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EDVO-Kit #

Clinical Diagnostic Immunoblot

Storage:

Store the entire experiment
in the refrigerator.

EXPERIMENT OBJECTIVES:

Dot-immunobinding Assay is a sensitive immunological assay used in research and clinical laboratories to quantify protein antigens. In this experiment, students will prepare a dot blot membrane to determine the presence of an antigen.

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.

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Experiment Components

- A Antigen
- B Antibody to antigen
- C IgG-Horseradish peroxidase conjugate
- D Peroxidase substrate
- E Hydrogen peroxide
- F Substrate Dilution Buffer
- G Tris buffered saline, pH 7.5
- H Instant nonfat dry milk

- Nylon Membranes
- Petri dishes, (60 mm diameter)

This experiment
is designed for
10 groups.

Store entire
experiment in the
refrigerator.

Requirements

- Forceps
- Graph paper
- Rulers
- Scissors
- Shaking platform (optional)
- Distilled Water

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.

Clinical Diagnostic Immunoblot

The introduction of radioimmunoassay (RIA) in the 1960s increased sensitivity of biological substance detection by many orders of magnitude. In principle, the method, which is measured by isotope emission, may be used with any determinant for which a specific binding molecule is available. The method was routinely applied to determine drugs, protein, nonprotein hormones, nucleic acids, and other antigens. In the 1970s and 1980s modifications were made to RIAs that maintained specificity and sensitivity while increasing ease of use and safety in handling reagents. Instead of radioactive molecules, an enzyme is attached to the antigen or the antibody and the amount of binding is measured as a function of enzymatic activity. This modified assay is called the enzyme-linked immunosorbent assay or ELISA. As in RIA, an adsorptive surface (e.g., plastic) can be used to insolubilize the antigen-antibody complex and the intensity of the enzyme reaction is measured. Chromogenic substrates made it easy to quantify enzyme reactions by measuring a color change. The main advantage of the ELISA reaction is to avoid isotopes and the long shelf-life of non isotopic reagents.

Recent modifications of ELISA include the use of different adsorptive materials and methods for measuring enzyme reactions. The most popular modification is Dot-immunobinding assay (Dot Blot assay). This assay is based on the adsorption of proteins onto membranes. The chromogenic product that precipitates on the membrane is measured by visual examination or measurement by automated equipment. The Dot Blot assay requires preparing an enzyme-labeled antibody (or antigen) that binds to the membrane bound antibody (or antigen). In the subsequent wash step, bound molecules are separated from unbound reactants.

In clinical Dot Blot assays, the antigen is first adsorbed on a membrane surface. The membrane is soaked in a "blocking buffer" to saturate it with a nonspecific protein. After a wash to remove excess blocking proteins the membrane is soaked in a solution of antibody to bring about the formation of the enzyme linked-antibody-antigen complex. Washing the membrane by soaking in an aqueous buffer is used to separate bound from unbound reactant. When the membrane is soaked in a substrate solution, the enzymatic reaction produces an insoluble product that precipitates as a dot on the membrane.

Two variations of this assay are direct or indirect ELISAs. In the direct, assay antigen is adsorbed to the membrane and the antibody molecule is covalently coupled to the enzyme. The enzyme catalyses the conversion of the substrate to a product which precipitates on the membrane surface at the site of the reaction. The indirect ELISA is used if the antibody concentration is low to efficiently couple an enzyme, or if the antibody

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Clinical Diagnostic Immunoblot

is in a biological mixture making its purification too costly or difficult. In the indirect "sandwich" ELISA, antigen is absorbed to the membrane. The membrane is soaked in primary antibody, i.e., antibody with specificity to the antigen. The membrane bound antigen antibody is then reacted by soaking the membrane in a solution of the secondary covalently linked enzyme-antibody.

In this experiment, the indirect assay will be utilized. The antigen will be reacted with its specific antibody. The secondary antibody-enzyme conjugate is anti-IgG conjugated to horseradish peroxidase. Simulated patient samples will be tested in duplicate and with control reactions. The procedures used in this experiment are based on the same principles involved in Dot-blot immunoassays routinely used in clinical tests including some that are available over the counter.

Experiment Overview

Experiment Procedures

EXPERIMENT OBJECTIVE:

In this experiment, you will prepare a Dot Blot membrane antibody test and determine the presence of antigen in simulated samples. This experiment is an indirect method, "sandwich technique" using antigen adsorbed to a nylon membrane, reacted with its specific antibody, and quantitated using an antibody-enzyme conjugate.



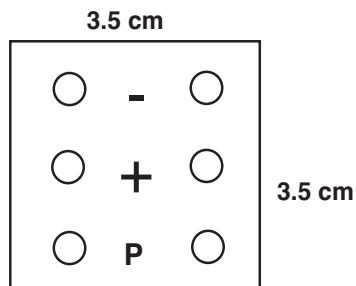
Wear gloves
and safety
goggles

LABORATORY SAFETY

1. Gloves and safety goggles should be worn routinely as good laboratory practice. **In all steps, use gloves and forceps to handle the membrane.**
2. **DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.**
3. Always wash hands thoroughly with soap and water after handling contaminated materials.



