EDVOTEK®

THE **BIOTECHNOLOGY EDUCATION** COMPANY®

Edvo-Kit #
1115

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Detecting Risk Factors for Alzheimer's Disease using Western Blot

Experiment Objective:

The objective of this experiment is for students to understand the theory and application of western blotting. Students will perform a western blot to determine simulated clinical trial participants' risk of developing Alzheimer's Disease.

See page 3 for storage instructions.

Table of Contents

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

Experiment Components

	Component	Storage	Check ✓
A	Pre-stained Protein Standard Markers (lyophilized)	Refrigerator	❑
B	Negative Patient Sample	Refrigerator	❑
C	Positive Patient Sample	Refrigerator	❑
	Immunochemical and Blotting Reagents		
D	Anti-ApoE4 Protein Antibody	Refrigerator	❑
E	Secondary Antibody Conjugate	Refrigerator	❑
F	Hydrogen Peroxide, stabilized	Refrigerator	❑
G	Peroxide Co-substrate	Refrigerator	❑
H	10x Blocking Buffer	Refrigerator	❑
I	Powdered Milk	Room Temp.	❑
J	10x PBS	Room Temp.	❑
•	10x Tris-Glycine-SDS Buffer (Chamber Buffer)	Room Temp.	❑
•	Tris-Glycine Powdered Buffer (Transfer Buffer)	Room Temp.	❑
•	Practice Gel Loading Solution	Room Temp.	❑
•	Nitrocellulose Membrane	Room Temp.	❑
•	Filter Paper	Room Temp.	❑
•	Large Filter Paper (Wick)	Room Temp.	❑
•	50 mL tubes	Room Temp.	❑
•	15 mL tubes	Room Temp.	❑
•	1.5 mL tubes	Room Temp.	❑

This experiment contains enough reagents for 6 lab groups, with 2 groups sharing a gel.

Enough membrane is provided to accommodate blotting of three 8 x 10 cm gels.

Components A - H should be stored in the refrigerator. All other components can be stored at room temperature.

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.

None of the experiment components are derived from human sources.

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Experiment Requirements *(NOT included with this experiment)*

- 12% Denaturing Polyacrylamide gels (3)
- Vertical Gel Electrophoresis Apparatus (Cat. #581 recommended)
- D.C. Power Supply
- Shaker Platform (optional)
- Automatic Pipettes with Tips
- Hot plate, heating block, or Bunsen burner to boil proteins
- Microtest (Microcentrifuge) Tubes
- Beakers
- Transfer Pipets
- Graduated Cylinders
- Plastic Wrap
- Scissors
- Trays or Containers
- Forceps
- Several Packs of Paper Towels
- Latex or Vinyl Lab Gloves
- Safety Goggles
- Methanol
- Distilled Water

Background Information

In 1906, the German psychiatrist Alois Alzheimer reported the first case of Alzheimer's Disease in a 50 year old woman. Since then, Alzheimer's Disease (AD) has grown to be one of the most recognizable and tragic neurodegenerative diseases. AD is characterized by memory loss and impaired neurological function. It is most commonly diagnosed in populations over 65 years of age, and the risk of developing AD increases with age.

AD is characterized by memory loss and a decline in cognitive ability. The first signs and symptoms of AD are only mild cognitive impairments, usually attributed to stress or fatigue. A diagnosis for AD must be based on sustained short-term memory loss, family history, and behavioral observations over time. Unfortunately, brain imaging techniques including PET and MRI scans are not yet at the point where a diagnosis of AD can be definitely given simply be observing a brain scan. The only definitive diagnosis of AD is post-mortem, by examining brain tissue and looking for molecular markers of AD.

Pathophysiology of AD

The brain controls the entire body, from breathing to playing soccer to listening to music. It is a complex organ and is composed of approximately 100 billion cells. The brain is divided into areas, known as lobes, based on location and function. The 5 major lobes of the brain are the brainstem, parietal lobe, cerebellum, frontal lobe, and temporal lobe. The brainstem is responsible for all of the major functions that keep a person alive, such as heartbeat, blood pressure, and breathing regulation. The parietal lobe is responsible for processing sensory information. The cerebellum regulates motor function and movement. The frontal lobe is responsible for executive decision making and higher order processing, such as reading and math. The temporal lobe is responsible for a variety of functions, including memory. Within the temporal lobe lies an area known as the hippocampus. The hippocampus is where all new memories are formed.

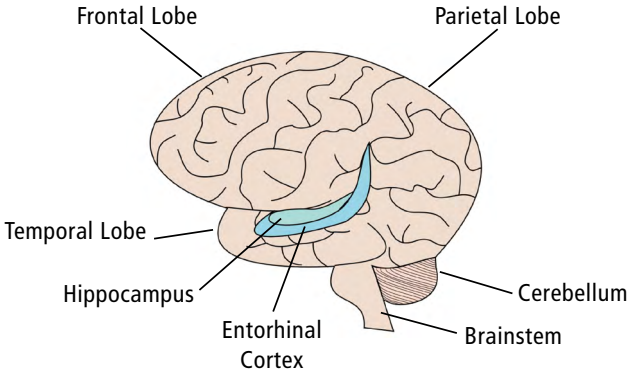


Figure 1: Areas of the Brain

In 1953, a temporal lobectomy (removal of the temporal lobe) was performed on Patient HM. Patient HM had intractable epilepsy, meaning he had seizures that could no longer be treated with medicine. The seizures were caused by overactive neurons in his temporal lobe, so his doctor decided to remove that area of his brain. While the surgery was successful and his seizures halted, he lost the ability to form new memories. All memories that he had before the surgery were intact, but he could not store any new information. This unfortunate surgery is how we now know that all new memories are formed in the hippocampus.

