samples as per your teacher's instructions.

4. Load 5µl PAGEmark™ Protein Marker to the first lane, followed by 10µl protein samples to the remaining lanes. The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa.

5. Run the gel at 30mA until the blue dye front is 0.5-2cm from the bottom of the gel.

6. Disassembly the gel carefully.

7. Wash the gel twice in distilled water, five minutes each.

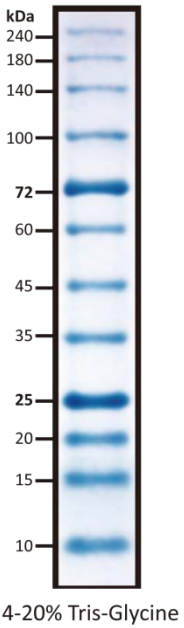
8. Remove all free water from the gel.

9. Add 50ml LabSafe GelBlue to cover the gel. Gently shake the gel for 60 minutes at room temperature.

10. Decant the LabSafe GelBlue and rinse the gel with distilled water. The gel can be stored in water. Longer destaining in water will give a clearer view of the protein bands.

Molecular Weight Determination:

Under appropriate conditions, one gram of a polypeptide binds 1.4 grams of SDS, resulting in the formation of a rod like particle with length proportional to the molecular weight of the polypeptide.



4-20% Tris-Glycine

The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa.

The molecular weights of the sample polypeptides can be estimated by the following procedure.

a. Following staining, place the gel on a glass plate and on top of a light box. Measure the distance traveled by the bromophenol dye front and each standard polypeptide and several unknown protein bands from the top of the resolving gel.

b. Calculate R_f (relative mobility) for each polypeptide from the formula:

   R_f = distance of protein migration / distance of dye migration

c. Plot R_f versus log M_r for the standard on semi-log paper or use a spreadsheet program such as Microsoft Excel.

d. Use the R_f value of the unknown sample polypeptide to estimate their molecular weight by interpolation.

RESULTS, ANALYSIS & ASSESSMENT

Q. What is the principle of electrophoresis separation of protein molecules?

A. Proteins are charged molecules and when placed under an electrical field the protein molecules migrate through a matrix, either towards the cathode or anode depending on the net charge of the protein molecules. During migration, proteins separate into different species depending on the size or the net charge of the protein molecules

Q. Describe the gel matrix you have used and how the matrix may be altered and its effects on the migration and separation of protein molecules.

A. The gel matrix used is a cross-linked polymer of acrylamide. The pore size of the gel matrix depends on the concentration of acrylamide and bis-acrylamide. The gel matrix pore size can be changed by altering the concentration of acrylamide and relative percentage of bis-acrylamide used during the polymerization step. As the concentration of acrylamide and the relative percentage of bis-acrylamide increase the gel matrix pore size reduces. As the gel matrix pore size reduces it becomes more difficult for the protein molecules to migrate through the matrix allowing greater separation between small protein molecules. For larger proteins, large pore sizes are required, i.e. less acrylamide.

Q. Describe non-denaturing and denaturing electrophoresis?

A. In non-denaturing protein electrophoresis protein is subjected to electrophoresis in its native form where as in denaturing electrophoresis protein is denature and native structure of protein molecule is destroyed with denaturing agents such as anionic detergent sodium dodecyl sulfate (SDS).

Q. Briefly describe the role of each reagent component solution used in this lab activity.

A. Acrylamide-polymerized to form gel matrix.

Bis-acrylamide – creates cross linking of polymerized acrylamide.

Ammonium Persulfate- initiates polymerization of acrylamide/bis-acrylamide.

TEMED – acts as catalyst to accelerate polymerization of acrylamide/bis-acrylamide.

Stacking Buffer (1M Tris-HCl, pH6.8) – for forming discontinuous stacking gel – a large pore size region for concentrating protein sample before entering separation zone.

Separating Buffer (1M Tris-HCl, pH8.8) – buffer for separating gel – for forming separation zone of smaller pore size and high alkaline medium.

