Western Blotting: Principle and Application for Detection of Proteins

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Abstract

Western blotting (protein blotting or immunoblotting) is a powerful and important procedure for the immunodetection of proteins post-electrophoresis, particularly proteins that are of low abundance. Since the inception of the protocol for protein transfer from an electrophoresed gel to a membrane in 1979, protein blotting has evolved greatly. The scientific community is now confronted with a variety of ways and means to carry out this transfer. This article describes the principle and various methods that have been used to transfer proteins from a gel to a membrane based on the principles of simple diffusion, vacuum-assisted solvent flow and electrophoretic elution.

Introduction

Western blotting also called Western blot and enzyme-linked immunosorbent assay (ELISA) are widely used in the protein detection. Since the inception of the protocol for protein transfer from an electrophoresed gel to a membrane by Towbin in 1979 (Towbin, 1979), protein blotting has evolved greatly. The name Western blotting is a pun on the name Southern blotting, a technique for DNA detection and the detection of RNA is termed northern blotting. Western blotting analysis can detect protein in a solution and thus can provide useful information. It uses gel electrophoresis to separate denatured proteins by mass. Later, protein is transferred from SDS-gel to a nitrocellulose membrane (electric transfer) and then combined with primary antibody and secondary antibody (antibody-enzyme conjugate, e.g., horseradish peroxidase (HRP). It is normally used with a high quality antibody directed against a desired protein. Researchers can examine the amount of protein in a given sample and compare levels between several groups. Immunocytochemistry can also be used for detection of proteins in tissues and cells using antibodies.

Principle of Western Blotting

Western blotting is a method in molecular biology/biochemistry/immunogenetics to detect protein in a given sample of tissue homogenate or extract. The proteins are separated first of all using SDS-polyacrylamidegel electrophoresis (SDS-PAGE) on basis of size. They are then transferred from the gel onto a membrane (typically nitrocellulose or PVDF membrane) and combined with antibodies specific to the protein. The secondary antiboy can be stained and pictured by a film. The film with the protein binds can be kept for a long time and scanned any time it needs to quantity the protein levels.

Steps of Western blotting

Tissue preparation

Typically, samples are taken from either tissue or from cell culture. The samples are cooled or frozen rapidly. They are homogenized using sonication or mechanical force. The resulting "whole cell homogenate" or "whole-cell fraction" can be used as such or subjected to centrifugation in a series of steps to isolate cytosolic (cell interior) and nuclear fractions. The prepared sample is then assayed for protein content so that a consistent amount of protein can be taken from each different sample. Samples are boiled for 1-5 minutes in a buffer solution (e.g. Laemmli's buffer), containing dye, a sulfurous compound - typically betamercaptoethanol, and a detergent known as sodium dodecyl sulfate or SDS. The boiling denatures the proteins, unfolding them completely. The SDS then surrounds the protein with a negative charge and the beta-mercaptoethanol prevents the reformation of disulfide bonds.

Gel electrophoresis

The proteins of the sample are separated according to molecular weight using gel electrophoresis. Gels have various formulations depending on the lab, molecular weight of the proteins of interest. Polyacrylamide gels are most common. Since the proteins travel only in one dimension along the gel, samples are loaded side-by-side into wells formed in the gel. Proteins are separated by mass into bands within each lane formed under the wells along with a ladder. 2-D gel (two dimensions) can also be used which spreads the proteins from a single sample out in two dimensions and proteins are separated according to isoelectric

point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

Electronic Transfer

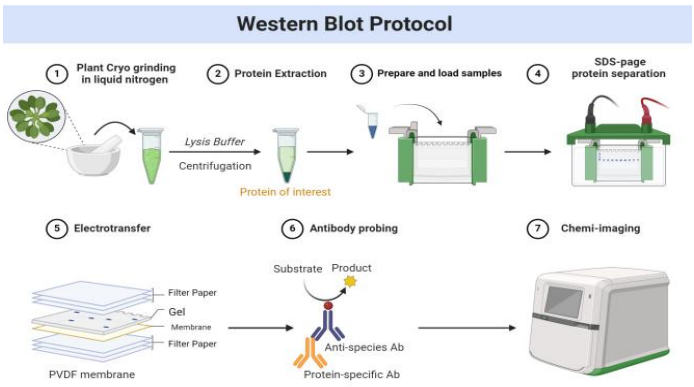
The polyacrylamide gel is good for separation of protein, but not suitable for the staining and further detection. Thus, they are moved further from gel to a membrane made of nitrocellulose or PVDF for antibody detection. The membrane is placed face-to-face with the gel, and current is applied to large plates on either side. The charged proteins move from gel onto the membrane maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Both varieties of membrane are chosen for their non-specific protein binding properties. Blocking is done in order to prevent non-specific protein interactions between it and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically bovine serum albumin (BSA) or non-fat dry milk, with a minute percentage of detergent such as Tween 20 or colloidal carbon.

Detection

During the detection process the membrane is probed for the protein of interest with antibodies, and links them to a reporter enzyme, which drives a colorimetric or photometric signal. It can take place by a two-step process or one-step detection. In two step process, firstly dilute solution of primary antibody (generally between 0.5 and 5 micrograms/ml) is used and incubated with the membrane under gentle agitation from 30 minutes to overnight. The solution is comprised of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. After rinsing the membrane to remove unbound primary antibody, it is exposed to secondary antibody which is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This step confers an advantage in that several secondary antibodies will bind to one primary antibody, providing enhanced signal. Most commonly, a horseradish peroxidase-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane. A radioactive label can also be used rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like Staphylococcus Protein A with a radioactive isotope of iodine. But, as non-radioactivity methods are safer, quicker and cheaper, it is preferable and radioactive label method should be avoided. Earlier, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This was also useful and advantageous for researchers and companies in terms of flexibility and detection process. But, a one-step probing system was required for high-throughput protein analysis. Thus, a probe antibody which recognizes the protein of interest as well as contains a detectable label proved to be more useful as it is ready for direct detection after a series of wash steps.

Analysis

After the unbound probes are washed away, the western blotting is ready for detection of the probes that are labeled and bound to the protein of interest. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.



Methods used to detect antigens on blots

Colorimetric detection

The colorimetric detection method depends on incubation of the western blotting with a substrate that reacts with the reporter enzyme (such as alkaline phosphatase or horseradish peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different colour that precipitates next to the enzyme and thereby stains the nitrocellulose membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

Chemiluminescence

Chemiluminescent detection method depends on incubation of the western blotting with a substrate and materials that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film and more recently by CCD cameras which captures a digital image of the western blotting. The image is analyzed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used. The new reagent, enhanced chemiluminescent (ECL) detection is considered to be among the most sensitive detection methods for blotting analysis.

Radioactive detection

Radioactive method is more sensitive. Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of interest. The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high and ECL provides a useful alternative.

Fluorescent detection

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera equipped which appropriate emission filters which captures a digital image of the western blotting and allows further data analysis such molecular weight analysis and a quantitative western blotting analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

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