Western Blotting

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Manuscript No: KN-V2-01/033
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INTRODUCTION

Towbin first described western blotting of proteins in 1979, and it has since become a standard and essential method for protein analysis. It is also known as immunoblotting or protein blotting. The specificity of the antibody-antigen interaction allows a target protein to be recognized among a complex protein mixture, such as cell or tissue lysate. It employs antibodies to detect particular protein targets linked to a membrane. It may be applied to produce semi-quantitative and qualitative information about a protein of interest (Antharavally et al., 2004).

SAMPLE PREPARATION

Detergent lysis

By treating lysed cells with detergents like Triton-X, TritonX-114, Tween-20, or digitonin, among others, it is possible to separate membrane-bound (hydrophobic) proteins from cytosolic (hydrophilic) proteins. This will generate two phases: a hydrophilic phase and a hydrophobic phase. This technique can be quite effective when it comes to enhancing for cytosolic and membrane proteins. But occasionally, the large amount of detergent required might cause issues for tests conducted later on. As a result, you might need to take out the extra detergent.

Ultrasonication

Ultrasonic homogenization is powerful technique that shears and disrupts cells. Sonication is advised for cell suspensions, bacteria, and some tissues. It generates ultrasonic sound waves using a probe. This method's ability to produce heat, which might cause protein denaturation if unchecked, is one of its drawbacks.

Enzyme digestion

Zymolyase, cellulases, chitinases, and lysozymes, respectively, are enzymes which may be employed to destroy the cell walls of yeast, plants, fungus, and bacteria.

Mechanical Homogenization

The breaking up of tissue (homogenization) and disruption of cells may require mechanical disruption in the lysis buffer. A French press, glass homogenizer, sonication, mortar and pestle, etc. can all be used to homogenize tissue. During this homogenization process, it is important to keep the tissue cold. You may do this by using ice or equipment with a cooling component. The sample preparation phase, which comes first in a Western blot, is crucial because it sets the stage for subsequent immunoblotting procedures to succeed. This initial step should provide a high yield of the target protein. Purified protein samples can be used for Western blot analysis. Tissue or cell extracts, on the other hand, are most frequently used since they comprise a variety of proteins. To release the proteins from tissue or cell extracts without triggering breakdown or proteolysis, the cells must first be properly disrupted or lysed (to minimize endogenous proteinase activity).

GEL ELECTROPHORESIS

According to their molecular weight proteins are separated using a procedure called SDS PAGE, or sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The method of separating protein molecules per their

electrophoretic mobility is often used in molecular biology, biotechnology, and forensics (Tovey & Baldo, 1987).

Principle of SDS-PAGE

As per the SDS-PAGE principle, a charged molecule in an electric field migrates to the electrode with the opposite sign. The relative mobility of charged species determines how the charged molecules separate from one another. Due to reduced resistance during electrophoresis, the smaller molecules migrate more quickly. The rate of migration is also influenced by the charge and structure of the proteins. Proteins are separated according to the length of their polypeptide chains after the impact of their structure and charge is eliminated by sodium dodecyl sulphate and polyacrylamide (Smith, 1984).

PROTEIN TRANSFER

Following electrophoresis, the proteins separated are "blotted" onto a solid support matrix, which is often a membrane made of PVDF or nitrocellulose. Dot blotting technique allows the material to be spotted directly onto the membrane in operations where protein separation is not necessary (Gooderham, 1984).Protein transfer from gel to membrane is necessary for two reasons, one is better handling capability offered by the membrane compared to a fragile gel and another one is better target protein accessibility on the membrane by macromolecules like antibodies. There are three ways to electro-transfer proteins from SDS-PAGE or native gels to membranes: Wet electroblotting (traditional wet or tank transfer) Semi-dry electroblotting (semi-dry transfer), Dry electroblotting (dry transfer).

WESTERN BLOT BLOCKING

Because blotting membranes have a high affinity for proteins, it's critical to inhibit any binding sites that may still be present after transfer to avoid test detection antibodies binding non-specifically later on. Since the target and antibodies are both proteins, the membrane can attach to both, so some unintended binding may occur. Consequently, the antibodies must only attach to the target protein and not the membrane. Antibody non-specific binding can be reduced by keeping vacant spots in inert protein or non-ionic protein from being occupied.

Western blot washing

Washing the membrane after blocking is crucial to get rid of extra or unattached chemicals between steps. Blots with a high background and poor quality/patchy appearance might result from insufficient or uneven washing. However, it's crucial to maximize the quantity and length of wash stages because overwashing might reduce the intended signal. To wash the membrane uniformly without causing any damage, make sure it is well coated with the suitable buffer and gently agitate the membrane. Tris-buffered saline (TBS) and phosphate-buffered saline (PBS) are two frequently used buffers; Tween 20 is frequently added to both (TBST and PBST).

ANTIBODY PROBING

To identify the desired protein in the membrane, the blot is blocked and then treated with one or more antibodies. The solution in which the membrane is floating has been added with the primary antibody. The primary antibody recognizes a particular protein's distinctive amino acid sequence. Labeled secondary antibody is added after sufficient washing to eliminate unbound primary antibody. The secondary antibody is often conjugated with an enzyme and recognizes the primary antibody.

PROTEIN DETECTION

The western blotting is now prepared for detection after the unbound probes have been removed by washing. Two different kinds of detection, Direct detection is the process of identifying and detecting the target using

a single antibody. Indirect detection involves the use of a primary antibody to probe the membrane and bind any target proteins that may be present, followed by the use of a secondary antibody to attach to the primary antibody and enable detection.

ANALYSIS AND IMAGING

When using X-ray film to obtain results from your western blot experiment, it is often necessary to expose several films to optimize exposure and developing time. Too short, and the sample bands could be hardly perceptible or invisible. If the background signal is there for too long, the bands will combine and the resulting blot will be extremely dark and difficult to see. Unbalanced concentration of controls or size markers relative to the goal can also cause issues, since one may become overexposed before the other is suitably visible. After that, films can be digitalized and subjected to additional study with analytic software. X-ray film detection techniques are classified as either qualitative or semi-quantitative.A target's presence or absence in a sample can be determined by comparing the bands in the sample lanes to size markers and controls. However, because of their bigger dynamic range, greater sensitivity, and higher resolution, digital imaging using CCD camera-based systems, for instance, offers a superior alternative if quantitation is necessary. By adjusting exposure periods, the signal-to-noise ratio may be maximized without requiring time-consuming, repetitive X-ray film exposures and develops. This may then be simply entered into software for data analysis and comparison.

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