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Western Blot Analysis

Teacher's Guidebook

(Cat. # BE-503)

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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 PAGE: Sample Loading Buffer (2X)
- 8 vials PAGEmark™ Blue PLUS Protein Marker
- 1 bottle Western Transfer Buffer (20X)
- 1 bottle Blocking Buffer (2X NAP-Blocker)
- 1 bottle MEM Washing Buffer (10X)
- 1 vial Simulated Sample 1
- 1 vial Simulated Sample 2
- 1 vial Antibody: BE Antibody 1 (Ab: BE-1)
- 1 vial Antibody: BE Antibody 4 (Ab: BE-4) (HRP Secondary)
- 1 bottle HRP Substrate
- 1 vial IMU Positive Control
- 1 vial IMU Negative Control
- 1 vial Sterile Water
- 6 Protein Binding Membranes
- 30 Centrifuge Tubes (1.5ml)

SPECIAL HANDLING INSTRUCTIONS

- Reagents/kit components should be stored as recommended

ADDITIONAL EQUIPMENT REQUIRED

- Protein Electrophoresis Equipment, SDS PAGE gels and electrophoresis buffer.
- Blotting Unit for Western Transfer
- Shaking Incubator
- Washing Trays 12cm x 12cm
- Methanol or Isopropanol
- Sharp pins

TIME REQUIRED

- 6-8 hours

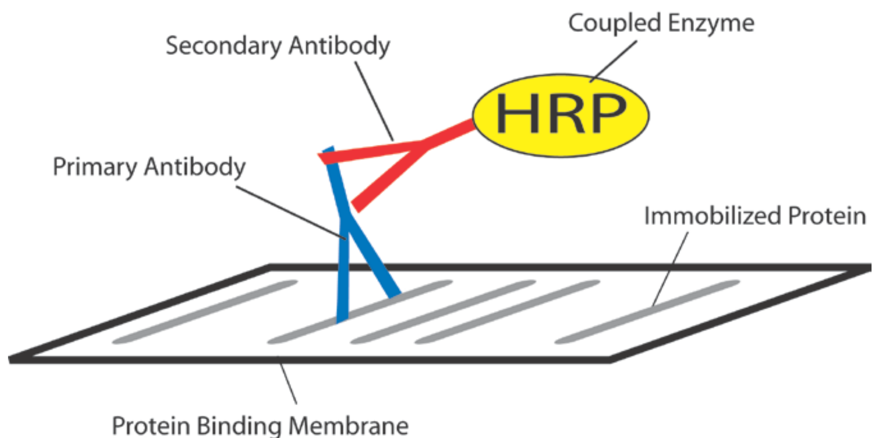
AIMS

- To understand the principle of SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting
- To run SDS-PAGE and prepare Western blots.
- To establish the importance of Western blotting in identifying the protein of interest.

INTRODUCTION

Western blotting is an important technique that is routinely used in research and diagnostic laboratories. Western blotting is combined with polyacrylamide gel electrophoresis, which separates proteins based on their molecular weight. Western blotting consists of the transfer of the separated proteins onto a membrane where they can be identified with specific antibodies. Western blotting is named after a similar technique, Southern blotting, which is the transfer of DNA to a membrane; a technique invented by the British biologist Edwin Southern. Northern blotting is a similar technique, but for RNA.

The key feature of Western 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).



The most common tags used in Western 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 Western blot and this is known as the blocking step. The blocking step is used to increase the specificity of the Western 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. 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 Western Blot Analysis experiment allows students to run their own Western 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. **NOTE**: You will need 8x10cm protein SDS-Polyacrylamide gels to match the supplied membrane, electrophoresis buffer, protein electrophoresis and Western blotting equipment for this experiment. Each group requires 5 lanes of a protein gel; so 2 groups can share one 10-well gel.

2. Allow all reagents stored in the cold to warm to room temperature.

3. Add 50µl of Sample Loading Buffer (2X) and 50µl Sterile Water to each vial of lyophilized antigen (Simulated Sample 1, Simulated Sample 2, IMU Positive Control and IMU Negative Control). Incubate at room temperature for 5 minutes and then mix well by vortexing.

