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Edvo-Kit #

272

Edvo-Kit #272

Immuno-electrophoresis

Experiment Objective:

In this experiment, students are introduced to the use of immuno-electrophoresis to separate and characterize a mixture of proteins and examine the specificity of an antigen-antibody interaction.

See page 3 for storage instructions.

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Experiment Components

Store entire experiment in the refrigerator.

Components	Check (✓)
A IgG	<input type="checkbox"/>
B Whole Serum	<input type="checkbox"/>
C Albumin	<input type="checkbox"/>
D Antibody to Whole Serum	<input type="checkbox"/>
E Antibody to IgG	<input type="checkbox"/>
F UltraSpec-Agarose™	<input type="checkbox"/>
G Electrophoresis Buffer (50x Concentrate)	<input type="checkbox"/>
• Microtest tubes	<input type="checkbox"/>
• 10 mL pipettes	<input type="checkbox"/>
• Filter paper	<input type="checkbox"/>
• Well cutters	<input type="checkbox"/>
• 60 mm petri plates	<input type="checkbox"/>

Experiment #272
is designed for
10 groups.

Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipettes with tips (5-50 µL)
- Microscope Slides (1" x 3")
- Distilled Water
- 400 to 600 mL Beaker
- 1000 mL Graduated Cylinder
- Microwave
- Humidifying chamber/container with lid (large enough to hold electrophoresis trays)
- Paper Towels
- Plastic Wrap or Foil
- 37°C Incubation Oven (Optional)

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

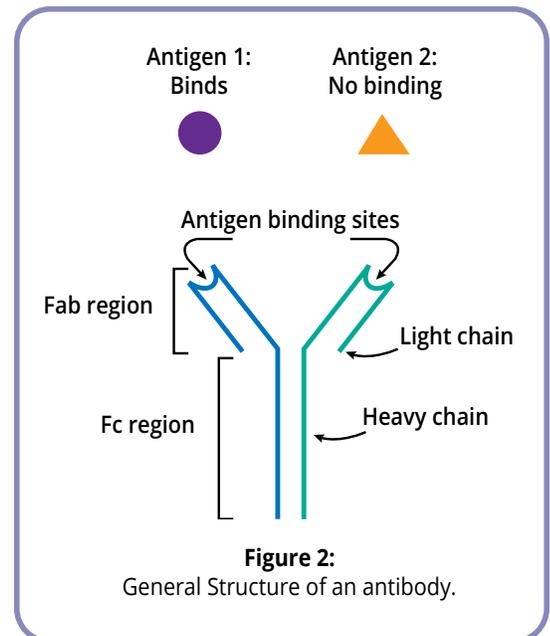
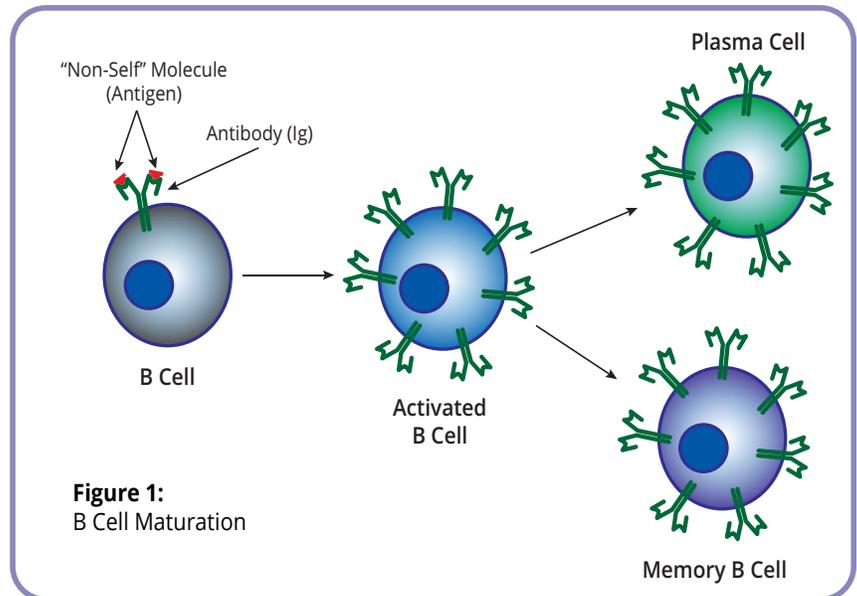
Background Information

An organism's immune system is responsible for fighting off infectious agents and foreign material. One line of this defense is the adaptive immune system, which has cells capable of synthesizing proteins that specifically target foreign material. These proteins are called antibodies and can distinguish between "self" and "non-self" molecules. Any molecule that binds to an antibody (also called immunoglobulins or Igs) is known as an antigen.

