EDVOTEK®

The Biotechnology Education Company ®

Revised and Updated

EDVO-Kit #

275

AIDS Kit II:
Simulation of HIV-1 Detection by Western Blot

Storage:
See page 3 for specific storage instructions.

EXPERIMENT OBJECTIVE:

The objective of this experiment is to understand the theory and applications of Western blots.

Table of Contents

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

Components & Requirements

This experiment is designed for 6 groups.

Experiment Components

		Storage
I.	**Samples for Electrophoresis:**	
A	Positive Control	Freezer
B	Negative Control	Freezer
C	Patient 1	Freezer
D	Patient 2	Freezer
E	Patient 3	Freezer
F	Standard Molecular Weight Dye Markers	Freezer
II.	**Components for Electrophoresis:**	
	UltraSpec-Protein Agarose™	Room Temp.
	10x Tris-Glycine-SDS Buffer (Chamber Buffer)	Room Temp.
	10x Tris-Glycine powdered Buffer (for gel prep only)	Room Temp.
	Practice Gel Loading Solution	
	1 mL Pipet	
	100 mL Graduated Cylinder (packaging for samples)	
III.	**Components for Membrane Transfer and Stain:**	
	Precut Western Blot Membranes (7 x 7 cm)	Room Temp.
	Precut Blotting Filter Papers (7 x 7 cm)	Room Temp.
	Protein InstaStain®	Room Temp.

Requirements

- Horizontal Gel Electrophoresis Apparatus, EDVOTEK® M12 or equivalent
- D.C. Power Supply
- Automatic Pipets with Tips
- Shaker Platform
- Incubation Oven (65° C)
- Burners or Hot Plates
- Microcentrifuge Tubes
- Beakers
- Pipets
- Graduated Cylinders
- Trays or Containers that can hold a 7 x 7 cm piece of membrane and 100 mL of liquid
- Disposable Lab Gloves
- Several Packs of Paper Towels
- Plastic Wrap
- Scissors
- Metric Rulers
- Methanol, 95-100%
- Glacial Acetic Acid
- Distilled Water

* Enough membranes are provided to accommodate 6 standard mini-gels (7 x 7 cm). EDVOTEK® Model M12 (Cat. #502) is recommended.

All 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.

THIS EXPERIMENT DOES NOT CONTAIN HUMAN DNA. None of the experiment components are derived from human sources.

Background Information

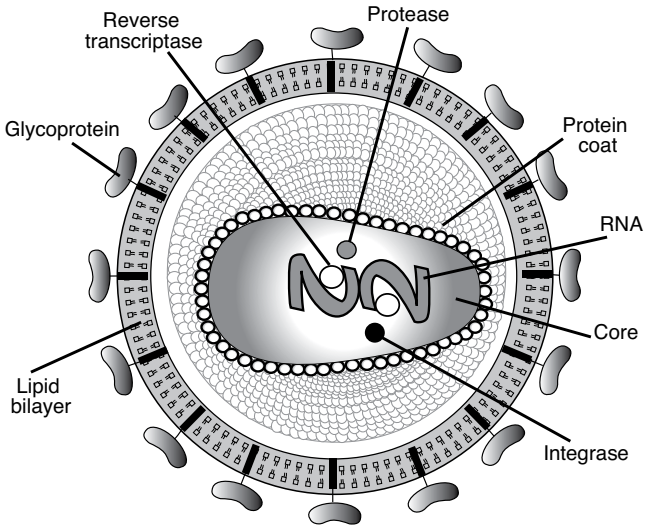
THE BIOLOGY OF HIV/AIDS

Acquired immune deficiency syndrome (AIDS) is a disease characterized by the progressive deterioration of an individual's immune system. The immunological impairment allows infectious agents such as viruses, bacteria, fungi and parasites to invade the body and propagate unchecked. In addition, the incidence of certain cancers dramatically increases in these patients because of faulty immunosurveillance. AIDS is a serious threat to human health and is a global problem. Intensive research is being done to advance methods of detection, clinical treatment and prevention.

THE HIV VIRUS

The AIDS etiologic agent is the human immunodeficiency virus type 1 (HIV-1), a retrovirus. HIV-1 contains an RNA genome and the RNA-dependent-DNA-polymerase termed reverse transcriptase. Members of the retrovirus family are involved in the pathogenesis of certain types of leukemias and other sarcomas in humans and animals. The structure and replication mechanism of HIV is very similar to other retroviruses. HIV is unique in some of its properties since it specifically targets the immune system, is very immunoevasive, forms significant amounts of progeny virus in vivo during initial stages of infection and can be transmitted during sexual activity.