How exactly memories are stored in the brain is still not well understood. Memories are formed in the hippocampus, which sends information to the frontal lobe. The frontal lobe then processes this information and makes executive decisions. The area of the brain that connects the hippocampus and the frontal lobe is called the entorhinal cortex. The entorhinal cortex is able to send information back and forth between the hippocampus and the frontal lobe, making it a potentially important area for consolidating memories. For instance, if someone forgets what

they ate for breakfast or repeats themselves in a conversation, that memory was likely not consolidated correctly in the brain. This may be because the memory never made it from the hippocampus to the frontal lobe. It is now well-accepted that AD begins in the entorhinal cortex and progressively spreads throughout the brain.

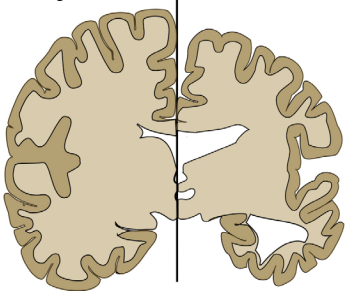


Figure 2: Healthy vs. Diseased Brain

What is happening within the entorhinal cortex during AD? Broad neuronal death is the major process that occurs during Alzheimer's Disease, and is caused by the accumulation of mutated and misfolded proteins. There are two major proteins that play a role in disease progression: A-beta and tau. A-beta is a small peptide (protein) that is cleaved from a larger protein known as Amyloid Precursor Protein (APP). When APP is cleaved by a certain protease, it forms A-beta peptides. A-beta peptides can all clump together to form plaques, or areas of dense A-beta accumulation. This can inhibit protein transport, mitochondrial activity, and many other processes that are critical to the health of a cell. Tau, on the other hand, is a microtubule protein. Microtubules are the "railroad tracks" of a cell, and tau binds to microtubules to stabilize them in the axon. Normally, tau is not found outside of the axon. However, during AD, tau is mislocalized to both the cell body and the dendrites. This mislocalized tau is also improperly regulated, and forms neurofibrillary tangles. These tangles are also very detrimental to the cell. When detecting AD in postmortem tissue, scientists use a stain for plaques and tangles. If these are present, it means that the patient had AD.

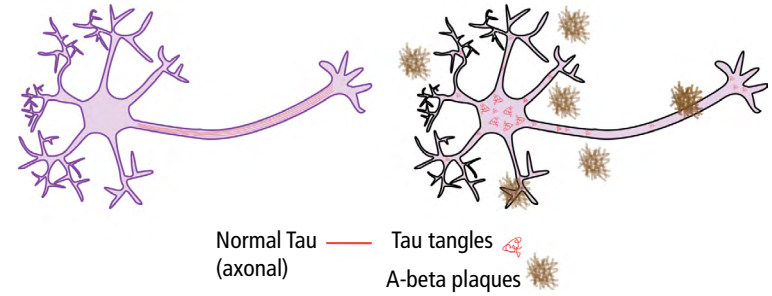


Figure 3: Healthy vs. Diseased Neurons

Between A-beta plaques and tau tangles, a diseased neuron becomes overwhelmed and dies off. Once a neuron dies, it can never regenerate. This is different from most other cells in the body. For example, if you cut your leg on a branch and begin to bleed, cells are able to come to the rescue, and you are eventually able to heal the skin back to normal. If there is an injury in the brain however, this does not happen and all functionality is lost. This is what makes brain injuries and neurodegenerative diseases so difficult to treat. It also makes them very difficult to treat, because neurons are very good at compensating for each other. If a few neurons die, it could be difficult to tell because so many neurons perform similar functions. Therefore, symptoms may not show until many neurons have died. Most clinical trials for drugs to treat AD begin upon diagnosis. However, it has been shown that by the time someone is diagnosed the disease has already irreversibly damaged the brain. Therefore, many scientists and research groups are working on tests which can detect and diagnose AD earlier, before the brain has massive neuronal loss.

Risk factors for AD

Age is the single greatest risk factor for developing AD. However, genetics also plays a strong role. Specifically, the variant of the gene ApoE can either increase or decrease a person's risk for AD. There are 3 different ApoE alleles: ApoE2, ApoE3, and ApoE4. ApoE3 is the most common allele, and does not seem to have an effect on a person's risk for developing AD. The E2 allele, however, can reduce someone's risk for AD, while E4 can increase it. Currently, there is no medication for AD, therefore testing is only done to enhance clinical trials. For instance, if a research

group is recruiting for a clinical trial for a drug to treat AD, they might group people according to their ApoE allele status to test their drug vs. placebo.

Scenario:

Your research group has found a compound that works to prevent AD progression in mice. The next step is a clinical trial in humans. In order to do all of the possible controls, you will want to have equal numbers of people with the ApoE4 allele in both your drug and your placebo group.

Because all 3 alleles are the same molecular weight, they cannot be separated by size. Additionally, primers are unable to be designed specifically for each allele because they differ in only a few amino acids. However, a western blot can be performed to see which protein is present because of the specificity antibodies have towards certain proteins.

