Review Article

Comprehensive Emphasis On Western Blotting For Protein Expression, Purification And Analysis

R. Senthilraj[1], B. Senthilnathan[2], R. Gowri[3], K. Rajaganapathy[4], M. Loganathan[5], K. Manvizhi[6], K. Masilamani[2*]

[1]Faculty of Pharmacy, Dr. M.G.R. Educational and Research Institute, Chennai 600077, India
[2]Faculty of Pharmacy, Sree Balaji Medical College and Hospital Campus, Bharath Institute of Higher Education and Research, Chennai 600044, India
[3]GRT Institute of Pharmaceutical Education and Research, Tirutani 631209, India
[4]Bioreach Labs (OPC) Pvt Ltd, Balaji Nagar, Madampakkam, Chennai 600126, India
[5]Jaya College of Paramedical Sciences, College of Pharmacy, Chennai 602024, India
[6]Department of Chemistry, Anand Institute of Higher Technology, Chennai 603103, India

***Corresponding author**
Dr. K. Masilamani, Faculty of Pharmacy, Sree Balaji Medical College and Hospital Campus, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu 600044, India
Email: masilamani.pharmacy@bharathuniv.ac.in
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Abstract

Western blotting is an important technique used to identify, quantify and determine the size of specific proteins. This technique makes it possible to identify the specific protein from a complex mixture. The central dogma is created by converting DNA into mRNA during transcription and helping to facilitate translations to induce protein synthesis. Blots, which frequently come after the use of gel electrophoresis are methods for putting DNA, RNA, and proteins onto a carrier for separation. For transferring DNA, the Southern blot and PCR methods are employed. The Northern blot, RT-PCR, and qPCR methods are utilized for transferring RNA. The transferring of protein can be utilized by the western blot method, which is now routinely used in drug discovery and scientific research, as well as for the identification of different medical disorders. This paper is based on key features of theoretical analysis of western blotting.

Keywords: Western blot, Protein expression, SDS-PAGE, Gel-electrophoresis, Protein purification.

INTRODUCTION

The central dogma is a process of the flow of genetic information from DNA to RNA to make a protein (Fig. 1). Blot is a method for the transfer of DNA, RNA, and proteins onto a carrier for separating them. For transferring DNA, the Southern blot and PCR and for RNA, the Northern blot, RT-PCR, and qPCR methods are utilized. The transfer of protein is utilized by the western blot technique (1-4).

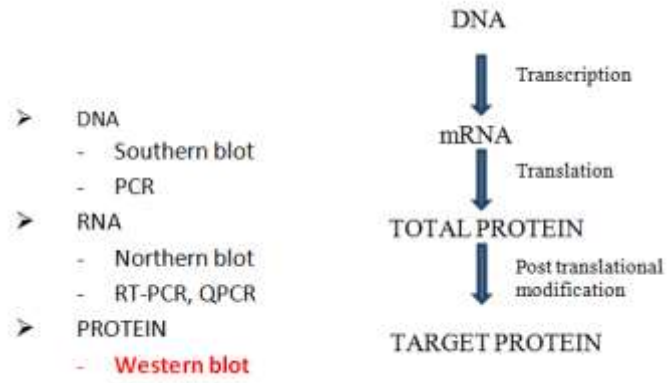
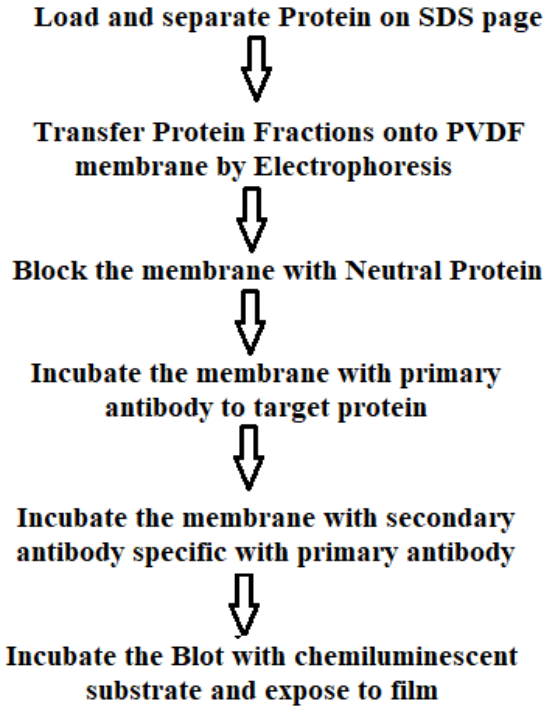


Fig. 1 Central Dogma of Gene and Protein Expression

Towbin et al. initially represented western blotting in 1979. Presently it is a standard methodology for macromolecule analysis as it generates qualitative and semi-quantitative information about the macromolecules. As antibody is used in this technique for selecting antigen, this can be called immunoblotting. It is commonly employed in cell and molecular biology and in biological science also (1-4). The charge and molecular weight of the macromolecule in a protein mixture can be easily determined. The western blot method has evolved as a customary tool for the identification of diseases and for new drug discovery and research. The western blot procedure is given in Fig. 2.