The HIV viral particle is surrounded by a lipid bilayer derived from the host cell membrane during budding. The viral proteins are identified by the prefix gp (glycoprotein) or p (protein) followed by a number indicating the approximate molecular weight in kilodaltons. The lipid bilayer contains gp120 and gp41. These two proteins are proteolytic products of the precursor gp160. The gp41 anchors gp120 in the bilayer. The protein gp120 is routinely used as a diagnostic marker for HIV in Western Blot Analysis. More recently other viral gp proteins are also included in the test. Beneath the bilayer is a capsid consisting of p17 and p18. Within this shell is the viral core. The walls of the core consist of p24 and p25. Within the core are two identical RNA molecules, 9800 nucleotides in length. Hydrogen bonded to each viral RNA is a cellular tRNA molecule. The viral RNA is coated by tightly bound molecules of p7 and p9. The core also contains approximately 50 molecules of reverse transcriptase.



There are several other viral proteins whose precise functions are not fully understood. The virus can be grown in tissue culture for diagnostic and research purposes. Several of the viral proteins have been cloned and generated in relatively large quantities.

Background Information

An individual can receive an inoculum of HIV through an abrasion in a mucosal surface (e.g., genital and rectal walls), a blood transfusion, or by intravenous injection with a contaminated needle. Virus or virally infected cells are found in body fluids such as semen and blood. The most important target for the virus is hematopoietic cells such as bone marrow derived monocytes, myelocytes and lymphocytes. Infection of immune system effector cells such as T cells and macrophages ultimately produce the most profound clinical consequences. Gp120 binds to the CD4 receptors on the surface of T helper (TH) cells. These receptors are membrane bound glycoproteins involved in T cell activation. Under normal conditions CD4 acts as a receptor for major histocompatibility class II (MHC II) membrane bound molecules that are present on the surface of macrophages and several other types of cells. TH cells are required for the body's overall immunological responses. The viral lipid bilayer fuses with that of the cells' membranes and the viral protein capsid becomes internalized via receptor mediated endocytosis. Subsequently, the rest of the CD4 receptors are down-regulated and gp120 appears on the T cell surface. Through a complex mechanism, the reverse transcriptase synthesizes a double stranded DNA copy of the genomic RNA template. The tRNA molecule acts as the primer of the first strand synthesis. The RNAse H activity of the reverse transcriptase degrades the RNA strand of the RNA-DNA duplex and the enzyme synthesizes a complimentary DNA strand. The DNA reverse transcripts (double-stranded DNA) migrate into the cell nucleus where they become covalently integrated into the cellular genomic DNA. The integration is partly catalyzed by viral proteins. The copy DNA integrates via specific, self-complimentary sequences at both ends called long terminal repeats (LTRs). These sequences also have important functions in viral transcription. The integrated copy DNA is called proviral DNA or the provirus. The provirus enters a period of latency that can last for several years. The proviral DNA is replicated along with the cellular DNA and can be inherited through many cell generations. The HIV proviral DNA contains the major genes common to all non-transducing retroviruses. These genes are gag, pol and env. HIV-1 also contains five or six smaller genes.

Unlike other cellular DNA polymerases, HIV DNA polymerase (reverse transcriptase) has a high error rate. These frequent mutations continually change the viral protein epitopes. This is believed to be the main mechanism of HIV immunoevasion. The gag gene is translated into a polypeptide that is cleaved by a viral protease into four proteins that form the inner shells. The protease is encoded in the pol gene. The pol gene encodes the reverse transcriptase and the integrase which is responsible for the genomic incorporation of copy DNA. The env gene encodes the surface glycoproteins the viral particles acquire as they bud from the cells. Viral replication causes the destruction of the TH cells.

Enzyme linked immunoadsorbent assay (ELISA) is an important immunochemical method used for the detection of low levels of antigens. ELISA is used for clinical screening for HIV in the blood supply. ELISA testing for HIV detects the patient's circulating IgG directed toward the viral antigens. A

Background Information

positive reaction in the ELISA requires more definitive testing for verification by the use of a Western Blot. One reason for this problem is that antibodies sometimes exhibit cross reactivity.

PROPERTIES OF PROTEINS

Denaturing SDS gel electrophoresis separates proteins based on their size. SDS (sodium dodecylsulfate) is a detergent which consists of a hydrocarbon chain bonded to a highly negatively charged sulfate group. SDS binds strongly to most proteins and causes them to unfold to a rodlike chain and makes them net negative in charge. In the absence of a denaturing agent such as 2-mercaptoethanol, 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 intact polypeptide chains are called denatured. Proteins which contain several polypeptide chains that are non-covalent bonds will be dissociated by SDS into separate, denatured polypeptide chains. Proteins can contain covalent cross-links 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. Treatment of proteins at 100°C for 3 minutes in the presence of high concentrations of reducing agents, such as 2-mercaptoethanol, will break disulfide bonds. This allows the SDS to completely dissociate and denature the protein.

