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# Dot Blot Analysis

## Teacher's Guidebook

(Cat. # BE-502)



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## **MATERIALS INCLUDED WITH THE KIT**

This kit has enough materials and reagents for 24 students (6 groups of 4 students each).

- 1 vial Simulated Sample 1
- 1 vial Simulated Sample 2
- 1 vial IMU Positive Control
- 1 vial IMU Negative Control
- 1 vial Antigen Binding Buffer
- 1 pack Protein Binding Membrane Strips
- 1 bottle Blocking Buffer (2X NAP-Blocker)
- 1 bottle MEM Washing Buffer (10X)
- 1 vial Antibody: BE Antibody 1 (Ab: BE-1)
- 1 vial Antibody: BE Antibody 4 (Ab: BE-4) (HRP Secondary)
- 1 bottle HRP Substrate
- 30 Centrifuge Tubes (1.5ml)
- 1 tube Sterile Water

## **SPECIAL HANDLING INSTRUCTIONS**

- The kit components should be stored as described on the components label.

## **ADDITIONAL EQUIPMENT REQUIRED**

- Shaking Incubator
- Plastic Washing Trays 12cm x 12cm

## **TIME REQUIRED**

- 4-6 hours

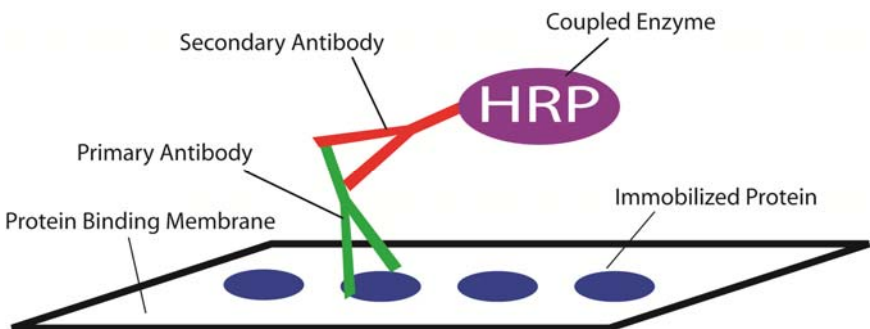
## OBJECTIVES

- To understand the principle of Dot Blotting.
- Use of Dot blotting for diagnostic tests.
- To establish the importance of Dot blotting in identifying the protein of interest.

## INTRODUCTION

Dot blotting is an important technique that is routinely used in research and diagnostic laboratories. Dot blotting is a simple technique to identify a known protein in a biological sample. The ease and simplicity of the technique makes dot blotting an ideal diagnostic tool.

The key feature of Dot blotting is the use of immunodetection to identify a specific protein, for example a protein marker for a disease. Once the proteins are immobilized on a protein binding membrane, usually nitrocellulose or PVDF (polyvinylidene fluoride), they can be probed with a primary antibody, an antibody specific for the protein of interest. Once bound the antibody is visualized, either with a specific tag coupled to the primary antibody or with a secondary antibody. The secondary antibody is a general antibody that recognizes the constant domain of immunoglobulin G and is species specific. So, if the primary antibody is a mouse antibody, the secondary antibody used will recognize all mouse antibodies. If a secondary antibody is used then this will carry the tag that allows visualization of the protein (see figure below).



The most common tags used in Dot blot are enzymes that catalyze a substrate to produce either light that is detected with radiography film, or color that is visualized on the membrane. The enzymes of choice are horseradish peroxidase (HRP) and alkaline phosphatase (AP).

An additional step is crucial to Dot blot and this is known as the blocking step. The blocking step is used to increase the specificity of the Dot blot technique by preventing non-specific interactions. If the membranes are not blocked then the antibodies can stick to non-specific proteins due to their charge. To prevent this, the membrane is placed in a protein mixture and the proteins block the charges that would attract the antibodies. Several blocking agents are used, including dried milk powder, bovine

serum albumin and casein, however modern blocking agents use synthetic and/or non-animal proteins to prevent any cross reaction with the animal antibodies. An example of a non animal blocker is the provided NAP-Blocker™.