Antibodies are produced by B Cells. B-Cells are a type of white blood cell, and they can both secrete antibodies into the blood and express antibodies on their cell surface. When antibodies bind to foreign material, they can inactivate them or signal for immune cells to attack the invaders. There are many different types of B-cells, and they can recognize antibodies either specific to one antigen or more generally to a class of antigens. When an antibody on the surface of a B-cell binds to an antigen, it signals that there is a foreign invader in the body. The B-cell interacts with a number of other cells to become activated and eventually divides into either memory B-cells or plasma cells. Memory B-cells stick around in the blood for a long time, so if the body is ever exposed to that antigen again it will be ready for it. On the other hand, plasma cells begin to divide rapidly to mass produce antibodies against the antigen that they recognized for a more immediate response (Figure 1). In this way, the body can both immediately attack the invader and be ready should they ever appear again.

Antibodies are Y-shaped molecules comprised of four linked polypeptide chains: two identical "heavy chains" and two identical "light chains". The antigen binding sites are located at the ends of the short arms of the Y. The amino acid sequence in this short arm is variable, allowing each antibody to recognize a unique part of an antigen (Figure 2). This short arm is so variable that there are billions of unique antibodies in the blood ready to bind antigens and fight infection.

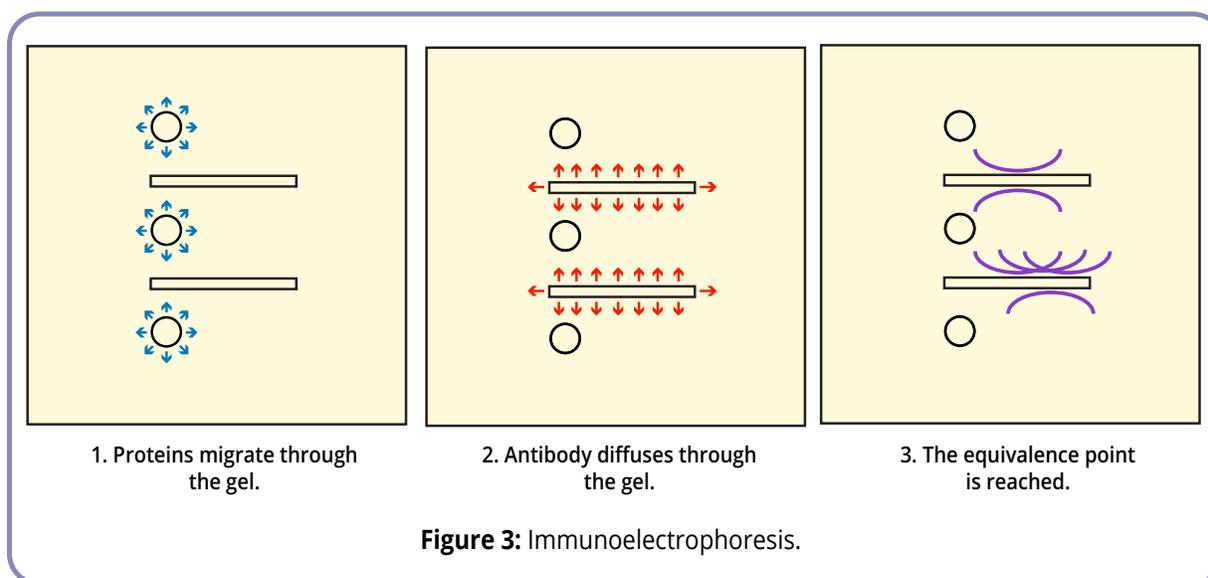
Antibodies are used widely in scientific and clinical research and provide a way to identify, and sometimes visualize, different antigens. In order to use antibodies in this kind of research, they need to be mass produced in different animals. Antibodies used in scientific research are produced as an immune response when animals (i.e. rabbits, mice, etc.) are injected with an antigen. If you take all of the antibodies produced by the animal's immune response, there will be a mixture of antibodies geared towards this specific antigen as well as antibodies against every other antigen that



Background Information, continued

the animal has ever been exposed to. General serum antibodies like this can be useful for gaining a picture of how many different antigens are in a particular sample, and they can be used as a reference for normal antibody levels. However, sometimes antibodies specific to a certain antigen are more useful. These can be obtained by isolating and culturing the individual immune cells that were produced when responding to an antigen injection. These antibodies, both general and specific, can be used for research to understand certain properties of proteins, or diagnostically to see if someone has certain antigens in their blood.

One way that scientists analyze antigen-antibody interactions is using a technique called immunoelectrophoresis. Immunoelectrophoresis is used in both clinical and research laboratories for separating and identifying proteins on the basis of their electrophoretic behavior and their immunological properties. Immunoelectrophoresis includes 2 steps. First, antigens are electrophoretically separated into an agarose gel. Second, antibodies diffuse throughout the gel and encounter the separated antigens (Figure 3).



Gel electrophoresis is a widely used analytical method that separates molecules based on charge, size, and shape. It is particularly useful for determining the size of biomolecules. In immunoelectrophoresis, samples of antigens are loaded into wells cut into the gel during casting. The gel, which consists of microscopic pores that act as a molecular sieve, is placed in a chamber containing a buffer solution and electrodes. Current is applied from a power source and, since biomolecules are charged, the antigens will migrate through the gel. If the antigens have a positive charge, they will migrate towards the negative electrode. If the antigens have a negative charge, they will migrate towards the positive electrode. The gel also acts as a microscopic sieve with molecules navigating through small pores in the gel. Small molecules can migrate faster through the gel than large molecules, allowing gel electrophoresis to separate small fast-moving molecules from large slow-moving ones. Biomolecules often have multiple charges and differing sizes, therefore they may migrate radially as opposed to strictly towards the positive or negative electrode.