4. Label six sets of 4 tubes either P1, P2, +, or -, respectively. Transfer 15µl of each sample from step 3 to the appropriate set of tubes. Supply each group with a single tube of Patient 1, Patient 2, IMU Positive and IMU Negative Control.

5. Add 250µl of sterile water to the lyophilized BE Antibody 1 and BE Antibody 4 (HRP Secondary), leave to stand for 5 minutes then mix with gentle pipetting up and down. OPTIONAL: Aliquot each antibody into 6 vials, with 40µl in each vial. Supply each group with 1 vial.

6. Make a 1X MEM Washing Buffer solution by adding 200ml 10X MEM Washing Buffer 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) bottle and then mix equal volumes of Blocking Buffer (2X NAP-Blocker) with 1X MEM Washing Buffer.

8. Make 1X Western Transfer Buffer by adding 50ml of 20X Western Transfer Buffer and 200ml methanol (or isopropanol) to a 1 liter container. Add 750ml DI water to make a final volume of 1 liter. Depending on the size or type of Western blot equipment you are using, you may require additional Western Transfer Buffer. Additional Western Transfer Buffer may be obtained from G-Biosciences (visit www.GBiosciences.com for information).

9. Prepared electrophoresis buffer as needed. Typical ingredient: 0.025M Tris base, 0.192M Glycine and 0.1% SDS.

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.

- 15µl each of Simulated Sample 1, Simulated Sample 2, IMU Positive Control and IMU Negative Control
- 1 vial PAGEmark™ Protein Marker (do not boil)
- 1 pin
- 1X MEM Washing Buffer (shared with class)
- 1X Blocking Buffer (NAP-Blocker) (shared with class)
- 1 vial BE Antibody 1
- 1 vial BE Antibody 4 (HRP Secondary)
- 1 bottle of HRP Substrate (shared with class)
- 3 50mL tubes
- 1 12cm x 12cm Washing Tray
- 1 Protein Binding Membrane
- Electrophoresis and Blot apparatus

PROCEDURE

SDS PAGE Protocol

1. Pierce the lids of the 4 sample tubes (Patient 1-2, IMU Positive and Negative Controls) with a sharp pin. Boil the protein samples for 3-5 minutes in a boiling water bath. After boiling, centrifuge the samples for 20 seconds to bring down the condensation from sides of tubes.

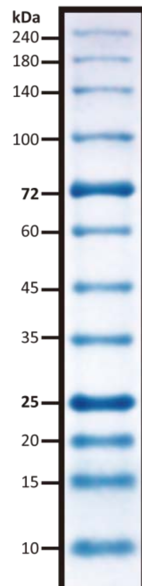
CAUTION: Be careful around the boiling waterbath.

2. Assemble SDS-PAGE gels in the electrophoresis equipment.

3. Load 5µl protein marker and 10µl of all the samples on the gel as per your teacher/ instructor's directions. Load as in the table below.

Lane #	1	2	3	4	5
Sample	Marker	Positive	Negative	Patient 1	Patient 2

4. The PAGEmark™ Protein Marker consists of a mix of twelve Prestained proteins of molecular weight 240, 180, 140, 100, 72, 60, 45, 35, 25, 20, 15 and 10kDa.
5. Run the gel at 30mA until the blue dye front is 0.5-2cm from the bottom of the gel.
6. Disassembly the gel carefully, as per your instructor's instructions and place the gel in a tray of transfer buffer.



4-20% Tris-Glycine

Western Blotting Procedure

CAUTION: Do not handle the membrane unless wearing gloves.

1. Follow the manufacturer's instructions for assembling of the Western blot apparatus. A general guideline is outlined below.

2. In a suitable tray, or large weigh boat, soak 2 sheets of filter paper, 2 fiber pads, and the Protein Binding Membrane (a nitrocellulose membrane) in 1X transfer buffer.

3. Open the plastic blot holder and place it in a plastic dish with the dark side down.

4. Place one fiber pad on the cassette, followed by one sheet of filter paper. Ensure no air bubbles are trapped.

5. Carefully, whilst wearing gloves, pick up the gel and lower it onto the filter paper. Ensure no air bubbles are trapped.