During electrophoresis, the SDS denatured proteins migrate 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 is obtained by the comparison of its position after electrophoresis to the positions of standard SDS denatured proteins electrophoresed in parallel.

WESTERN BLOT ANALYSIS

Western Blot Analysis involves the direct transfer of protein bands from an agarose or polyacrylamide gel to a charged nylon membrane for analysis. Following an electrophoresis experiment, the gel is removed from the tray and the nylon membrane is placed directly on the gel. (Nylon membranes are much stronger and more pliable than gels and can undergo many manipulations without tearing.) Protein bands are transferred to the surface of the nylon membrane and are adsorbed on the membrane by hydrophobic bonds. This transfer is achieved electrophoretically in specially designed chambers, by capillary flow or by the application of vacuum.

Background Information

The total protein transferred can then be visualized by staining the membrane with protein dyes. Visualizing a specific protein within a mixture of proteins is usually detected by immunochemical methods. It cannot be detected by protein staining because the amount may be too low and the banding of the protein mixture may block it from view.

For immunological detection of specific protein, the unstained membrane is placed in a blocking buffer that contains detergent and protein that bind to all unoccupied sites on the nylon membrane. The membrane is then incubated in buffer that contains antibody to one (or more) of the blotted proteins. The antibody binds to the adsorbed protein (antigen) and subsequent washings removes unbound antibody. A secondary antibody that is covalently linked to an enzyme such as alkaline phosphatase or horseradish peroxidase is used for detection. The conditions for cross-linking the enzyme to the secondary antibody does not appreciably affect the antigen binding specificity, the affinity of the antibody, or the catalytic activity of the enzyme.

The membrane is then incubated in a solution of the secondary antibody where it will bind selectively to the bound antigen-primary antibody complex. Following this treatment, the membrane is washed to remove the unbound secondary antibody-enzyme complex and is then incubated in a solution containing a phosphatase or peroxidase substrate. The products of the enzymatic reaction yield chromogenic products that are easily visible on the nylon membrane.

In this experiment, students will use a modified Western Blot Analysis to detect a specific protein.

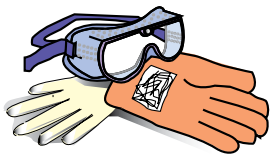
The Experiment

Experiment Overview and General Instructions

EXPERIMENT OBJECTIVE:

The objective of this laboratory is to understand the concepts and methodology involved with Western blots. The experiment will test for the presence of simulated viral proteins from hypothetical cell cultures infected with serum from HIV seropositive individuals.

LABORATORY SAFETY



1. Wear gloves and goggles routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

Protein-Agarose Gel Electrophoresis

Note: When preparing the 2.5% protein agarose gel, make sure to use the 1x Tris-glycine buffer prepared by your instructor (DO NOT use the 1x Tris-glycine SDS electrophoresis running buffer).

A. PREPARATION OF 2.5% PROTEIN AGAROSE GEL

1. To make 2.5% agarose in 1X Tris-glycine Buffer, determine volume of agarose required for your gel tray. Refer to Table A, below, to determine volume required.

Table A: 2.5% Protein Agarose Gel

Size of EDVOTEK Casting Tray	Amt of Agarose	+	Volume 1X Tris-glycine Buffer	=	Total Volume
7 x 7 cm	0.5 gm		20 mL		20 mL
7 x 14 cm	1.0 gm		40 mL		40 mL

2. Add the required amount of protein agarose powder to the required volume of Tris-glycine buffer. Swirl to disperse clumps.

Protein-Agarose Gel Electrophoresis

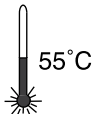
3. With a marking pen, indicate the level of the solution volume on the outside of the flask.

4. Heat the mixture to dissolve the agarose powder. The final solution should be clear (like water) without any undissolved particles present.

   a. Microwave method:
   - Cover flask loosely with plastic wrap to minimize evaporation. Do not cover tightly.
   - Heat the mixture on High for 1 minute.
   - Swirl the mixture and heat on High in bursts of 25 seconds until all the agarose is completely dissolved.

   b. Hot Plate method:
   - Cover the flask with foil to minimize evaporation.
   - Heat the mixture to boiling with occasional stirring. Boil until the agarose is completely dissolved.

5. Cool the agarose to 55°C with swirling to promote even dissipation of heat. If detectable evaporation has occurred, add hot distilled water to bring the volume of the solution up to the original volume as marked on the flask.



After the agarose solution has cooled to 55°C:

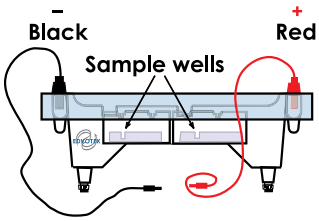
CAUTION:
Melting at high temperatures or without stirring will result in scorching the agarose.