After the antigens are separated by electrophoresis, the gel is removed from the electric field and antibodies are introduced into narrow troughs parallel to the separated antigens. Next, the gel is incubated (either at room temperature or 37°C) and radial diffusion takes place. This means that both antigen and antibody can diffuse through the gel. When the solutions of antibody and antigen encounter each other, they may create a line of precipitation. This line of precipitation occurs only at a specific ratio of antibody to antigen and is known as the equivalence point.

Background Information, continued

The equivalence point is a concentration of both antigen and antibody that maximizes binding. If the antibody concentration is much higher than the antigen concentration, the antigen will be a limiting reagent and binding will not be maximal. If there is much more antigen than antibody, there will not be enough antibody to bind all the antigen and the precipitation will not be as pronounced. Therefore, during immunodiffusion, antigens and antibodies will form precipitate arcs when they encounter each other at the point of equivalence (Figure 4). Each antibody and antigen's equivalence point will be different because each will have different binding properties. Therefore, if an antibody (or multiple antibodies in whole serum) binds to multiple antigens, there will be multiple precipitation arcs—one for each antigen.

In research, immunoelectrophoresis is used to monitor antigen and/or antibody purifications, to detect impurities, and to analyze soluble antigens from plant and animal tissues. However, the most common use of immunoelectrophoresis is for clinical diagnosis of problems related to antibody levels in the blood.

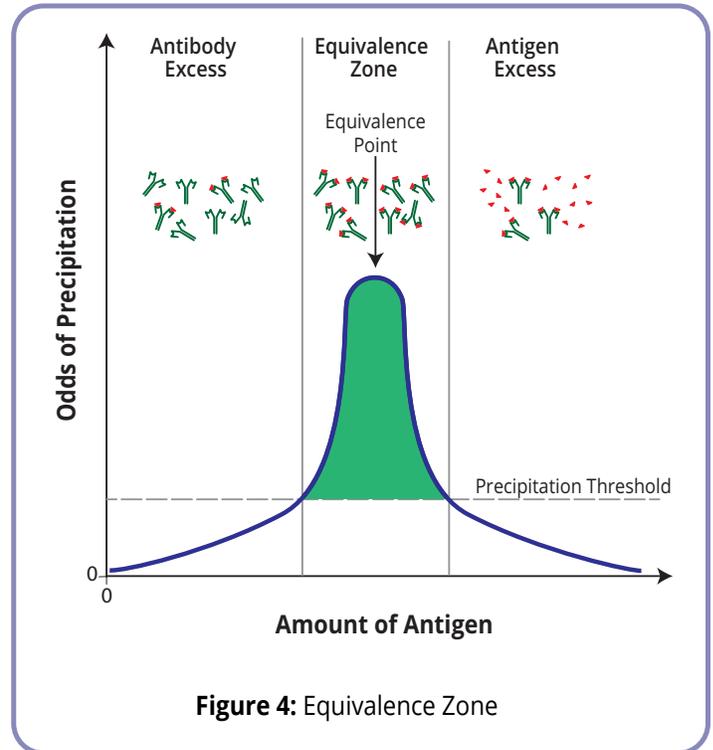


Figure 4: Equivalence Zone

There are many clinical scenarios in which immunoelectrophoresis is used as a diagnostic technique. Perhaps the most common is in multiple myeloma. Multiple myeloma is a type of cancer which affects plasma cells. Plasma cells, as mentioned above (Figure 1), are responsible for mass producing antibodies after a B cell encounters an antigen. During myeloma, a mutation in plasma cells causes them to divide uncontrollably and occupy the bone marrow. The bone marrow produces most blood cells, including red blood cells that carry oxygen and iron to all parts of the body. When the cancerous plasma cells (myeloma cells) occupy the bone marrow, there is less room for red blood cells to be produced, causing anemia. Oftentimes, multiple myeloma can be initially detected by a combination of anemia (iron deficiency) and an increase in antibody (immunoglobulin) levels. These two symptoms suggest, but do not confirm, multiple myeloma.

To test for myeloma, the composition of the different immunoglobulins is analyzed by immunoelectrophoresis. The patient samples are run and analyzed against whole serum antibodies and specific antibodies. If the increase in immunoglobulins is caused by a general increase in many different immunoglobulin types (Figure 5A), that would suggest that the body is simply fighting an infection.