Western Blot Analysis

In Western Blot analysis, the first step is running a denaturing polyacrylamide gel. Similarly to how agarose gel electrophoresis can separate DNA fragments based on size, polyacrylamide gel electrophoresis can separate proteins based on size. Unlike DNA, which always consists of nucleotides arranged in a double-helix structure, the amino acids of proteins can take on large and complex configurations. In order for these proteins to be accurately resolved by their size, they must first be denatured, or unraveled from their 3-dimensional structure.

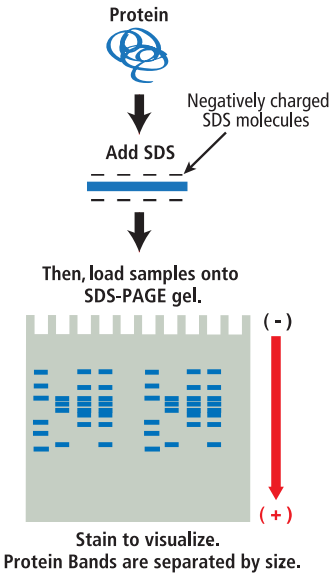


Figure 4:
SDS Gel Electrophoresis

Denatured proteins have lost their specific folding patterns and biological activity, but their amino acid chain remains in tact. In most cases, the proteins are denatured through boiling in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. SDS is a detergent consisting of a hydrocarbon chain bound to a negatively charged sulfate group. SDS binds to amino acids, giving the entire protein a net negative charge. Additionally, SDS binding causes proteins to unfold and helps in the denaturation process. However, even with SDS, some proteins have very strong bonds between amino acids, including 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 2-mercaptoethanol, will break disulfide bonds. This allows the SDS to completely dissociate and denature the proteins.

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. This is because the polyacrylamide gel serves as a sort of maze for the proteins to travel through. The smaller the proteins, the easier and faster they transport through the gel. The molecular weight of an unknown protein is obtained by the comparison of its position on the gel after electrophoresis to the positions of a standard SDS protein ladder.

The second step of Western Blot Analysis involves the direct transfer of protein from a polyacrylamide gel to a charged nitrocellulose sheet. It is advantageous to transfer proteins to a membrane because membranes are much stronger and more pliable than gels. The charged nitrocellulose membrane binds proteins with a high affinity, and proteins can migrate out of the gel and onto the sheet. After transfer, total protein can be visualized by staining the membrane with protein specific dyes, or specific proteins can be detected by immunochemical methods.

For immunological detection, the membrane is placed in blocking buffer which contains detergents and blocking proteins that bind to all unoccupied sites on the membrane. The membrane has a limited protein-binding capacity, so by saturating the sites with a non-specific protein mixture, such as milk proteins, ensures that no accidental contamination of the membrane occurs. The membrane is then incubated in buffer that contains antibody to one or more of the blotted proteins. The primary antibody binds specifically to the adsorbed protein antigen. Subsequent washings will remove excess, unbound antibody. A secondary antibody against the primary antibody is then applied. This secondary antibody recognizes the primary antibody and is often linked to an enzyme such as horseradish peroxidase for detection. It is critical that all non-bound secondary antibody is washed off, to be sure that the detection is specific to secondary antibody that is bound to the primary antibody. Finally, the membrane is incubated with a solution containing peroxidase substrates that develops a brown color when it interacts with the horseradish peroxidase on the secondary antibody.

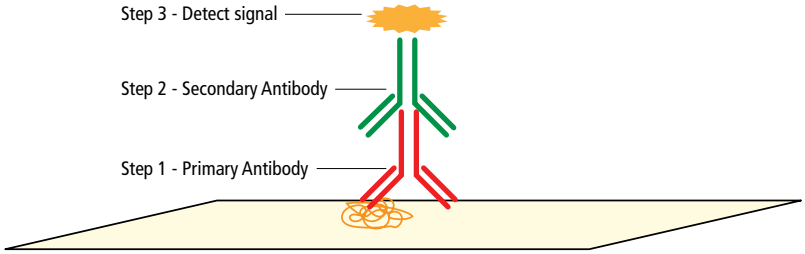


Figure 5: Western Blot Detection

In this experiment, you will be running a western blot with simulated patient samples to determine which of your clinical trial participants are positive for the ApoE4 protein, and thus allele.

Experiment Overview

EXPERIMENT OBJECTIVE

The objective of this experiment is for students to understand the theory and application of western blotting. Students will perform a western blot to determine simulated clinical trial participants' risk of developing Alzheimer's Disease.

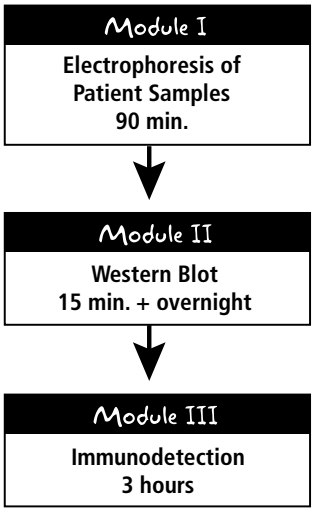
LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn 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.