6. Seal the ends of the gel tray with rubber dams or tape.

7. Pipet the cooled agarose solution into the bed. Make sure the bed is on a level surface. Place comb(s) in appropriate slots.

8. Allow the gel to solidify. It will become firm and ready for electrophoresis in approximately 20 minutes.

Protein-Agarose Gel Electrophoresis



B. PREPARATION OF ELECTROPHORESIS APPARATUS

1. Remove comb(s) and dams or tape. Place gel tray in electrophoresis chamber with the wells closest to the negative electrode.

2. Cover gel with prepared 1x Tris-Glycine-SDS Electrophoresis Buffer. Refer to Table B, below, to determine volume required.

Table B: 1x Tris-Glycine-SDS Electrophoresis (Chamber) Buffer

EDVOTEK Model #	Concentrated Buffer (10x)	+	Distilled Water	=	Total Volume
M6+	30 mL		270 mL		300 mL
M12	40 mL		360 mL		400 mL
M36	100 mL		900 mL		1000 mL

C. PREPARATION OF PROTEIN SAMPLES

The samples for electrophoresis were shipped in lyophilized (freeze-dried) form.

- If samples A - E have already been rehydrated and heated by your lab instructor, proceed with Protein-Agarose Gel Electrophoresis as outlines on page 11.

- If the samples A – E have not been rehydrated and heated by your lab instructor, follow the heating procedure (Steps 1-2) to heat the samples.

1. Bring a beaker of water, covered with aluminum foil, to a boil. Remove from heat.

2. Make sure the sample tubes A through E have been rehydrated and are tightly capped and well-labeled. The bottom of the tubes should be pushed through the foil and immersed in the boiling water for 5 minutes. The tubes should be kept suspended by the foil.

3. Proceed to loading the gel while the samples are still warm.

Note: Upon completion of loading the samples for electrophoresis, the unused portions of the protein samples should be frozen. Remove the samples from the freezer and follow steps 1-3 above to re-heat and run the samples when using them at a later time.

Protein-Agarose Gel Electrophoresis

NOTE:
Change pipet tips between each loading. Make sure the wells are clear of practice gel load solution. Wear gloves and safety goggles.

D. LOADING OF PROTEIN SAMPLES

Load 20 µL of each of the protein samples in the following manner (7 x 7 cm gel):

Lane	Tube	Protein sample
1	A	Positive control
2	B	Negative control
3	C	Patient 1
4	D	Patient 2
5	E	Patient 3
6	F	Standard Molecular Weight Dye Markers

E. RUNNING THE GEL

1. After the samples are loaded, carefully snap the cover all the way down onto the electrode terminals.

2. Insert the plug of the black wire into the black input of the power supply (negative input). Insert the plug of the red wire into the red input of the power supply (positive input).

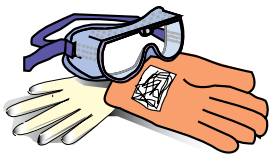
3. Set the power supply at the required voltage and run the electrophoresis for the length of time as determined by your instructor. When the current is flowing, you should see bubbles forming on the electrodes.

4. Proceed with electrophoresis until the blue tracking dye has traveled at least 4 - 4.5 cm from the wells. Proceed to the step setting up the Western Blot.

Table C: Time and Voltage

Volts	Recommended Time	
	Minimum	Optimal
125	25 min	60 min
70	60 min	1.5 hrs

Capillary Western Blot Analysis



Wear Gloves - Do Not Touch The Nylon Membrane With Bare Hands.

F. SETTING UP THE WESTERN BLOT

1. Place a piece of plastic wrap on your bench top. Be sure it is smooth and flat. The filter paper, gel, membrane, and paper towels will be placed onto it to make the blotting sandwich.
2. Wearing gloves, carefully remove the cover sheets from the (white) Western Blot membrane. Using forceps, transfer the membrane to a plastic tray.
3. Pre-wet the membrane (7 x 7 cm) by immersing it in approximately 20 mL of 95-100% Methanol for 10 seconds. Pour and save the Methanol.
4. Immediately immerse the membrane in distilled water for about 5 minutes to remove the Methanol.
5. Pour off the water and immerse the membrane in diluted Transfer Buffer. Let the membrane sit until needed for the gel, at least 10 minutes.
6. Remove the gel from the tray and immerse the gel into a separate tray that contains the Transfer Buffer. Soak for 10 to 15 minutes.
7. Saturate 1 piece of 7 x 7 cm filter paper with Transfer Buffer. Place the filter paper on the plastic wrap on your bench.
8. Wear gloves and carefully remove the gel from the Transfer Buffer and place upside down on the filter paper. Roll a pipet over the surface to remove air bubbles that may be trapped under the gel.
9. Pipet 1 to 2 mL of Transfer Buffer over the top of the gel and place the Western Blot membrane over the gel. **NOTE: If the membrane appears to have a smooth surface on one side and a rough surface on the other side, make sure the rough surface is in direct contact with the gel.** Roll a pipet over the surface to remove air bubbles.
10. Use a pencil to lightly trace the location of each of the bands in lane 6. Beside each mark, indicate the color of the respective band. (B1 = Blue 1, B2 = Blue 2, P = Purple, and R = Red).
11. Saturate 1 piece of filter paper with Transfer Buffer and cover the membrane with the wet filter paper. Roll a pipet over the surface to remove air bubbles.
12. Add the second piece of dry filter paper to the top of the stack. Remove air bubbles.
13. Evenly place a stack of 7 x 7 cm paper towels 4 to 6 cm in thickness on top of the stack.
14. Place a plastic tray or plate on top of the stack. Place a light weight beaker (400 mL size) on top. Allow the protein transfer to proceed overnight or for a minimum of 4 hours.