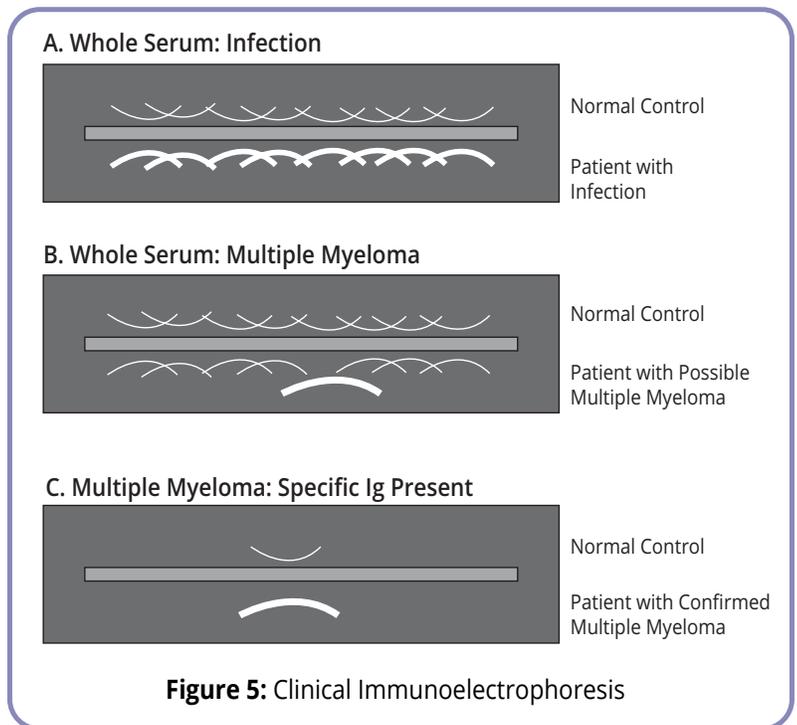


Figure 5: Clinical Immunoelectrophoresis

Background Information, continued

The anemia would have been caused by a lack of iron intake. If it seems like one band is stronger than the rest (Figure 5B), individual immunoglobulins can be tested. If the increase is specific to one type of immunoglobulin, or a monoclonal increase (Figure 5C), it likely means that one type of plasma cell has been dividing uncontrollably, leading to myeloma.

In this experiment, you will use immunoelectrophoresis to separate a mixture of whole serum antigens or specific antigens and analyze their binding to either a mixture of whole serum antibodies or specific antibodies.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students are introduced to the use of immunoelectrophoresis to separate and characterize a mixture of proteins and examine the specificity of the antigen-antibody interaction.

LABORATORY SAFETY

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:

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

Before starting the Experiment:

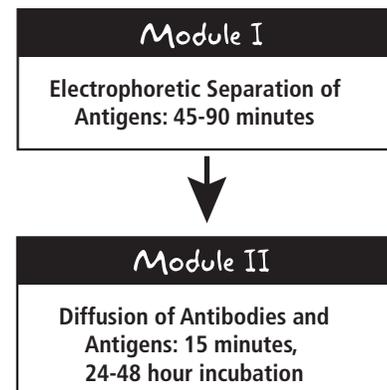
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

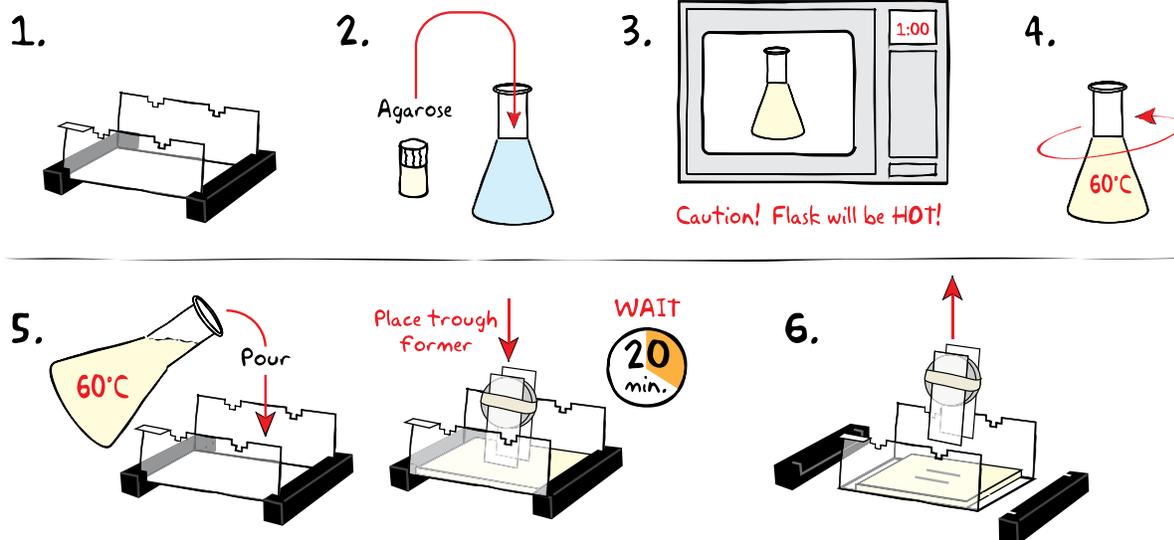
- Record your observations.

