INMUNOELECTROPHORESIS

This protocol is based on the EDVOTEK® protocol "Radial Immunodiffusion".

10 groups of students

1. 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.

2. EXPERIMENT COMPONENTS for 10 groups of students

COMPONENTS	STORE
A IgG	4-8ºC
B Whole serum	4-8ºC
C Albumin	4-8ºC
D Antibody to whole serum	4-8ºC
E Antibody to Ig	4-8ºC
F UltraSpec-Agarosa™	4-8ºC
G Electrophoresis (25x concentrate)	4-8ºC
Microtest tubes	
10 ml pipettes	
Filter paper	
Well cutters	
Petri plates (60 mm)	

NOTE: Store all components at the indicated temperatures upon receipt.

NOTE: No human material is used in this practice.

NOTE: 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.

2.1 Requirements

- Horizontal Electrophoresis Apparatus
- Power Supply
- Automatic Micropipet and Tips (5-50 µl)
- Waterbath
- Microscope Slides (1" x 3")
- Distilled Water
- 400 to 600 ml Beaker
- 1000 ml Graduated Cylinder
- Bunsen Burner, Heat Plate, or Microwave

- Container with Lid (large enough to hold electrophoresis trays)
- Paper Toweling
- Plastic Wrap or Foil
- 37°C Incubation Oven

NOTE: Make sure glassware is clean, dry, and free of soap residue. For convenience, you can buy additional disposable transfer pipettes to the steps of extraction and washing liquids.

3. BACKGROUND INFORMATION

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. Proteins such as rabbit serum proteins, which are antigens, when injected into another animal such as a goat (the host), elicit the production of antibodies in the host. The interaction between the antigen and its antibody, which is also a protein, is both strong and highly specific. If solutions of antigen and antibody are mixed in different ratios, it is found that at a specific ratio, known as the **equivalence point**, the binding is maximized and a precipitate of antigen-antibody complex is formed.

In the clinical laboratory, immunoelectrophoresis is used diagnostically. It is utilized in examining certain serum abnormalities, especially those involving immunoglobulins, urine protein, cerebrospinal fluid, pleural fluids and other body fluids. In research, this procedure may be used to monitor antigen and/or antibody purifications, to detect impurities, analyze soluble antigens from plant and animal tissues, and microbial extracts.

Gel electrophoresis is a widely used analytical method that separates molecules based upon charge, size and shape. It is particularly useful for determining the size of biomolecules. Samples of proteins are loaded into wells made in 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 Direct Current (D.C.) power source. Since biomolecules are charged, they will migrate through the gel.

In immunoelectrophoresis, the proteins are first separated by horizontal agarose gel electrophoresis on the basis of their different charge-to-mass ratios. The gel with the separated proteins (antigens) is then removed from the electric field and antibodies to the proteins are introduced into narrow troughs parallel to the separated antigens. Diffusion of both antigen antibody takes place and, at a particular locus, the equivalence point is reached resulting in precipitation.

The purpose of this experiment is to demonstrate the use of immunoelectrophoresis to separate and characterize a mixture of serum proteins as well as to examine the specificity of the antigen-antibody interaction.

4. EXPERIMENTAL PROCEDURES

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.

4.1 Laboratory safety

No human material is used in this experiment.

1. Gloves and safety goggles should be worn at all times as good laboratory practice.

2. NOT PIPETTE WITH THE MOUTH, use appropriate devices.

3. Exercise caution when working with equipment using together heat and mix of reagents.

4. Wash hands with soap and water after working in the laboratory or after using biological reagents and materials.

5. Be careful when using any electrical equipment in the laboratory.

6. Boiling agarose can splatter and cause severe burns. When heating agarose, always wear safety goggles and use hot gloves.

7. Prior to turning the power supply on, be sure that the chamber is level and the work surface is dry.

If you are unsure of something, ASK YOUR INSTRUCTOR

4.2 Approximate time requirements for pre-lab and experimental procedures

Your schedule will dictate when the gels should be poured. Since the gels are very thin, solidification requires approximately 10 minutes. Gels should not be removed from the gel trays.

4.3 PreLab Preparations

Notes preparations teacher practice

The class size, length of classes of practices and equipment availability are factors that must be considered in the planning and implementation of this practice with their students. These guidelines can be adapted to fit your specific circumstances.