Capillary Western Blot Analysis

NOTE:
Areas of the membrane touched by ungloved hands will leave oil residues and will not bind protein during transfer. Many gloves contain powder which will increase the background on the membrane. Put on gloves and wash them under tap water to remove any residual powder.

G. PROCESSING AND STAINING OF THE BLOT MEMBRANE

1. Remove the tray, paper towels and filter paper from the top of the membrane. Leave the membrane in place on top of the gel.
2. Using a pencil, lightly trace the outline of each well and number according to loading sequence. **(Remember the gel is upside down.)**
3. Using forceps, remove the membrane from the gel and place it on a clean piece of filter paper. **The side that was in contact with the gel should be facing up.**
4. With a pencil, on one of the lower corners write "F" (for front). On the other corner, write your group number or initials. Place the membrane in a 65°C incubation oven for 10 minutes to fix the samples.

OPTIONAL STOPPING POINT

After the membrane has been fixed, the membrane can be held for several days before staining.

REMEMBER:
The Western HIV/AIDS diagnostic test establishes the presence of the viral coat protein gp120 by antibody interaction and also confirms the size of the protein band to be 120,000 daltons ±10%.

No human or viral HIV/AIDS or derivative materials are used in this experiment.

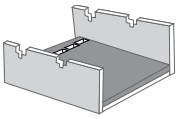
5. Very briefly wet the membrane in Methanol so that there are no dry areas.
6. Transfer the membrane to distilled water for about 5 minutes.
7. Immerse the membrane in 50-75 mL of the "Staining Solution". The membrane should be completely covered with liquid.
8. Wearing gloves, gently float one card of the Protein InstaStain® with the stain side (blue) facing the liquid.
9. Let the membrane soak undisturbed in the liquid for 15 minutes with occasional agitation. NOTE: If the color of the membrane appears very light, continue with the staining procedure until the membrane has a dark blue appearance.
10. Discard the Protein InstaStain®. With forceps, transfer the membrane to a tray of Methanol and gently move the membrane in the alcohol to destain. Look for the positive control to appear. When the bands are evident, immediately transfer the membrane to a tray of distilled water.
11. After several minutes, remove membrane from the distilled water, lay on a piece of filter paper.
12. Compare the three patient samples to the positive and negative controls.

EDVO-Kit # 275

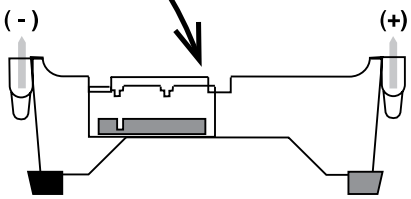
AIDS Kit II: Simulation of HIV-1 Detection by Western Blot

The Experiment

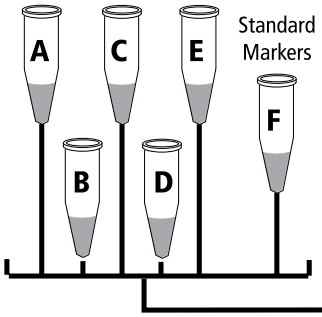
Prepare agarose gel in casting tray



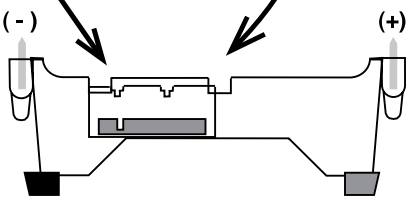
1. Remove comb and submerge gel under buffer in electrophoresis chamber.