After the Experiment:

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



Module I: Electrophoretic Separation of Antigens



CASTING THE AGAROSE GEL

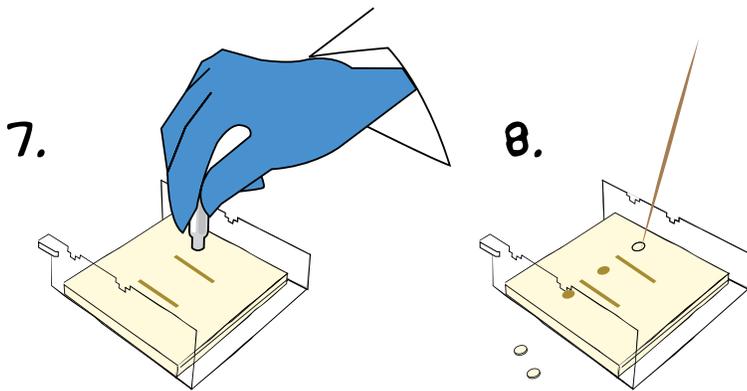
NOTE: Gels may be prepared by your instructor ahead of class.

- PREPARE** the gel-casting tray by sealing the ends of the tray with the rubber end caps.
- MIX** 1.0 g agarose powder with 100 mL diluted electrophoresis buffer in a beaker or flask.
NOTE: The whole class should make up one large batch of agarose and then divide for individual gels.
- DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 60 seconds. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
- COOL** agarose to 60°C with careful swirling to promote even dissipation of heat.
- POUR** the cooled agarose solution into the prepared gel-casting tray (Table A). Before the agarose solidifies, **PLACE** trough former into the center of the tray to simultaneously form the two troughs. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
NOTE: Make sure the trough former is parallel with the sides of the gel tray and perpendicular to the end caps.
- REMOVE** end caps and trough former. Take particular care to prevent damage to the gel.



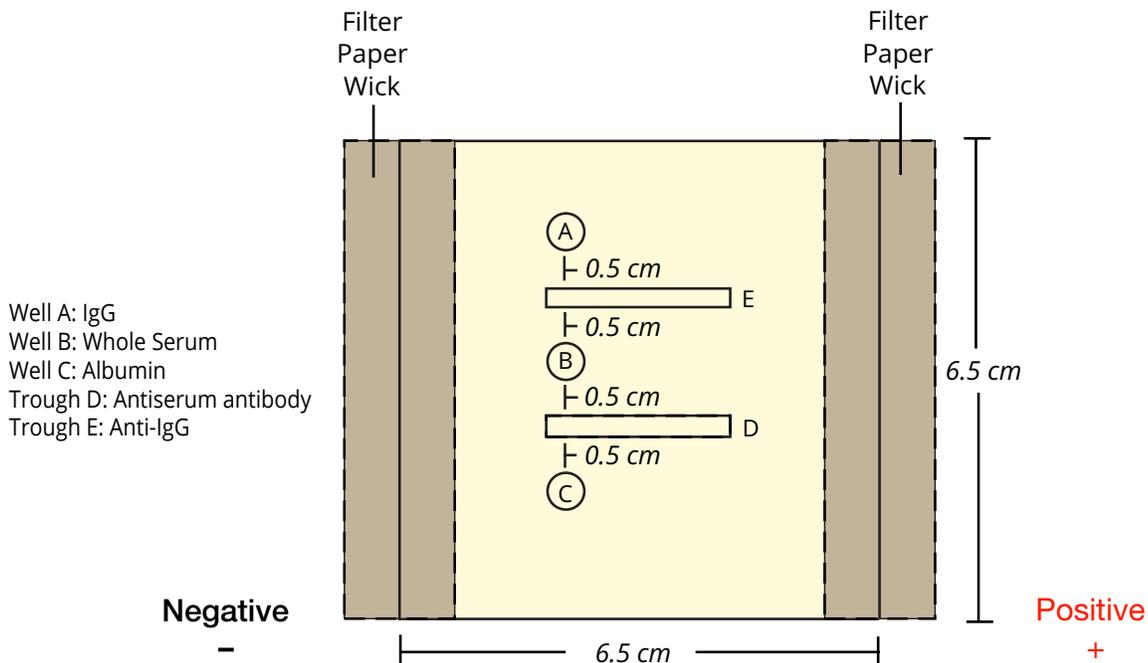
Table A	
Thin 1.0% UltraSpec-Agarose Gels	
Size of Gel Casting tray	Volume of molten agarose
7 x 7 cm	9 mL
7 x 14 cm	19 mL