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.

Registration laboratory activities

Students must register in their book practices the activities listed below.

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.

General instructions
A. PREPARATIONS BEFORE PRACTICE
PREPARING ELECTROPHORESIS BUFFER

1. Prepare electrophoresis buffer by diluting the entire contents (40 ml) of the bottle containing concentrated buffer (component G) in 960 ml distilled water for a total of 1000 ml.

   This buffer will be used to prepare agarose as well as for electrophoresis.

PREPARING THE AGAROSE GEL

2. Prepare the agarose gel solution as follows:

   a. Add entire contents of UltraSpec Agarose™ bottle to 100 ml diluted electrophoresis buffer in 400 to 600 ml beaker. With a marker, mark the upper level of the buffer on the outside of the beaker.

   b. Heat the mixture to dissolve the agarose powder. The final solution should be clear (like water) with out any undissolved particles.

      Microwave method:
      - Cover flask with plastic wrap to minimize evaporation.
      - 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.

      Hot plate or burner method:
      - Cover the flask with foil to prevent excess evaporation.
      - Heat the mixture to boiling over a burner with occasional swirling. Boil until all the agarose is completely dissolved.

   c. 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.

   d. Keep the agarose at 60°C until gels are to be poured.

3. Before pouring the gel solution into the casting tray, construct a trough former using two microscope slides and the bottom half of a small (60 mm) petri plate.

   - Tape one microscope slide to the bottom of the petri plate (see **Figure 1**).
   - 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 countertop.

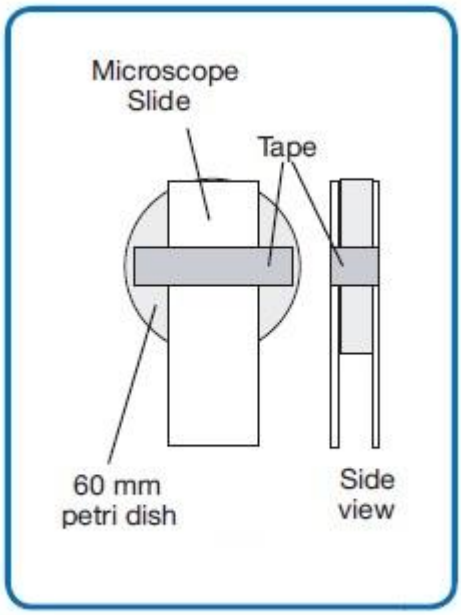

Figure 1

4. For gel trays approximately 7 cm x 7 cm (EDVOTEK® trays)
    - Pipet 9 ml of molten agarose into the gel tray.
    - Spread the agarose with the pipet to ensure even and complete coverage.
    - Before the molten agarose solidifies, place the trough former in the middle of the tray to simultaneously form the two troughs. See Template on **Figure 2**.

For gel trays approximately 7cm x 14cm (EDVOTEK® M12).

- Pipet 19 ml of molten agarose into the gel tray.
- Spread the agarose and place the trough former in the middle of the tray.

5. Aliquot samples as desired. Enough tubes have been provided to aliquot all five components for 10 groups.
6. Prepare a humidifying chamber as follows:
    - Line the bottom of a plastic container with paper towels.
    - Add distilled water to saturate the towels, but do not allow excess water to pool in the container.
    - Cover with lid, plastic wrap, or foil.
7. 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 **Figure 2**).