LABORATORY NOTEBOOKS

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:

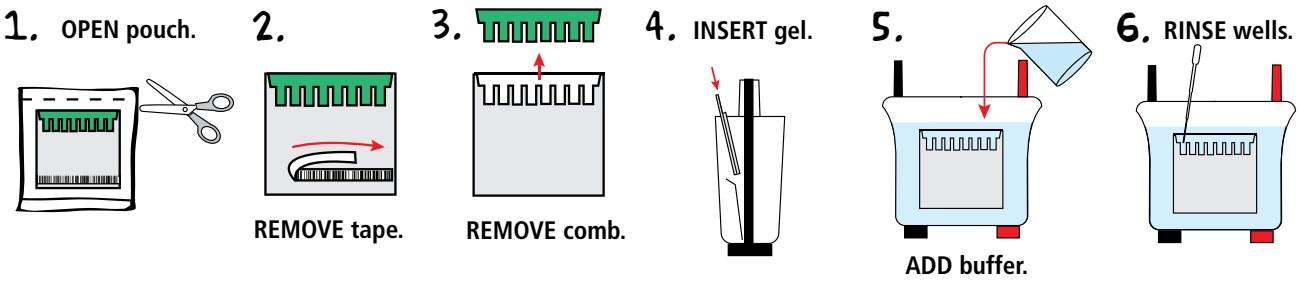
- Record (draw) your observations, or photograph the results.

After 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.

Module I: Electrophoresis of Patient Samples

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.

Wear gloves and safety goggles

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 or sample loading.

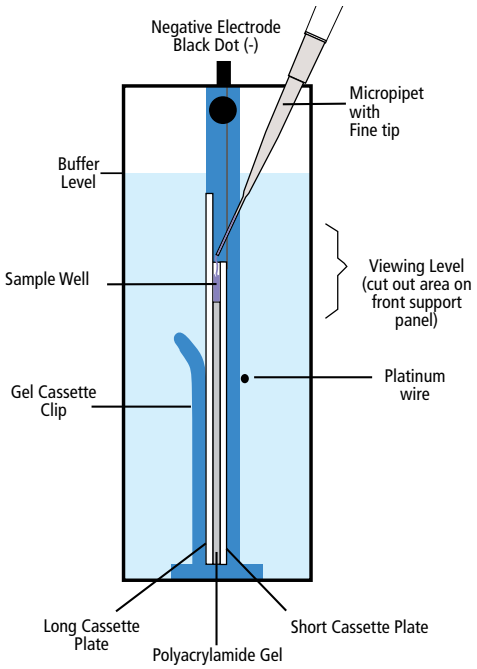
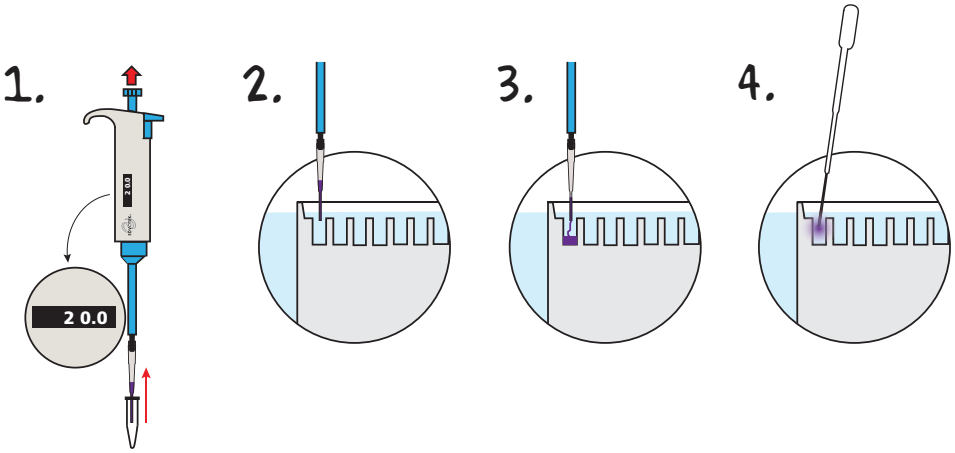


Figure 6: A polyacrylamide gel cassette in the EDVOTEK® Vertical Electrophoresis Apparatus, Model #MV10.

Module I: Electrophoresis of Patient Samples, continued

PRACTICE GEL LOADING (OPTIONAL)

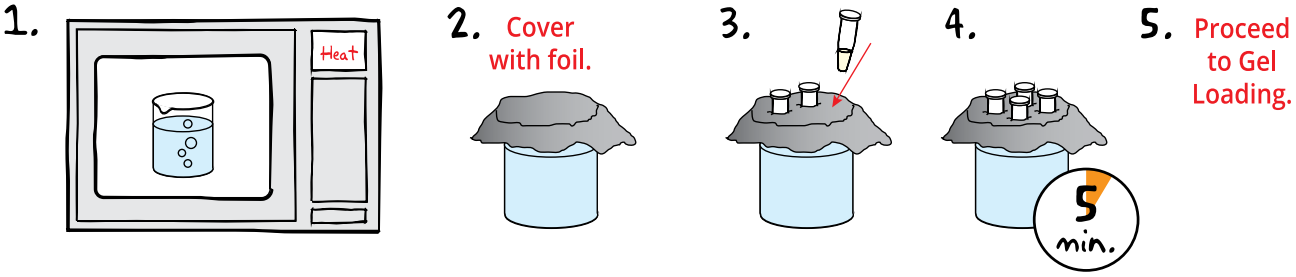



Wear gloves and safety goggles

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 I: Electrophoresis of Patient Samples, continued

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. The samples can be aliquoted into individual microcentrifuge tubes or placed at a classroom pipetting station for students to share.