Sample loading buffer- contains SDS and reducing agents for denaturing protein sample.

Electrophoresis Running Buffer- for establishing electrical conductance between the anode and the cathode electrode for running electrophoresis

Protein Marker -for determination of molecular weight of sample proteins.

Q. Briefly describe the role of SDS in protein electrophoresis.

A. SDS binds and denatures protein molecules forming long rod like structures where the length of the protein is proportional to the molecular weight.

Q. Describe the role of reducing agents in protein electrophoresis.

A. Reducing agents break inter and intra molecular disulfide bonds in the protein molecules to allow complete linearization.

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b. Determination of protein subunits or aggregation structures.
c. Estimation of protein purity.
d. Protein quantitation.
e. Monitoring protein integrity.
f. Comparison of the polypeptide composition of different samples.
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- 1 bottle 10ml Detergent Solution (10% SDS) (shared with whole class)
- 1 bottle 10ml Stacking Buffer (shared with whole class)
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- 1 vial 100µl TEMED (shared with whole class)
- 8µl PAGEmark™ Protein Marker
- 10µl Sample Loading Buffer (2X)
- 50µl Tissue Extract in sample loading buffer
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- 50ml LabSafe GelBlue

PROCEDURE

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1M Stacking Buffer	X	X	0.6ml	
10% SDS	0.5ml		0.5ml	
10% APS	50µl		50µl	
Distilled water	To 5ml		To 5ml	
TEMED	5µl		5µl	

2. Clean the gel plates, spacers and stand as per manufacturer's instruction. Assembly the gel plates and spacers on the gel stand for casting the gel. Make sure the gel plates and spacers are aligned at the bottom.

3. Label two 15ml tubes for "Separating Gel" and "Stacking Gel". Add all the reagents, except TEMED, to the tube according to the table above. Mix the solution thoroughly. Do not add TEMED until you are ready to cast the gel.

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5. Slowly add 1ml 70% isopropanol to prevent evaporation and create an even surface on top of the gel. Let the gel polymerize for 30-40 minutes.

6. Pour off the isopropanol and rinse the top layer of the resolving gel with 0.5ml distilled water.

7. Add TEMED to the “stacking gel” solution and mix well. Slowly fill up the top of the gel.

8. Carefully put the comb between the two glass plates without introducing air bubbles. Let the stacking gel polymerize for 30minutes. Now the gel is ready.

II. Running and Staining Electrophoresis Gel

1. Boil the protein samples for 5minutes in a 100°C water bath. Do NOT boil the protein marker

2. Vortex the sample briefly and centrifuge for 30 seconds to bring down the condensation.

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6. Disassembly the gel carefully.

7. Wash the gel twice in distilled water, five minutes each.

8. Remove all free water from the gel.

9. Add 50ml LabSafe GelBlue to cover the gel. Gently shake the gel for 60 minutes at room temperature.

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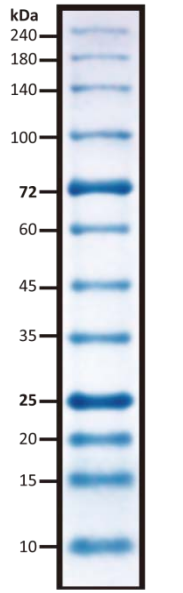

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b. Calculate R_f (relative mobility) for each polypeptide from the formula:

   R_f = distance of protein migration / distance of dye migration

c. Plot R_f versus log M_r for the standard on semi-log paper or use a spreadsheet program such as Microsoft Excel.

d. Use the R_f value of the unknown sample polypeptide to estimate their molecular weight by interpolation.

RESULTS, ANALYSIS & ASSESSMENT

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Q. Describe the gel matrix you have used and how the matrix may be altered and its effects on the migration and separation of protein molecules.

Q. Describe non-denaturing and denaturing electrophoresis?

Q. Briefly describe the role of each reagent component solution used in this lab activity.

Q. Briefly describe the role of SDS in protein electrophoresis.

Q. Describe the role of reducing agents in protein electrophoresis.

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