### ***How Are Antibodies Made (Primary Antibody)?***

When animals are exposed to antigens, they generate an immune response and produce antibodies (proteins) that recognize and bind tightly to the specific antigens. Each antibody recognizes only a single antigen. Scientists have learned to use the immune response of animals to make antibodies that can be used as tools to detect and diagnose diseases. Animals such as goats, rabbits, and mice can be injected with an antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease-causing agent, the antibodies can be used to develop diagnostic tests for the disease. In an immunoassay, the antibodies used to recognize antigens like disease agents are called primary antibodies.

### ***Enzyme Labeled Antibodies (Secondary Antibodies)***

Secondary antibodies recognize and bind to primary antibodies in immunoassays (e.g. Dot and Western blots). Secondary antibodies are prepared in the same manner as primary antibodies and the antigen is antibodies from a different species, normally a fragment containing the constant (conserved) domain.

Specific enzymes, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), are then chemically coupled to the constant domain of the antibody, away from the antigen binding domain. The enzymes are able to catalyze a chemical substrate to produce either a chemiluminescence (light) or colorimetric (color) product that can be detected. This experiment uses HRP and a colorimetric substrate known as 3,3',5,5'-tetramethylbenzidine (TMB).

This Dot Blot Analysis experiment allows students to run their own Dot blot and use it as a diagnostic tool. The kit is provided with simulated clinical samples and students will probe the samples for a protein that is over expressed when the patient is infected, allowing them to identify infected patients.

## TEACHER'S PRE-EXPERIMENT SET UP

1. Allow all reagents stored in the cold to warm to room temperature.
2. Add 200 $\mu$ l of Antigen Binding Buffer to each vial of lyophilized antigen (Simulated Sample 1, Simulated Sample 2, IMU Positive Control and IMU Negative Control). Mix well by vortexing.
3. Transfer 20 $\mu$ l Simulated Sample 2 to a clean tube and add 180 $\mu$ l Antigen Binding Buffer to dilute the sample 1 in 10. Use this diluted sample for all subsequent experiments.
4. Label six sets of 4 tubes either P1, P2, positive, or negative for patient 1, patient 2, IMU Positive and IMU Negative Control, respectively. Transfer 10 $\mu$ l of each sample from step 2 and 3 to the appropriate set of tubes. Supply each group with a single tube of Simulated Sample 1, Simulated Sample 2, IMU Positive Control and IMU Negative Control.
5. Add 250 $\mu$ l of sterile water to the lyophilized primary and secondary antibody pellets, leave to stand for two minutes then mix with gentle pipetting up and down. OPTIONAL: Aliquot each antibody into 6 vials, with 40 $\mu$ l in each vial. Supply each group with 1 vial.
6. Make a 1X MEM Washing Buffer solution by adding 200ml MEM Washing Buffer (10X) to a 2-liter container. Bring up to final volume of 2 liters with DI water  
*The MEM Washing Buffer is TBS (Tris buffered saline) supplemented with a mild detergent to aid in membrane washing.*
7. The morning of the experiment, gently shake the supplied Blocking Buffer (2X NAP-Blocker) and mix equal volumes of Blocking Buffer (2X NAP-Blocker) with 1X MEM Washing Buffer.

## MATERIALS FOR EACH GROUP

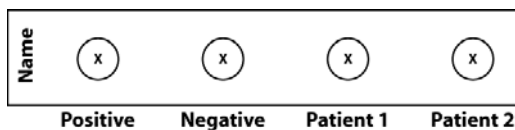
Supply each group with the following components. Several components will be shared by the whole class and should be kept on a communal table.