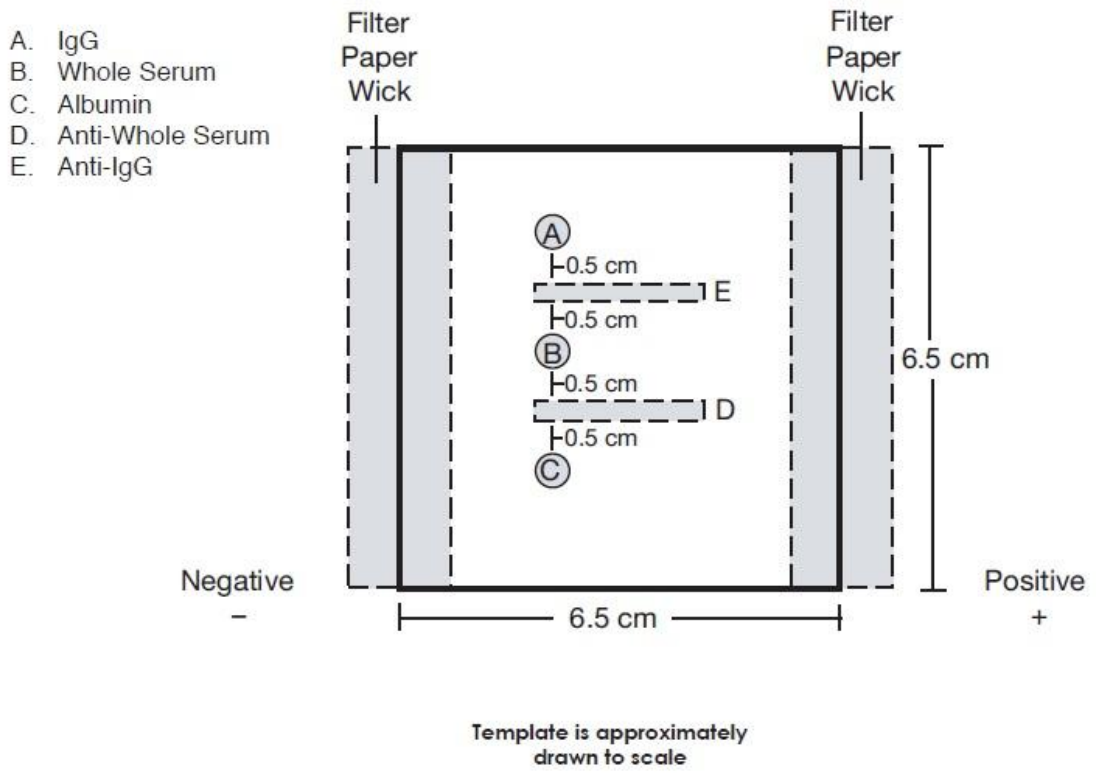

Figure 2

4.4 Material that should receive each group

Distribute the following to each student group, or set up a work station for students to share materials.

- Electrophoresis Unit
- electrophoresis Buffer
- Agarose Gel
- Albumin Sample
- IgG Sample
- Whole Serum Sample
- Anti-Serum Sample
- Anti-IgG Sample
- Well Cutter
- Filter Paper Wicks

4.5 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 humidity chamber in a 37°C incubation oven will expedite the formation of precipitin arcs.

5. STUDENT EXPERIMENTAL PROCEDURES

ELECTROPHORETIC SEPARATION OF ANTIGENS

1. Cut wells where indicated in **Figure 2** using well cutter. The distance between the troughs and the edge of each well should not be more than 0.5 cm.