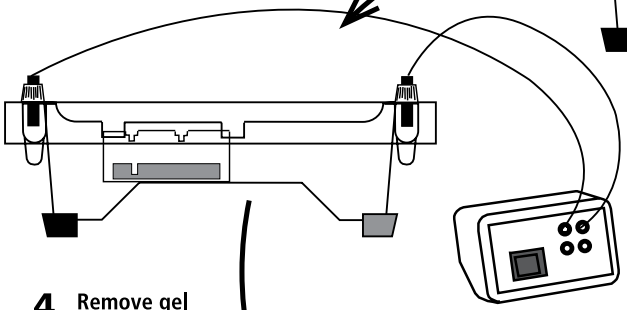
Samples for Electrophoresis

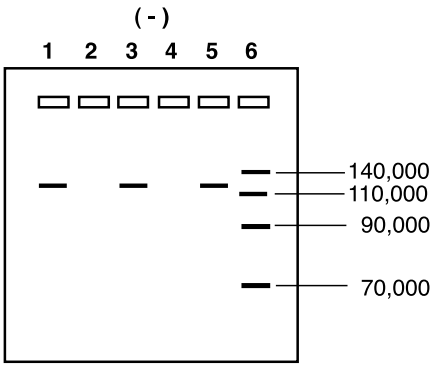


2. Load 20 µl of each sample into consecutive wells.

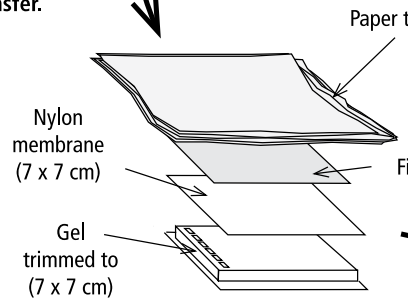


3. Snap on safety cover, connect leads to power source and initiate electrophoresis.





4. Remove gel and initiate Southern Blot transfer.



5. Stain and analyze results transfered to membrane.

Molecular Weight Estimation of Simulated Viral gp120 Protein

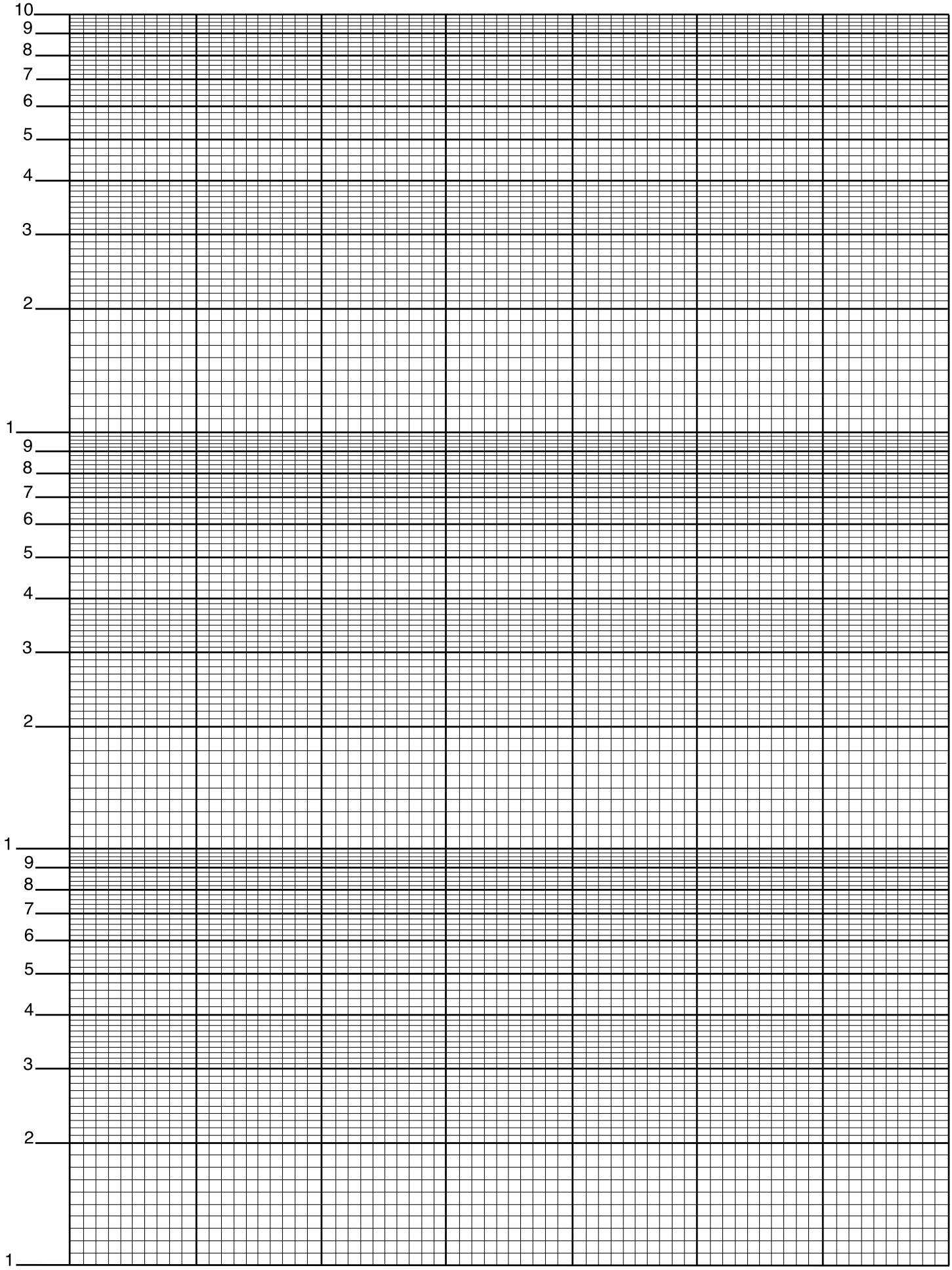
1. Measure the distance of each band traced on the membrane for the standard dye markers and the positive viral samples. Each measurement should be from the bottom of the well to the bottom of each band.