Module I: Electrophoretic Separation of Antigens



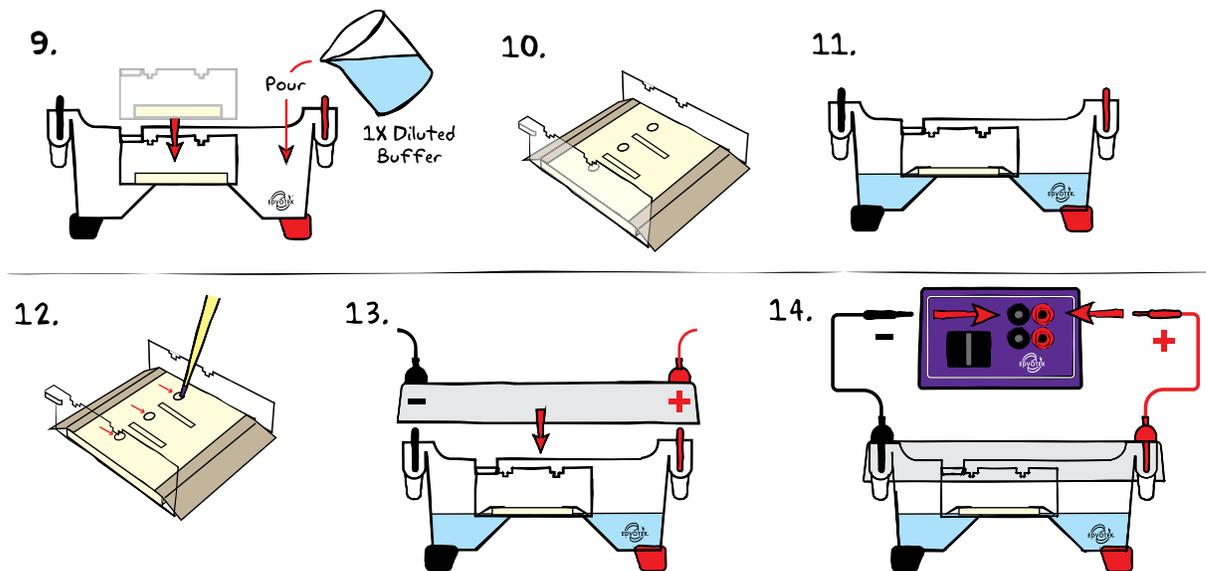
PREPARING THE GEL

7. **CUT** wells A, B, and C, as indicated in Template 1, using a well cutter. The distance between the troughs and the edge of each well should not be more than 0.5 cm.
8. Gently **REMOVE** the round agarose gel plugs with a toothpick or spatula.



Template 1:
Template for Immunoelectrophoresis.
(Drawn approx. to scale.)
Filter paper wick instructions
are on the next page.

Module I: Electrophoretic Separation of Antigens



RUNNING THE GEL

9. **TRANSFER** the gel (still on the tray) into the electrophoresis chamber. **POUR** diluted electrophoresis buffer into the side wells of the apparatus but **DO NOT** submerge the gel.
10. Gently **LAY** the filter paper wicks over the ends of the gel. (They should overlap about 3 to 4 mm.) **ALLOW** them to become saturated with electrophoresis buffer.
11. **PRESS** lightly on the wicks to ensure good contact between the gel and the electrophoresis buffer. The wicks should be submerged in the buffer. If necessary, add more buffer, but **DO NOT cover the gel with buffer**.
12. **PIPETTE** 20 µL of samples A, B, and C into wells as indicated by Table 1a. **CHANGE** pipette tips between samples. If needed, reference Figure 1 for a proper sample loading diagram.
13. **PLACE** safety cover on the unit.
14. **CONNECT** the leads to the power supply with the black lead in the black (negative) input and the red lead into the red (positive) input. **TURN ON** and set the power supply. Reference Table B for the recommended voltage. When current is flowing properly, bubbles should form on the electrodes.
15. **RUN** the electrophoresis until the blue dye has migrated to the ends of the troughs (see Figure 6). The exact time required is dependent upon the voltage (see Table B).

NOTE: The samples contain dye which will migrate at different rates. Terminate electrophoresis when the first dye reaches just past the end of the troughs. Do not allow the samples to migrate off the end of the gel.

Table 1a: Well Loading

Well A	IgG
Well B	Whole Serum
Well C	Albumin

Table B
Time & Voltage Guidelines

Volts	Recommended Times	
	Minimum	Maximum
150	15 min.	30 min.
125	30 min.	45 min.
75	35 min.	70 min.