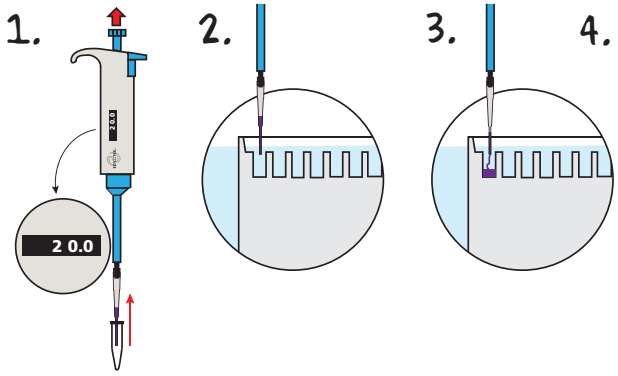
Wear gloves and safety goggles

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 I: Electrophoresis of Patient Samples, continued

LOADING THE PROTEIN SAMPLES



Two student groups can share one gel. The first group should load samples into wells 1 - 5. The other group sharing the gel should load samples into wells 6 - 10 (see Table 1).

1. Using a fresh fine tip micropipette tip, **MEASURE** 20 µL of the first sample as indicated in Table 1. Sample A is the protein standard markers and should go in the first lane of your group's half of the gel.
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 for remaining samples in Table 1, changing the tip between each new sample.

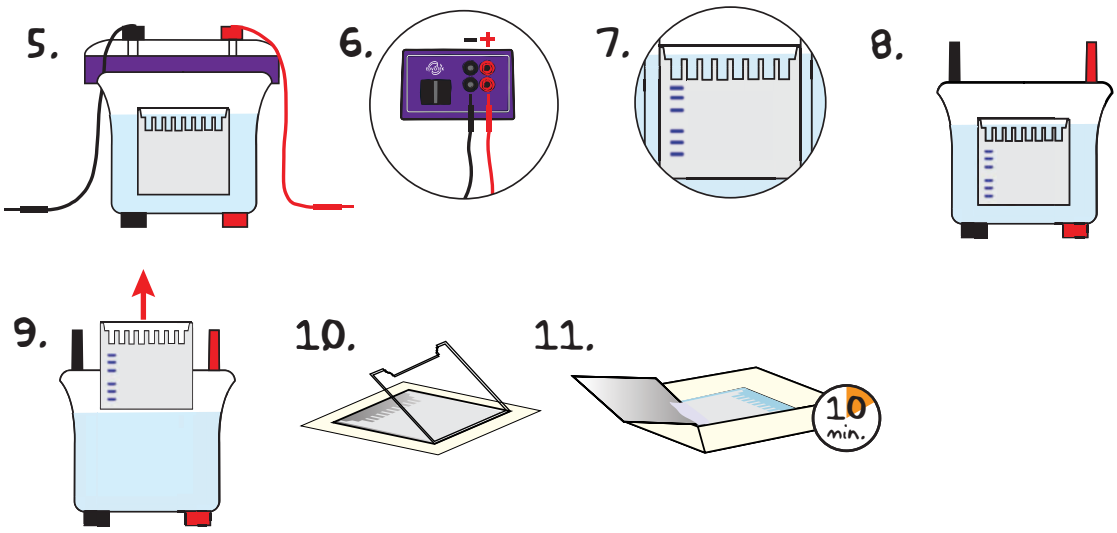
NOTE: Be sure to change pipette tips between loading each sample!

Table 1: Gel Loading

Lane	Sample
1	Protein Standard Markers (Group 1)
2	Patient 1 (Group 1)
3	Patient 2 (Group 1)
4	Patient 3 (Group 1)
5	Patient 4 (Group 1)
6	Protein Standard Markers (Group 2)
7	Patient 1 (Group 2)
8	Patient 2 (Group 2)
9	Patient 3 (Group 2)
10	Patient 4 (Group 2)

Module I: Electrophoresis of Patient Samples, continued

RUNNING THE GEL



5. Once all samples have been loaded, carefully **PLACE** the cover onto the electrode terminals.
6. **CONNECT** the electrical leads to the power supply.
7. **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. ***Note: When the current is flowing, you should see bubbles forming on the electrodes.***

Table A — Time and Voltage Guidelines

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

8. After the electrophoresis is finished, **TURN OFF** the power supply, disconnect the leads, and carefully **REMOVE** the cover.
9. **REMOVE** the gel cassette from the electrophoresis apparatus and **BLOT** off excess buffer with a paper towel.
10. **REMOVE** the front plate by:
    a. Lay cassette down on the table and locate the gap between the front and back plates on the corners of the cassette.
    b. Use a spatula, or a similarly thin object, to torque the plates apart at the upper left corner.
    c. Repeat in all 4 corners of the plate and, if necessary, in the center of the left and right sides of the cassette.
    d. Continue to repeat b and c until the 2 plates loosen apart.
    e. Carefully remove the top plate.
11. **PLACE** the gel in transfer buffer and carefully **REMOVE** the gel from the back plate. **SOAK** for 10 minutes. The gel is now ready for the Western Blot Procedure.