Student Experimental Procedures



Application of Samples

In all steps, use gloves and forceps to handle the membrane.

SAMPLE APPLICATION

1. Place a piece of membrane on a paper towel.
2. Using a micropipet, apply 5-10 μ l of the samples to the membrane. Each membrane should have negative and positive controls and patient samples.

All samples are applied in duplicate as shown in diagram on left.

- Negative control
 - Positive control
 - Patient sample
3. Allow the membrane to dry completely for 10-15 minutes at room temperature or 5 minutes in a 37°C incubation oven.



Optional Stopping Point: Experiment can be stopped after step 3 and resumed during next lab period.

BLOCKING STEP

4. Two 60 mm diameter petri dishes will be used for steps 5 through 30. Label the petri dishes #1 and #2.
5. Pipet 10ml of blocking buffer into petri dish #1.
6. Immerse the membrane in the blocking buffer and incubate at room temperature for 10 minutes with frequent mixing or place on a shaking or rotating platform.

Student Experimental Procedures

Useful Hint!



If steps 10 and 19 are done manually, the plate should be shaken at 5 to 10 second intervals. The more frequently the plate is shaken, the more likely a reproducible result will occur.

Remember!



All reagents in this experiment can be discarded down the drain.

FIRST ANTIBODY

7. Pipet 5ml of diluted anti-albumin antiserum into petri dish #2.
8. Remove the membrane from the blocking buffer. Drain excess buffer from the membrane by holding the membrane with forceps and touching the corner of it to a clean paper towel.
9. Immerse the membrane in the diluted antiserum in petri dish #2.
10. Incubate at room temperature for 10 min. with frequent mixing or place on a shaking or rotating platform. See Useful Hint at left.

FIRST RINSE

11. Rinse and dry petri dish #1. Pipet 10ml of Tris buffer containing saline (TBS) into the petri dish.
12. Lift the membrane from the diluted antiserum in petri dish #2. Drain excess antiserum from the membrane.
13. Immerse the membrane in the TBS (dish #1). Incubate at room temperature for 5 minutes with frequent mixing or place on a shaking or rotating platform.
14. Lift the membrane from petri dish #1. Pour off the TBS.
15. Pipet 10ml of fresh TBS into petri dish #1. Immerse the membrane and incubate at room temperature for 5 minutes with frequent mixing or place on a shaking or rotating platform.

SECOND ANTIBODY

16. Discard the antiserum in petri dish #2. Rinse and dry the petri dish. Pipet 5ml of IgG-horseradish peroxidase (antibody-enzyme conjugate) into the petri dish.
17. Remove the membrane from the TBS (dish #1). Drain excess TBS from the membrane.
18. Immerse the membrane in antibody-enzyme conjugate (dish #2).
19. Incubate at room temperature for 10 minutes with frequent mixing or place on a shaking or rotating platform. See Useful Hint at left.



Student Experimental Procedures



Wear safety goggles and gloves.

Note:

Your instructor will prepare the peroxidase substrate approximately 15 minutes before you need it for Step 24 in the experimental procedure. Obtain the peroxidase substrate from your instructor.

SECOND RINSE

20. Rinse and dry petri dish #1. Pipet 10ml of fresh TBS into the dish.
21. Lift the membrane from the antibody-enzyme conjugate (dish #2). Drain excess conjugate from the membrane.
22. Immerse the membrane in the TBS (dish #1). Incubate at room temperature for 5 minutes with frequent mixing or place on a shaking or rotating platform.
23. Lift the membrane from petri dish #1. Pour off the TBS. Pipet 10ml of fresh TBS into petri dish #1. Immerse the membrane into the fresh TBS. Incubate at room temperature for 5 minutes with frequent mixing or place on a shaking or rotating platform.

SUBSTRATE

24. Rinse and dry petri dish #2. Pipet 10ml of peroxidase substrate into the dish.
25. Lift the membrane from the final TBS wash solution and drain excess TBS from the membrane.
26. Immerse the membrane in the peroxide substrate (dish #2). Allow the membrane to soak in the substrate for 5-10 minutes, or until the color intensity of the reaction is maximal.
27. Discard the TBS in petri dish #1. Rinse and dry the petri dish. Pipet 10 ml of distilled water into the dish.
28. Lift the membrane from the substrate solution and drain excess substrate from the membrane.
29. Immerse the membrane in the distilled water. Soak the membrane in the water for one minute.
30. Lift the membrane from the water and drain excess water from the membrane.
31. Place the membrane on a paper towel. Record the dots of precipitated substrate in Samples A & B. Compare to the two controls.