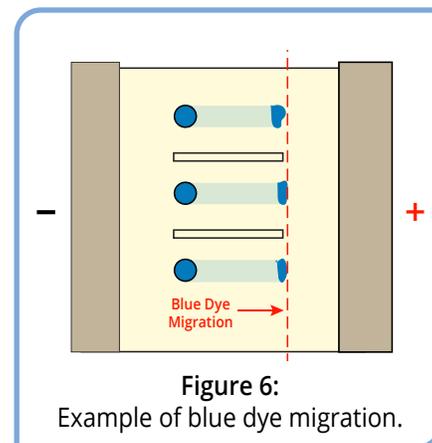
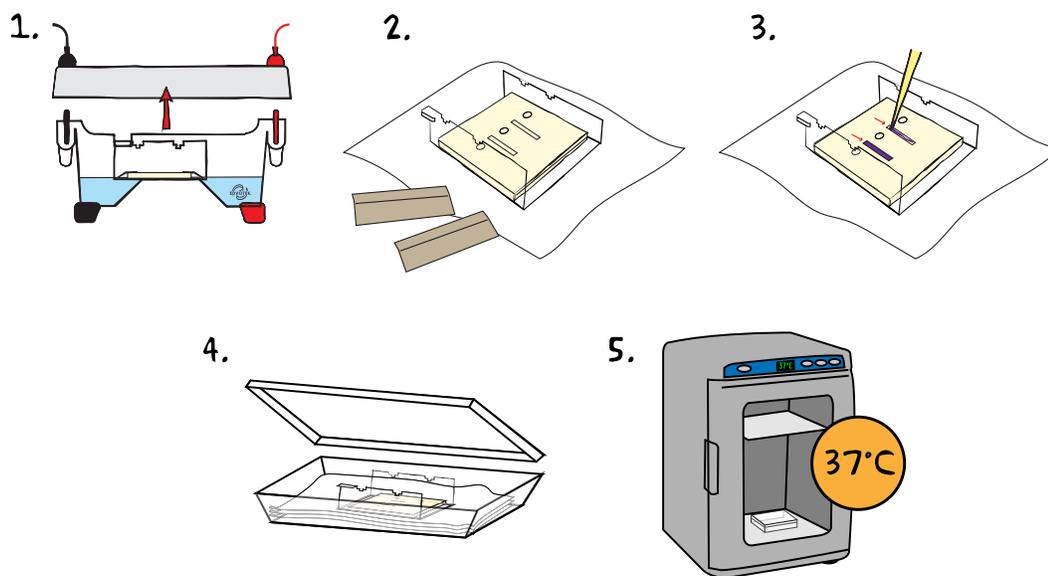


Figure 6:
Example of blue dye migration.

Module II: Diffusion of Antibodies and Antigens



1. After electrophoresis is completed, **TURN OFF** the power, unplug the power source, **DISCONNECT** the leads, and **REMOVE** the cover.
2. **DISCARD** the filter paper wicks and **REMOVE** the gel tray from the apparatus. **PLACE** the tray on a level surface.
3. **PIPETTE** 50 μ L of each antibody to the appropriate trough (see Table 1b). Use the pipette tip to carefully **SPREAD** the antibody solution along the entire length of the trough. **CHANGE** pipette tips between samples.
4. **PLACE** the tray in a closed humidifying chamber containing moistened paper towels.
5. **ALLOW** diffusion to take place over a 24 to 48 hour period, or until visible precipitates form in the gel. The chamber can be placed in a 37°C incubator or remain at room temperature.

Table 1b: Trough Loading

Trough D	Antiserum antibody
Trough E	Anti-IgG

Study Questions

1. Why is a blue dye added to the protein solutions for electrophoresis?
2. Why do precipitates form arcs?
3. How does immunodiffusion assay differ from immunoelectrophoresis assay?
4. How many arcs were observed from the whole serum sample? What does each arc represent?
5. What results can you expect if the gel were stained with a protein stain?
6. How is immunoelectrophoresis used in a clinical setting?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation For:	What to do:	When:	Time Required:
Module I: Electrophoretic Separation of Antigens	Prepare electrophoresis buffer.	Up to one week before performing the experiment.	30 min.
	Prepare gels.	On the day of the lab.	30 min.
	Cut filter paper wicks.	Anytime before performing the experiment.	15 min.
	Aliquot protein samples.	Anytime before performing the experiment.	15 min.
Module II: Diffusion of Antibodies and Antigens	Aliquot antibody samples.	Anytime before performing the experiment.	15 min.

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

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Pre-Lab Preparations

MODULE I: ELECTROPHORETIC SEPARATION OF PROTEINS

Preparing Diluted Electrophoresis Buffer

Depending on class size and how many gels you need to run, prepare the 1X Electrophoresis buffer as follows in Table 2. This buffer will be used for agarose gel preparation and electrophoresis chamber buffer.

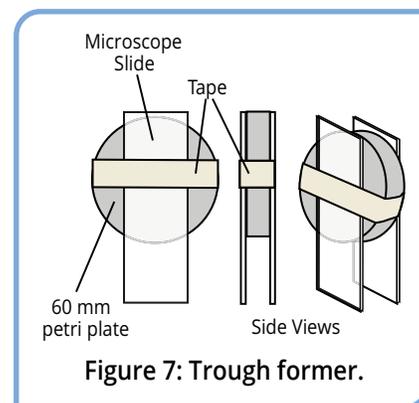
Table 2: 1X Electrophoresis Buffer Dilutions

Volume of 50X Buffer	Volume of Distilled Water Needed	Final Volume of 1X Buffer
20 mL	980 mL	1 L
40 mL	1,960 mL	2L
60 mL	2940 mL	3L

Creating the Trough Former

Before casting agarose gels, construct a trough former. For each trough former, you will need two microscope slides, the bottom half of a small (60 mm) petri plate, and tape.

1. Tape one microscope slide to the bottom of the petri plate (see Figure 7).
2. Tape the second microscope slide to the opposite side of the petri plate so it is even with the first slide. From the side view, the slides should be even and parallel. The trough former should be able to stand unpropped when placed on a counter top.



Preparing Agarose Gels

Each group requires 1 thin 7 x 7 cm agarose gel (9 mL of molten agarose per group). You may choose to have students make their own batch of agarose for gel casting (using the instructions on page 9) or you can prepare the agarose in bulk for the class using the instructions below.