6. Next using the forceps, lower the Protein Binding Membrane onto the gel. Ensure no air bubbles are trapped.

7. Finally, lay the final piece of filter paper, then the fiber pad onto the stack and close the cassette.

8. Transfer the cassette to the Western blot apparatus and transfer the proteins according to your teacher/ instructor's directions.

NOTE: The orientation of the "sandwich" is essential. The wrong orientation will result in the proteins moving away from the membrane and being permanently lost. The gel should be nearest the cathode (the negative terminal).

9. On completion of transfer, open the cassette and carefully remove the Protein Binding Membrane with forceps and place in a 12x12cm washing tray with 1X MEM Washing Buffer.

10. *If sharing a membrane with another group use a razor blade to cut the membrane in half. Cut through the prestained marker lane.*

Protein Detection

1. Wash the Protein Binding Membrane twice for 5 minutes in 20ml 1X MEM Washing Buffer by placing the tray on a shaker.

2. Discard the MEM Washing Buffer and add 20ml 1X Blocking Buffer (NAP-Blocker) to block the non-specific sites. Incubate at room temperature for 30-60 minutes with gentle shaking. This can be left at 4°C overnight.

3. Prepare the primary antibody by adding 40µl BE Antibody 1 to 20ml 1X Blocking Buffer (NAP-Blocker).

4. Discard 1X Blocking Buffer (NAP-Blocker) and add the BE Antibody 1 solution to the Protein Binding Membrane and incubate for 30-60 minutes at room temperature with gentle shaking.

5. Discard the antibody solution and wash 3 times with 20ml MEM Washing Buffer for 10 minutes each.

6. Make secondary antibody by mixing 40µl BE Antibody 4 (HRP Secondary) with 20ml 1X Blocking Buffer (NAP-Blocker) in a 50mL tube. The secondary antibody has a horseradish peroxidase tag.

7. Discard the wash and add the secondary antibody solution to the Protein Binding Membrane and incubate at room temperature for 30-60 minutes with gentle shaking.

8. Discard the antibody and wash 3 times with 20ml MEM Washing Buffer for 10 minutes each.

9. Discard MEM Washing Buffer and add 5ml of HRP Substrate to the Protein Binding Membrane. Let shake for 5 minutes or until color develops at room temperature.

10. 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

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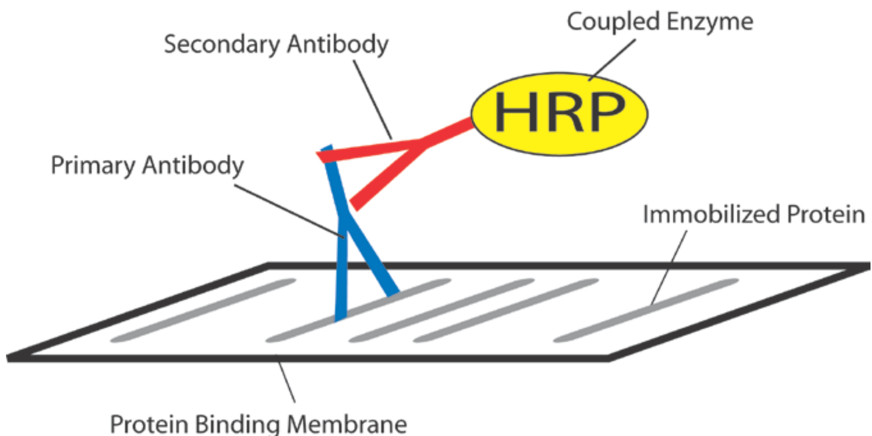

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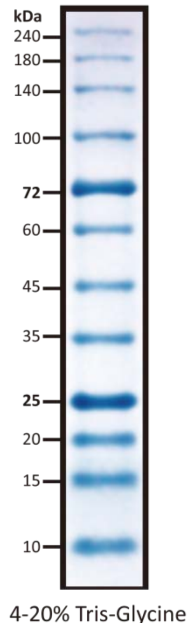

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