PRESERVATION AND STORAGE OF THE MEMBRANE (OPTIONAL)

32. If you wish to preserve/store the membrane, it must first be dried. Stand the membrane on its edge (i.e. using a test tube rack as a support to dry) for a minimum of 20-30 minutes. Store the membrane in a plastic bag.

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. Explain the binding of proteins to a nylon membrane? What evidence in the experiment is there that it is a surface phenomenon?
2. What are the similarities and differences between this technique and enzyme-linked immunosorbent assay (ELISA)?
3. What are advantages of the ELISA reaction over RIA?



Notes to the Instructor

APPROXIMATE TIME REQUIREMENTS

1. Prelab preparation and dispensing of biologicals and reagents will require approximately 1 hour.
2. Students should be able to complete the experiment in approximately 1.5 to 2 hours.

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).

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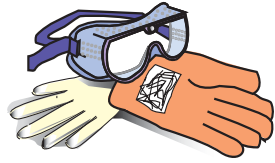
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Wear gloves when handling the nylon membranes.

PreLab Preparations

1. Cut Nylon membranes into 3.5 x 3.5 cm pieces to fit into 60 mm petri dishes. Distribute one piece of membrane per group.
2. In a 1 Liter beaker or flask, dilute 10x TBS (G) by mixing 90ml of 10x TBS with 810ml of distilled water. This is 1x TBS. Each group will require 65ml of 1x TBS.

Prepare Samples

1. Negative Control
 - Label 10 tubes "Negative Control"
 - Aliquot 0.03ml (30 μ l) of 1x TBS per tube.
2. Positive Control
 - Label 10 tubes "Positive Control"
 - Aliquot 0.03ml (30 μ l) of Component A, Antigen per group.
3. Patient Sample
 - Label 10 tubes "Patient Sample"
 - Aliquot 0.03 ml (30 μ l) of Component A, Antigen or 1x TBS per tube. (Some students can receive antigen or TBS to show variation in the class results.)

Prepare Blocking Buffer

- Mix the instant nonfat dry milk (H) in 200ml of 1x TBS.
- Aliquot 10ml per lab group.

Prepare Diluted Anti-antigen Antiserum

- Dilute all of the anti-antigen antiserum (B) with 50ml of blocking buffer. Rinse tube B with a portion of the blocking buffer to ensure that none of the anti-antigen antiserum remains in the tube.
- Aliquot 5ml per lab group.

Prepare Peroxidase Conjugate

- Dilute all of the IgG-Horseradish peroxidase conjugate (C), with 50ml of blocking buffer. Rinse tube C with a portion of the blocking buffer to ensure that none of the IgG-Horseradish peroxidase conjugate remains in the tube.
- Aliquot 5ml per lab group.



Pre-Lab Preparations

7. Prepare Substrate (prepare within 15 minutes of its use)

- Dissolve all of the peroxidase substrate (D), in 75ml of Substrate Dilution Buffer (F).
- Add 7.5ml hydrogen peroxide (E).
- Aliquot 10ml per group.

QUICK REFERENCE

Each group will receive:

Nylon membrane (3.5 x 3.5 cm)	1 piece
Negative Control	0.03 ml (30 μ l)
Positive Control (30 μ l)	0.03ml
Patient Sample (Antigen or 1x TBS)	0.03 ml (30 μ l)
Anti-antigen antiserum	5 ml
Blocking buffer	10 ml
IgG-Horseradish Peroxidase conjugate	5 ml
TBS	65 ml
Prepare within 15 minutes of use: Peroxidase Substrate	8 ml

Helpful Hints & Reminders

1. Always wear gloves and handle the membrane with forceps.
2. When draining excess solution or water from the membrane, hold the membrane vertically with forceps and touch the lower edge to a piece of clean absorbent paper towel.
3. The precipitation of product can be checked by lifting the membrane from the substrate and looking at the intensity of the dot.
4. The dried membrane with dots of precipitate can be stored by wrapping in plastic wrap. Visualization of the dried membrane may be enhanced by re-wetting the membrane with distilled water.
5. Different students can be given different patient samples (+ or -) yielding variations in the results. For example, patient samples can be either positive or negative.

Anticipated Results

Dots of precipitated product will appear within minutes of immersing membrane in the enzyme substrate solution.

This experiment can also be modified to quantify various dilutions of the primary antibody. In such an experiment the instructor would dilute Sample A (positive) two fold and four fold. The procedure is identical for the various steps. The diameter of the dots will decrease with decreasing antigen concentration. A plot of dot diameter squared vs. antigen concentration can be prepared on regular graph paper. A linear relationship should be apparent. From this graph extrapolate the diameter squared of the unknown concentration to the intersect on the graph and read the approximate concentration of the unknown. It should correlate with 0.1 mg/ml antibody.



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