Module II: Western Blot Procedure

1. **PRE-SOAK** the wick, blotting paper, and nitrocellulose membranes in transfer buffer for 5-10 min. When handling nitrocellulose membranes, be sure to handle them by their edges. Carefully **SLIDE** the membrane out of the blue cover and transfer using forceps to the transfer buffer.
2. **PLACE** the large gel plate on top of a container approx. 16 x 9 x 4 cm (L x W x D). **ADD** transfer buffer to the tray and **PLACE** the presoaked wick onto the gel plate such that the ends are submerged in 2 cm of transfer buffer (see Figure 7).

Wear gloves when handling nitrocellulose membrane to avoid transferring oil from your skin which will interfere with the protein transfer.

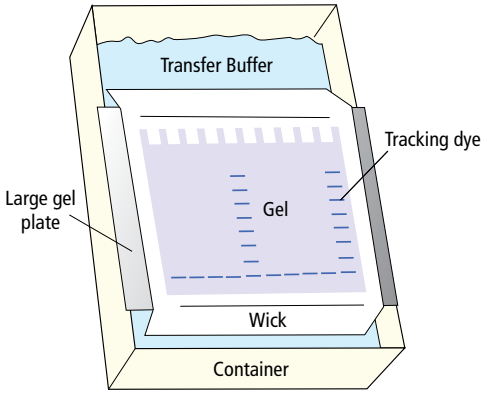


Figure 7: Top view of the blot transfer assembly.

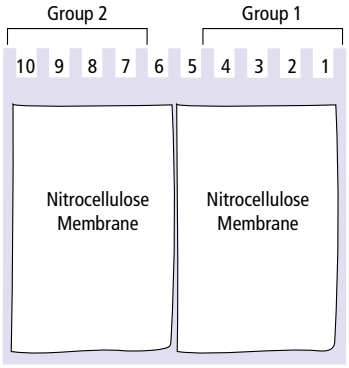


Figure 8: Placing the nitrocellulose membranes.

3. **FLIP** the gel so that the ladder is now on the right, and **PLACE** gel flat on top of wick. Take care not to rip the gel. **SMOOTH** over top of gel to remove air bubbles.
4. **PLACE** nitrocellulose membranes on top of the groups' respective gel lanes (Figure 8).
5. **PLACE** the two pieces of blotting paper (from step 1) on top of the membrane. **SMOOTH** over to remove all air bubbles underneath.
6. **PLACE** a 6 cm stack of paper towels on top of blotting paper. Finally, **PLACE** a 1 kg weight on top of stack to complete assembly, as shown in Figure 9.

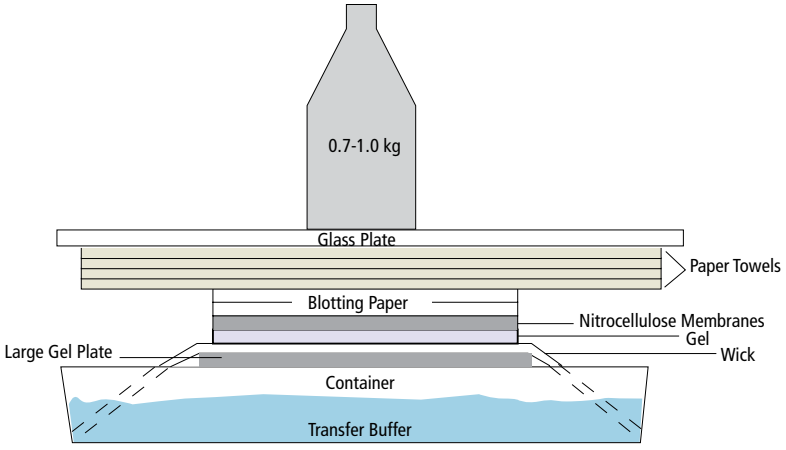
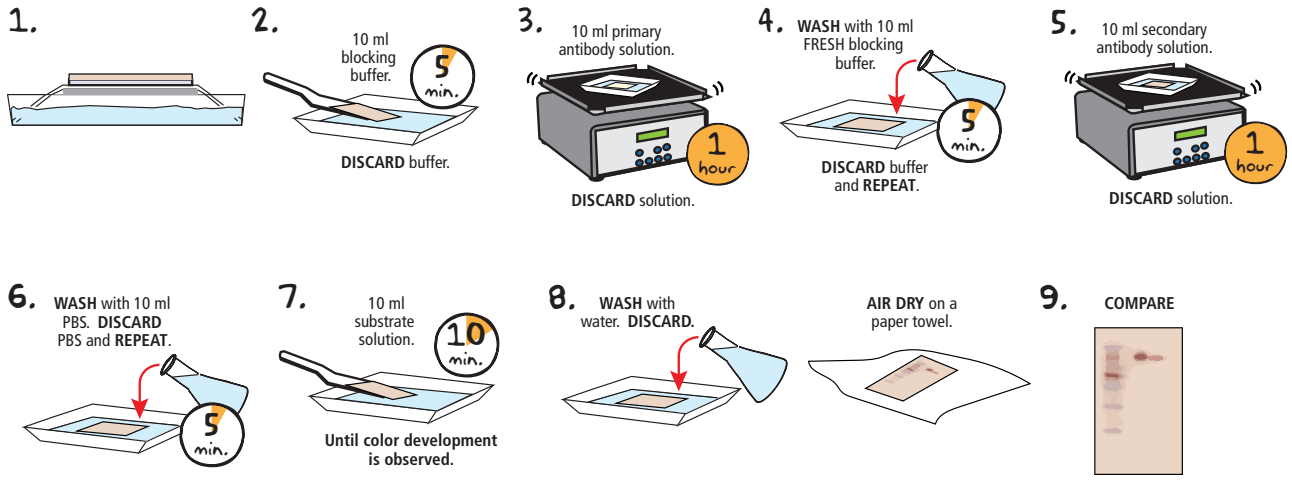


Figure 9: Side view of the blot transfer assembly.