Fig-2 Procedure and Steps for Western Blot

The western blot technique is based on the relative mass and charge separation of proteins by gel electrophoresis. The proteins are exposed to target primary antibodies. Usually, a secondary protein recognizes the target macromolecule



to be coupled to the first protein. The SDS polyacrylamide gel electrophoresis was created in 1970 by Ulrich Laemmli to separate molecules. Towbin (1979) represented the transfer of proteins from polyacrylamide-urea gels to nitrocellulose sheets. Burnette coined the name western blot to explain the method using polyacrylamide gels and dodecyl sulfate (SDS-PAGE). The western blot is currently a vital tool in the biological and drug discovery fields (5-9).

CELL LYSIS AND EXTRACTION OF PROTEIN

The western blot may be a versatile methodology for locating proteins in cell cultures, bacteria, yeast, and animal and plant tissues. For western blot, macromolecule extraction is needed, which may be accomplished in one of two ways. The primary approach is mechanical, and the second one is chemical. The mechanical techniques follow sonication, blending with abrasives, and breakdown with glass or bimetallic beads (5-9). Chemical strategies utilize buffers that may solubilize the proteins; to achieve this, ionic detergents like metallic element deoxycholate, dodecyl sulfate and cetyltrimethylammonium bromide are used. Chemical procedures additionally use non-ionic or zwitterionic detergents; the selection is based on the extraction potency and speed of lysis. SDS denatures the proteins. In the experiments requiring macromolecules, an anionic triton x-100 can be used to lyse cells slowly; preventing macromolecule denaturation and breakdown. During this methodology, zwitterionic detergents, such as 3-[(3-cholamidopropyl) dimethylammonium]-1-propane sulfonate are often used without changing the net charge or charge of the solubilized molecule. As a result of its effectiveness in solubilizing proteins, the radio-immuno-precipitation assay (RIPA) can be used. Proteolytic enzyme inhibitors and lysis-buffer solutions are used to take care of stable macromolecule structures (10-14). Table 1 & 2 lists the various chemicals and buffers that are used for western blots.

Table 1: Chemicals, Buffers used in the Western Blotting-cell lysis to the protein extraction

Ionic Chemicals	**Remarks**	**Non-Ionic or Zwitterionic**	**Remarks**	**Buffers / Methods**	**Remarks**
Sodium dodecyl sulfate (SDS)	Responsible for the speed of the lytic effect. It can denature the proteins.	Triton X-100	Responsible for the lysis of cells smoothly, slowly & avoids protein denaturation and complex breakdown	**RIPA**	Responsible for avoiding protein denaturation and protein complex breakdown
Deoxycholate	Responsible for the speed of the lytic effect. In contrast, it can denature the proteins.	3[(3-cholamidopropyl) dimethylammonium] -1-propane sulfonate		**Tris-Triton**	Extracting the cytoplasmic proteins
Cetyl trimethylammonium bromide (CTAB)	Responsible for the speed of the lytic effect. It can denature the proteins.	---			

Table 2. Classification of detergents.

Type	**Detergents**

Ionic	Sodium dodecyl sulfate, deoxycholate
Non-ionic	Triton X-100, digitonin, tween 20 & 80
Zwitterionic	CHAPS
Chaotropic	Urea

PROTEIN QUANTIFICATION

Prior to electrophoresis, the protein concentration was determined to homogenize the deposited protein in gel wells. Protein overload can saturate immunodetection, producing non-specific results. Numerous techniques are used to quantify proteins. The bicinchoninic acid assay (BCA), Lowry protein assay, and Bradford protein assay are the most frequently utilized methods for protein quantification in western blot (15-18). These colorimetric assays rely on colour changes that occur when protein amino acids interact with specific reagents. Lowry protein assay method uses copper and Follin's reagent to characterize the protein, which is detected at a wavelength of 750 nm. The Bradford protein assay involves the measurement of color intensity in the determination of protein concentration. Using a dye called Coomassie brilliant blue, which reacts in the presence of proteins, the colour shift is seen in the range of 465 to 595 nanometers. The bicinchoninic acid assay (BCA) is an extremely sensitive technique that combines the reaction of proteins with Cu^{2+} ions in an alkaline medium in the presence of a reagent called BCA that detects Cu+ ions very sensitively. Its variation is minimum. The macromolecular structure of the protein, as well as four specific peptides (cysteine, cysteine, tryptophan, and tyrosine), are said to be responsible for the purple-blue in the samples that can be identified at a wavelength of 562 nm.