   The sizes for the standard dye markers are (in daltons):

Blue 1	140,000
Blue 2	110,000
Purple	90,000
Red	70,000

2. Plot using a semilog graph paper the distance travelled by each band on the x-axis and their molecular weight on the y-axis.

3. Determine the molecular weight of the viral protein by the extrapolating from the standard curve.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Experiment Results and Study Questions

LABORATORY NOTEBOOK RECORDINGS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

STUDY QUESTIONS

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Why are the electrophoretically fractionated proteins transferred to a membrane for detection?

2. Would higher or lower percentage gels favor transfer to a membrane? Would larger or smaller proteins transfer better?

3. What is the purpose of the negative and positive controls?

4. What is the difference between a Western, Northern and Southern Blot?

Notes:

Instructor's Guide

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

Pre-Lab Preparations

PREPARATION OF MEMBRANES
(Any time before the lab - required first day)

Wear rinsed and dried lab gloves. Powders from gloves will interfere with the procedure.

1. Keep protective cover sheets around the membranes and make sure the cover sheets and membrane are all aligned. **Keep the membrane covered this way during all the following steps.**

2. Divide the covered membrane into six 7 x 7 cm squares by drawing pencil lines on the upper cover sheet.

   If you are using gels that are smaller or larger than 7 x 7 cm, you must adjust the dimensions of your membrane squares accordingly. You may also have to alter the sizes of the filter paper and towels the students prepare. Larger gels may necessitate fewer groups.

3. Cut the covered membranes on the lines to produce six squares. Make sure the sheets are aligned before cutting.

4. Store the prepared, covered membranes at room temperature until ready to use.

PREPARATION OF BUFFERS
(On the day of the lab)

Preparing the Tris-Glycine Buffer (for gel preparation only)

NOTE: *In an effort to aid the environment, EDVOTEK has reduced the shipping weight and plastic necessary for shipment of liquid buffer. This powdered buffer can be dissolved on site and used for gel preparation.*

1. Add the powder contents of the Tris-Glycine Buffer to a flask or beaker (500 mL size or larger).

2. Add 300 mL distilled or deionized water to the powdered buffer. Swirl and stir to dissolve the powder (a stir plate, if available, may be useful). This is 1x buffer, ready for use in preparing the gel(s).

3. Continue with gel preparation as indicated in the kit instructions (p. 9)

Transfer Buffer (required first day)

1. To 350 mL of distilled water, add 50 mL of 10X Tris-Glycine-SDS liquid concentrate.

2. Add 100 mL of 95 - 100% methanol to the buffer. Mix. Keep tightly covered at room temperature until ready to use.

Pre-Lab Preparations

Electrophoresis Buffer, Tris-glycine-SDS Buffer

1. Add 1 part EDVOTEK® 10X Tris-Glycine-SDS buffer to every 9 parts distilled or deionized water.
2. Make enough 1X buffer for all electrophoresis units used. The approx. volume of 1X electrophoresis buffer required for EDVOTEK Horizontal Electrophoresis Chambers are listed in Table A on page 9.

Notes:
Store any unused portion of reconstituted sample at -20°C and repeat steps 2 - 4 when using samples at a later time.

This experiment kit contains practice gel loading solution. If you are unfamiliar with gel electrophoresis, it is suggested that you practice loading the sample wells before performing the actual experiment.

PREPARATION OF LYOPHILIZED PROTEIN SAMPLES FOR ELECTROPHORESIS

(On the day of the lab - Required first day)

1. Add 130 µL of distilled or deionized water to each tube (A-E). Incubate the samples at room temperature for 5 minutes. Vortex or mix vigorously.
2. Bring a beaker of water, covered with aluminum foil, to a boil. Remove from heat.
3. Make sure the sample tubes A through E are tightly capped and well labeled. The bottom of the tubes should be pushed through the foil and immersed in the boiling water for 5 minutes. The tubes should be kept suspended by the foil.