STOPPING POINT

Allow transfer to take place overnight (12-15 hours).

Module III: Immunodetection



1. **DISMANTLE** the stack above the membrane. Carefully **REMOVE** the membrane from the gel with forceps or a spatula. **VERIFY** that the transfer occurred by the presence of prestained protein standard markers on the membrane.
2. **TRANSFER** the group's membrane to a small tray or small sealable plastic bag containing 10 mL blocking buffer (membrane should be submerged) and occasionally agitate for 5 minutes. **DISCARD** the blocking buffer.
3. **ADD** 10 mL of primary antibody solution which has been prepared by your instructor. **INCUBATE** for one hour at room temperature on a rotating or shaking platform. **DISCARD** the primary antibody solution.
4. **WASH** the membrane for 5 minutes in 10 mL blocking buffer. **DISCARD** the blocking buffer. **REPEAT** wash.
5. **ADD** 10 mL of secondary antibody solution which has been prepared by your instructor. **INCUBATE** for one hour at room temperature on a rotating or shaking platform. **DISCARD** the secondary antibody solution.
6. **WASH** the membrane for 5 minutes in 10 mL PBS. **DISCARD** the PBS. **REPEAT** wash.
7. **ADD** 10 mL substrate solution which has been prepared by your instructor. **INCUBATE** for 5-10 minutes or until color development is observed. **DISCARD** the substrate solution.
8. **WASH** membrane with water and then air dry on a paper towel. Alternatively, you can blot the edge of the membrane onto a paper towel.
9. **COMPARE** the patient samples to determine which are positive for ApoE4 and which are negative.

NOTE:
The lid from a micropipette rack (200 µL size) works well for a tray to incubate the membrane.

NOTE:
Substrate is prepared by your instructor just prior to use.

Study Questions

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

1. What is Alzheimer's Disease?
2. What are the major risk factors for Alzheimer's Disease?
3. Why is it difficult to find drugs to treat Alzheimer's Disease?
4. Why do we need to do a Western Blot to determine ApoE allele status?

Instructor's Guide

NOTES TO THE INSTRUCTOR

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

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).

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

Preparation For:	What to do:	When:	Time Required:
Module I: Electrophoresis of Patient Samples	Reconstitution of Lyophilized Proteins.	Anytime before performing the experiment.	10 min.
	Prepare Tris-Glycine-SDS Buffer (electrophoresis buffer).	Anytime before performing the experiment.	10 min.
Module II: Western Blot Procedure	Prepare Tris-Glycine buffer (transfer buffer).	Anytime before performing the experiment.	10 min.
	Prepare Membranes and Blotting Paper.	Anytime before performing the experiment.	10 min.
Module III: Immunodetection	Prepare buffers for immunodetection.	Anytime before performing the experiment.	15 min.
	Reconstitute and dilute antibodies.	Up to 2 hours before the lab.	10 min.
	Prepare Peroxidase Substrate.	During the PBS washes following the secondary antibody.	10 min.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

Pre-Lab Preparations - Module I

ELECTROPHORESIS OF PATIENT SAMPLES

This experiment requires three 12% Denaturing Polyacrylamide Gels to be shared by the 6 student groups. The Protein Standard Markers (Component A) and Components B and C must be reconstituted.

Component B is a negative sample while Component C is a positive sample. You may choose to have each group load two of each (4 samples/patients total), or aliquot out each and spread them around the class so that the class data can be pooled to see which "participants" are positive for ApoE4. Enough material is provided for 12 positive samples and 12 negative samples. These sample are already denatured and do not require boiling.

Protein Denaturation and Reconstitution of Lyophilized Proteins

1. Add 125 µL distilled water to the tube for Component A (Protein Standard Markers). Vortex for 30 seconds, or until completely dissolved.
2. Add 245 µL distilled water to each of the tubes for Components B and C. Vortex each tube for 30 seconds, or until completely dissolved.
3. 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 instructions on page 12.
4. After boiling, aliquot 20 µL of the samples to each group. Label either with a simulated patient number or using the following labels:
   - Protein Standard Markers (20 µL Component A)
   - Patient 1 (20 µL Component B)
   - Patient 2 (20 µL Component C)
   - Patient 3 (20 µL Component B)
   - Patient 4 (20 µL Component C)

NOTE: This experiment 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. Refer to instructions entitled "Practice Gel Loading Instructions".

Tris-Glycine-SDS Buffer (Electrophoresis Buffer Only)

1. Add 1 part EDVOTEK® 10X buffer to every 9 parts distilled water.
2. Make enough 1X buffer for the 3 electrophoresis units (2 liters for three EDVOTEK® units).

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.