- 30µl each of Simulated Sample 1, Simulated Sample 2, IMU Positive Control and IMU Negative Control
- 1X MEM Washing Buffer (shared with class)
- 1X Blocking Buffer (NAP-Blocker) (shared with class)
- 1 vial Antibody: BE Antibody 1
- 1 vial Antibody: BE Antibody 4 (HRP Secondary)
- 1 bottle of HRP Substrate (shared with class)
- 4 strips of Protein Binding Membrane
- 2 50mL tubes
- 1 12cm x 12cm washing tray

## PROCEDURE

### *Spotting Sample Protocol*

1. Using forceps remove the nitrocellulose membrane strip from the protective cover. Place the strip on a clean flat surface (e.g. clean white paper). Label with your name at the left end of the strip with a **pencil**. (see figure 1)
2. Using a 5-10µl pipette, remove 5µl of the sample and spot onto the membrane. With the tip end held 0.5cm/ ¼" above the area to be spotted, slowly push the sample out of the tip to form a hanging drop. Touch the drop onto the center of the area to be spotted and allow the sample to enter the membrane. Spot all the samples on the strip as in the figure below.



3. Once all students in your group finished spotting and all liquid has absorbed onto the membrane, place all membrane strips in a 12x12cm washing tray with forceps.

***Protein Detection***

1. Add 20ml 1X Blocking Buffer (NAP-Blocker) to block the non-specific sites. Incubate at room temperature for 30-60 minutes with gentle shaking.
2. Prepare the primary antibody by adding 40 $\mu$ l BE Antibody 1 to 20ml 1X Blocking Buffer (NAP-Blocker).
3. Discard the Blocking Buffer. Add the primary antibody solution to the membrane and incubate for 30-60 minutes at room temperature with gentle shaking.
4. Discard the antibody solution and wash 3 times with 20ml MEM Washing Buffer for 10 minutes each.
5. Make secondary antibody by mixing 40 $\mu$ l BE Antibody 4 (HRP Secondary) with 20ml 1X Blocking Buffer (NAP-Blocker) in a 50mL tube. The secondary antibody has a horseradish peroxidase tag.
6. Discard the MEM Washing Buffer and add the secondary antibody solution to the membrane and incubate at room temperature for 30-60 minutes with gentle shaking.
7. Discard the antibody solution and wash 3 times with 20ml 1X MEM Washing Buffer for 10 minutes each.
8. Discard the MEM Washing Buffer and add 5ml of HRP Substrate to the membrane. Let shake for 5 minutes or until color develops at room temperature.
9. Pour off the substrate and add DI water to stop the color reaction. Record your results.



## RESULTS, ANALYSIS & ASSESSMENT

1. Which of the patients is carrying the highest level of infection?

*Patient 1 has a higher level of the detected protein, which in this experiment is indicative of infection. Increased levels of the protein represents infection.*

2. Describe the circumstances in which only one antibody is required for Dot blotting.

*The primary antibody can be directly labeled with a tag, such as horseradish peroxidase or alkaline phosphatase. This eliminates the requirement for a secondary antibody*

3. What is the function of the blocking step?

*The blocking step is required to prevent non-specific binding of the antibodies to the membrane, which could result in false positives.*

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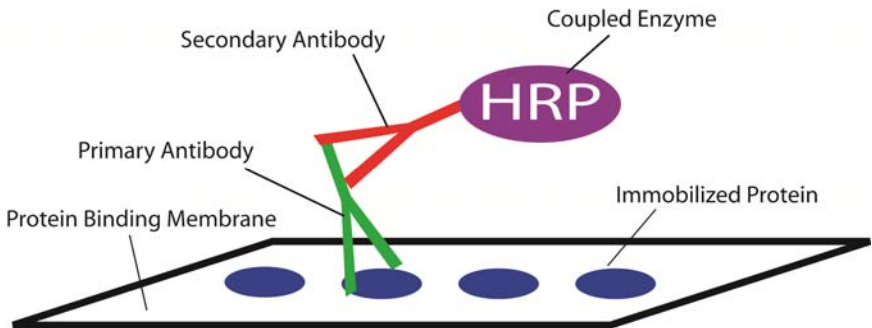
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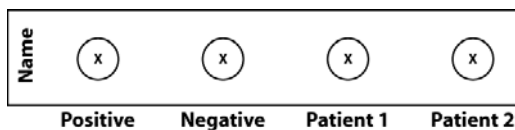
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