Bulk Agarose Gels for the Class (1.0%)

1. Use a 250 mL flask to prepare the diluted gel buffer.
2. Pour 1.0 gram of UltraSpec-Agarose™ into 100 mL of diluted electrophoresis buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. Heat agarose solution in microwave or on hotplate to dissolve.
5. Cool the agarose to 60°C in a waterbath. If evaporation has occurred, add sufficient distilled water to bring the volume up to the original volume as marked on the beaker.
6. Keep the agarose at 60°C until gels are to be poured. Each group will require 9 mL of molten agarose.

FOR MODULE I Each Student Group should receive:

- Diluted electrophoresis buffer
- 9 mL molten agarose
- Trough former
- 2 Filter paper wicks
- 25 µL of Components A, B, and C

Additional Materials

- Prepare electrophoresis wicks by cutting filter paper into strips approximately 7 cm x 3 cm. The ends of the wicks must be long enough to extend into electrophoresis buffer in the chamber (see Template 1 on page 10).
 - If you are using an M12 unit (holds two 7 x 7 cm gels or one 7 x 14 cm gel), it is recommended that you use a 7 x 14 cm gel casting tray. If you only have 7 x 7 cm gel casting trays, use a transfer pipet to wet the area between the two gels and cut a third wick to connect the gels. Make sure the wick is soaked with buffer but that the gels are not submerged.
- Aliquot 25 µL of components A, B, and C per gel. (Enough tubes have been provided to aliquot all components for 10 groups.)

Pre-Lab Preparations

MODULE II: DIFFUSION OF ANTIBODIES AND ANTIGENS

Preparing a Humidifying Chamber

1. Line the bottom of a plastic container with paper towels.
2. Add distilled water to saturate the towels, but do not allow excess water to pool in the container.
3. Cover with lid, plastic wrap, or foil.

**FOR MODULE II
Each Student Group
should receive:**

- 55 μ L of Components D & E
- Humidifying chamber
- Paper towels
- Distilled water

Additional Materials

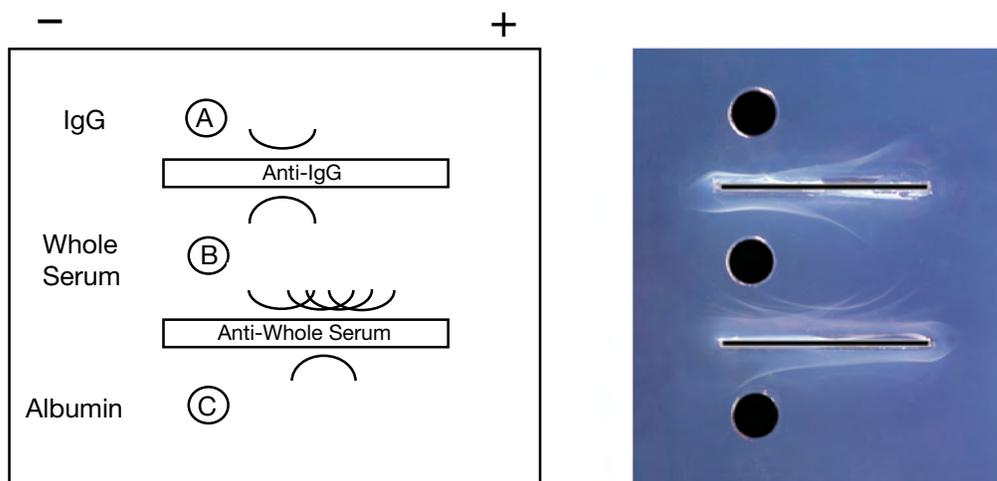
- Aliquot 55 μ L of components D and E per group. (Enough tubes have been provided to aliquot all components for 10 groups.)

Avoiding Common Pitfalls

1. Follow the instructions carefully when preparing gels. Make sure the agarose is completely dissolved.
2. The gels should be made on the day of the lab. They will solidify quickly because they are very thin.
3. The spacing of the wells and troughs is critical to the success of the experiment.
4. Make neat, clean wells with the well cutters.
5. Do not submerge the gel in electrophoresis buffer when running the gel. Use the wicks to make contact between the gel and buffer.
6. Do not add antibody to the troughs until after the electrophoretic separation of the proteins.
7. When adding antibody to the troughs, slowly and carefully spread the solution over the entire area of the trough.
8. Placing the humidifying chamber in a 37°C incubation oven will expedite the formation of precipitin arcs.

Expected Results

1. Note the formation of arcs of white precipitate in the gel.
2. Identify the number of proteins in the whole serum from the number of arcs of precipitate. Four to six arcs can be expected, corresponding to various albumins found in Whole Serum.
3. Identify albumin and IgG in the whole rabbit serum from comparison with the pure albumin and the pure anti-IgG segments. The prominent precipitant arc corresponds to the complex formed by the reaction of the anti-IgG with pure IgG.



Results may vary somewhat from those depicted in the above schematic.
Drawing not depicted to scale.

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