Pre-Lab Preparations - Module II

WESTERN BLOT PROCEDURE

(Prepare Any Time Before the Lab - Required First Day)

Tris-Glycine Buffer (Transfer Buffer Only)

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

1. Add the powder contents of the Tris-Glycine Buffer to a flask or beaker (larger than one liter).
2. Add 800 mL distilled or deionized water to the powdered buffer. Swirl and stir to dissolve the powder (a stir plate, if available may be useful).
3. Add 200 mL of 95-100% methanol. Mix, cover tightly, and store in the refrigerator until ready to use.

Nitrocellulose Membranes

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

1. Keep both upper and lower 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. If you are using gels that are smaller or larger than the 8 x 10 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 less groups.
3. Cut six membranes for the groups to share. Each membrane should be roughly the size of half a gel.

Additional Blotting Items

1. Cut two pieces of blotting paper to fit gel.
2. Cut wick to width of gel and length to overhang from edge of tray to within 1/8" of tray bottom.

REQUIREMENTS FOR THE FIRST DAY

(Reagents for Two Groups)

- One 12% Denaturing Polyacrylamide gel
- Protein samples (aliquots if desired)
- Practice gel loading solution (optional)
- Diluted electrophoresis buffer
- 100 mL of diluted transfer buffer
- 2 nitrocellulose membranes
- 2 filter paper pieces
- Wick
- Paper towels and plastic wrap
- Small plastic boxes for soaking membranes and gels
- Pipette
- 0.7 - 1.0 kg weight
- Small dish (less than width of gel) for transfer

Pre-Lab Preparations - Module III

IMMUNODETECTION

NOTE:
The blocking buffer will likely precipitate during storage. Warm at 37°C for 5-10 minutes or until the precipitate has dissolved.

Prepare Buffers for Immunodetection
(Prepare on the Day of the Lab - Required Second Day)

1. Dilute 10x Blocking Buffer (Component H) by adding 540 mL distilled water. The blocking buffer will likely precipitate during storage. Warm at 37° C for 5-10 minutes or until the precipitate has dissolved.
2. Prepare the "Complete Blocking Buffer" by adding the Powdered Milk (Component I) to 600 mL diluted blocking buffer. Store in fridge until use. Dispense 30 mL to each group in 50 mL tube.
3. Dilute the bottle of 10x PBS solution (Component J) by adding the contents (30 mL) to 270 mL distilled water. Dispense 25 mL for each group.

Reconstitution and Dilution of Lyophilized Antibodies

1. Measure out 60 mL Complete Blocking Buffer into a 100 mL beaker and label "Anti-ApoE4 Antibody". Remove 300 µL of the Complete Blocking Buffer and add it to the vial of Anti-ApoE4 Antibody (Component D). Pipet up and down 5 times to resuspend and transfer back to the 100 mL beaker. Aliquot 10 mL for each group in 15 mL tubes.
2. Measure out 60 mL 1X PBS into a 100 mL beaker and label "Secondary Antibody". Remove 300 µL of the 1X PBS and add it to the vial of Secondary Antibody Conjugate (Component E). Pipet up and down 5 times to resuspend and transfer back to the 100 mL beaker. Aliquot 10 mL for each group in 15 mL tubes.

Peroxidase Substrate
(Prepare During the Lab Experiment, 15-30 minutes before the last incubation)

1. Dispense 68 mL of the diluted phosphate buffered saline (PBS) to a clean flask or beaker.
2. Add Peroxide Co-substrate (Component G) to the 68 mL of diluted PBS. Mix thoroughly by swirling or using a magnetic stir bar. There is usually undissolved material remaining.
3. Then, add 7.5 mL of Hydrogen peroxide (Component F). Mix well.
4. Dispense 12 mL of the peroxidase substrate for each group in 15 mL tubes.

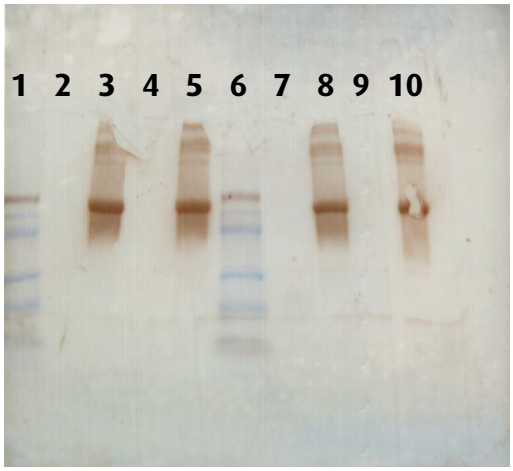
QUICK REFERENCE:
The substrate is prepared for the peroxidase enzyme, which is attached to the anti-IgG peroxidase conjugate (secondary antibody).

Prepare the substrate 15-30 minutes before students require it for plate development (last incubation).

REQUIREMENTS FOR THE SECOND DAY
(Individual Groups)

- Approximately 35 mL complete blocking buffer
- 25 mL diluted PBS
- 10 mL secondary antibody conjugate
- 10 mL Anti-ApoE4 Antibody
- 12 mL prepared substrate

Expected Results and Analysis



Lane	Contents
1 or 6	Protein Standard Markers
2 or 7	Patient 1 (negative)
3 or 8	Patient 2 (positive)
4 or 9	Patient 3 (negative)
5 or 10	Patient 4 (positive)

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