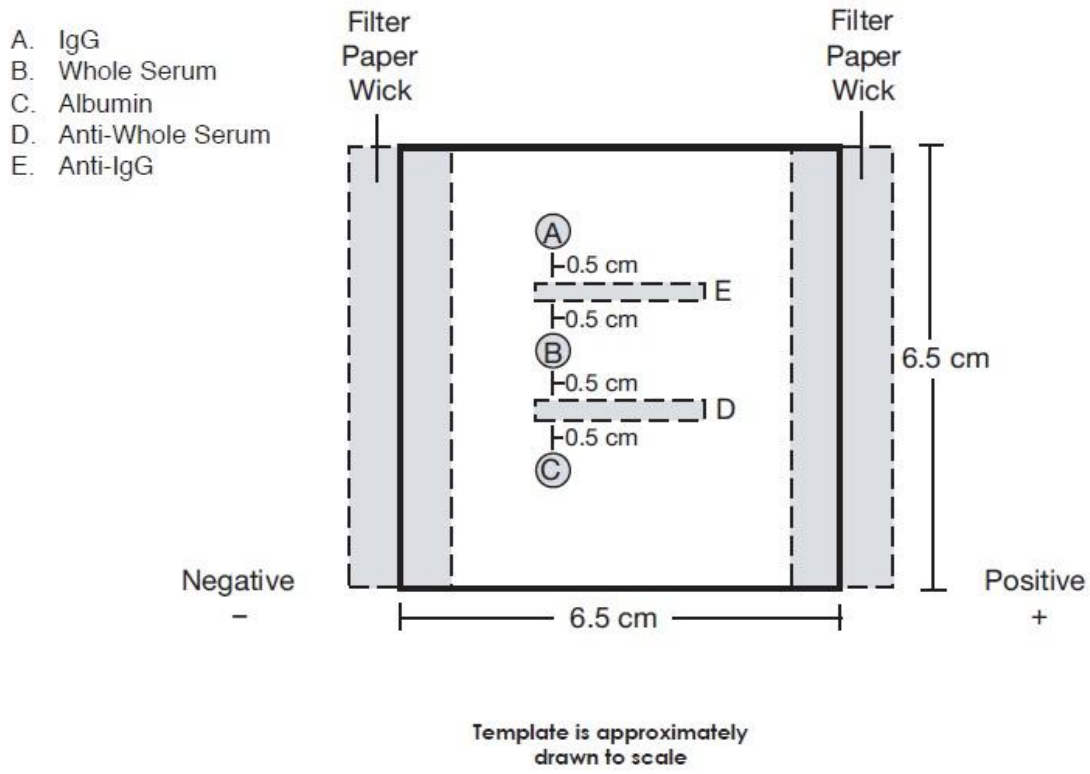

Figure 2

2. Remove agarose gel plugs with a toothpick or spatula.
3. Transfer the tray containing the gel to the electrophoresis apparatus.
4. Gently lay the filter paper wicks over the ends of the gel, (They should overlap about 3 to 4 mm) and allow them to become saturated with electrophoresis buffer.
5. The wicks should be submerged in the buffer. Press lightly on the wicks to ensure good contact between the gel and the electrophoresis buffer.

   If necessary, add more buffer, but do not cover the gel with buffer.
6. Load 20 µl of each antigen (A, B, and C) in the wells as indicated by the diagram. Change pipet tips between samples.
7. Carefully, snap on the lid of the electrophoresis apparatus, insert the leads into the power supply with the black lead in the black (negative) input and the red lead into the red (positive) input.
8. Turn on and set the power supply for the required voltage (50V to 125V). When current is flowing properly, bubbles should form on the electrodes.

9. Run the electrophoresis until the blue dye has migrated to the ends of the troughs. The exact time required is dependent upon the voltage (see Time and Voltage Guidelines at **Table 1**).

TABLE 1

Time and Voltage guidelines		
Voltage	**Recommended time**	
	Minimum	**Optimal**
50	60 min	2,0 hours
70	40 min	1,5 hours
125	30 min	45 min

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.

10. After electrophoresis is completed, turn off the power, unplug the power source, disconnect the leads, and remove the cover.
11. Discard the filter paper wicks and remove the gel tray from the apparatus. Set the tray on a level surface and proceed with the diffusion steps.

NOTE: Do not add the antibody samples in the troughs until after electrophoretic separation.

DIFFUSION OF ANTIBODIES AND ANTIGENS

1. Add 50 µl of each antibody to the appropriate trough (see **Figure 2**).

   Use the pipette tip to carefully spread the antibody solution along the entire length of the trough.
2. Place the tray in a closed humidifying chamber containing moistened paper towels.
3. 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 incubation oven or remain at room temperature.

6. EXPERIMENTAL RESULTS

6.1 Experimental Results and Analysis

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.

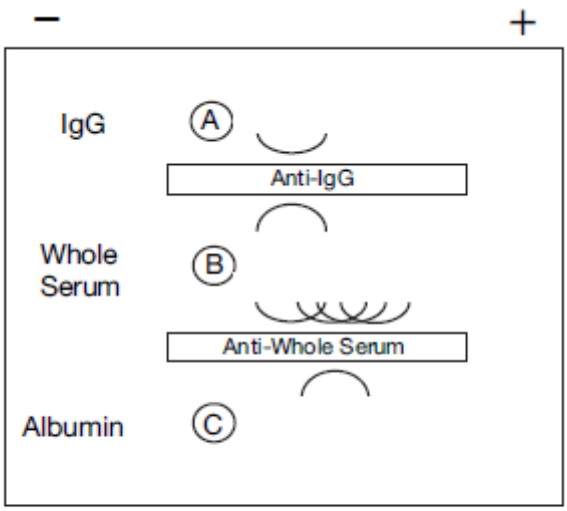

Figure 3: Expected results.

6.2 Study Questions

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

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?