Note: DO NOT boil the Standard Molecular Weight Dye Markers (Comp. F).

4. Remove the sample and tap or briefly microcentrifuge to get condensate at the top of the tubes back into the sample.
5. Aliquot 20 µL of each sample (A-E) for each lab group. Have students load samples onto the gel while the samples are still warm to avoid aggregation.

Time and Voltage

Volts	Recommended Time	
	Minimum	Optimal
125	25 min	60 min
70	60 min	1.5 hrs

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 the table to the left.

Run the gel until the bromophenol blue tracking dye migrates 4 - 4.5 cm from the wells.

Pre-Lab Preparations

CAUTION:
Do not use Methanol with acrylic materials. Methanol will destroy acrylic.

REPARATION OF STAINING SOLUTION

Several days before the lab or on the day of the lab (required second day).

1. To 200 mL of distilled water, add 250 mL absolute methanol, add 55 mL glacial acetic acid. Mix thoroughly. Label this "Staining Solution". Keep tightly covered at room temperature until ready to use.
2. Pour the solution into a glass tray. Do not use acrylic. Alcohols such as methanol will crack acrylic.

EACH LAB GROUP SHOULD RECEIVE THE FOLLOWING:

First Day

- **Boiled Components A-E (pre-aliquoted) & un-boiled Component F**
- **Components to prepare agarose gel (see Student Experimental Procedure)**
- **Practice gel loading solution (optional)**
- **20-30 mL 95-100% methanol**
- **Diluted Tris-Glycine-SDS electrophoresis buffer**
- **80 mL Diluted transfer buffer**
- **1 Western Blot membrane**
- **3 Filter paper pieces**
- **Paper towels and plastic wrap**
- **Small non-acrylic boxes for soaking membranes and gels**
- **20-30 mL Distilled water**
- **Pipet**
- **400 mL Beaker (weight for blot)**

Second Day

- **50-75 mL Staining Solution**
- **(1) Piece of Protein InstaStain®**

Avoiding Common Pitfalls

Potential pitfalls and/or problems can be avoided by following the suggestions and reminders listed below.

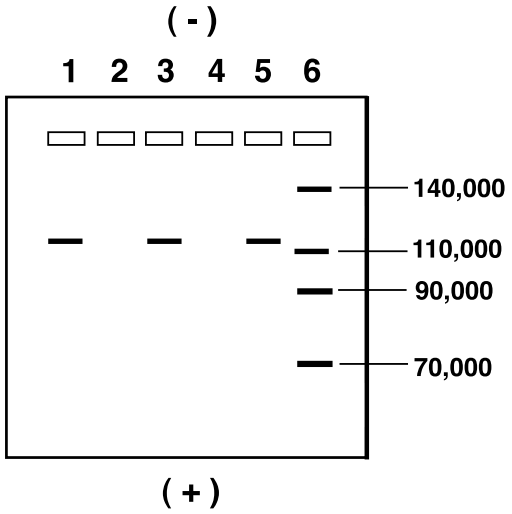
- To ensure that protein bands are well resolved, make sure the gel formulation is correct and that electrophoresis is conducted for the optimal recommended amount of time.
- Correctly dilute the concentrated buffer for preparation of both the gel and electrophoresis (chamber) buffer. Remember that without buffer in the gel, there will be no protein mobility. Use only distilled or deionized water to prepare buffers. **Do not use tap water.**
- For optimal results, use fresh electrophoresis buffer and Staining Solution prepared according to instructions.
- Before performing the actual experiment, practice sample delivery techniques to avoid diluting the sample with buffer during gel loading.
- To avoid loss of protein bands into the buffer, make sure the gel is properly oriented so the samples are not electrophoresed in the wrong direction off the gel.
- The protein marker used in this experiment is a dye that will not transfer to the membrane. It must be traced onto the membrane before blotting.

Experiment Results and Analysis

Samples containing the simulated HIV protein and the positive control should show a protein band. The positive control, Patients 1 and 3 show bands. The negative control and Patient 2 should not have any visible bands.

The idealized schematic shows the relative positions of the protein bands, but is not drawn to scale. The molecular weight of the viral glycoprotein for the positive control and the positive patients can be extrapolated from the standard curve. Students should plot the distance in millimeters traveled by each of the standard proteins on the x-axis and the respective molecular weights on the y-axis using semi-log graph paper.

Lane	Sample		Mol. Wt. (daltons)
1	A	Positive control	120,000
2	B	Negative control	—
3	C	Patient # 1 - positive	120,000
4	D	Patient # 2 - negative	—
5	E	Patient # 3 - positive	120,000
6	F	Standard dye markers	
		B-1 (Blue 1)	140,000
		B-2 (Blue 2)	110,000
		P (Purple)	90,000
		R (Red)	70,000



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