ELECTROPHORESIS GELS

There are other ways to separate proteins, but the most popular one uses cellulose acetate sheets and starch gel. But the pore size cannot be controlled. By adjusting the ratios of acrylamide, bisacrylamide and polyacrylamide in gel composition the pore size can be altered. Low molecular weight proteins (less than 50 kDa) with a higher percentage of acrylamide (10–20% T) are best separated using gels with smaller pore sizes, whereas high molecular weight proteins (greater than 100 kDa) are best separated using gels with a lower percentage of acrylamide (less than 10% T). Free radicals are produced as a result of the addition of tetramethyl ethylene diamine and ammonium persulfate, which fastens the polymerization of acrylamide. Running buffers for polyacrylamide gels are adaptable and enables fast separation and identification of proteins (19-21).

Types of gels

Proteins may be separated by polyacrylamide gel electrophoresis under denaturing or non-denaturing conditions. Non-denaturing polyacrylamide gels (ND-PAGE) maintain the three-dimensional form of the macromolecule structure whereas charge, size, and form create the basis for separation. To preserve the macromolecule structure, a solution of non-reductive and non-denaturant nature like tri-glycine at a pH range of 8 to 9.5, tris-borate at 7 to 8.5 and tris-acetate at a 7.2 to 8.5 can be used. In denaturing polyacrylamide gels, associated with anionic (SDS), a reductant (beta-mercaptoethanol) and heat produce dissociate macromolecule structures in peptides (SDS-PAGE) (22-24). Beta-mercaptoethanol and heat, disrupt disulfide bridges (Cys-S-S-Cys) and thiol group (Cys-SH) in peptide chains, allowing macromolecule dissociation. SDS binds to proteins and provides them a charge, breaking hydrophobic bonds. Once the gel gets polymerized, an acceptable macromolecule concentration (20–40 g) is added to the comb well. The thickness and capability of the well will control the quantity and concentration of the macromolecule supernatant (0.75-1.5 mm).

Discontinuous and continuous buffer systems

Based on the type of buffers used, there are two kinds of electrophoresis systems: the continuous buffer system of Weber and Osborn, within which an equivalent running buffer is employed in the single apparatus, and the discontinuous buffer system, or Laemmli system, within which two kinds of gels are employed: a "resolving gel" (small pore) that contains polyacrylamide and a "stacking gel" (big pore) that contains four-dimensional amide. The running buffer and the buffers utilized in every gel of the discontinuous system are distinct, have totally different pH scales and ionic strengths, and are used one by one in every gel. Before being transferred to the resolution gel, the pH scale promotes glycine ionization for migration across the gel and favours size-based macromolecule separation, the discontinuous system produces a low conductivity and voltage difference zone that suits macromolecule concentration. In this system, macromolecule quality is maintained by chloride ions (Cl-) within the gel and glycine ions (Gly-) in the sample buffer (25).

Sample preparation

Before electrophoresis, the samples are heated in a resolution called loading buffer between 70 and 95°C to allow them for suitability of weight, density and color to assist them to load in the well and forestall leaks. The reducing substances dissolve the disulfide bonds between these substances. It links DTT or beta mercaptoethanol. Proteins may be separated by denaturing into the primary structure with a charge. In addition, the protein is ready to urge to its binding site which results in reduction and denaturation. Proteins with a lower relative molecular mass move to the gel's bottom. Macromolecule motion across the gel may be studied with help of markers. The marker helps in the identification of macromolecules of interest (26).

Transfer of proteins to the membrane

During the immunodetection approach utilizing antibodies, proteins should be transferred to a membrane for easy handling. The transfer may be achieved by applying an electric field like polyacrylamide gel electrophoresis; however, the proteins move in the direction of the positive conductor (27). The transfer of macromolecule to the membrane may be achieved in 2 ways: semi-dry or wet. Each approach has the gel and membrane in shut proximity; however, the wet methodology needs additional transfer of buffer and an extended exposure amount at a lower temperature (2–12 h at 4° C) than the semi-dry method (7-30 min at area temperature).

In addition to separating SDS from the protein, methanol also significantly increases the rate at which proteins bind to the membrane. However, these outcomes may vary depending on the protein. Microporous surfaces like nitrocellulose membranes or polyvinylidene difluoride (PVDF) are frequently used for the transfer of proteins. Important characteristics of these membranes include high binding capacity, simplicity in both short and long-term storage of immobilized molecules and reproducibility. The PVDF membrane is more durable and enables greater protein binding than nitrocellulose. The nitrocellulose membrane has a lower background noise level. Contrary to nitrocellulose membranes, PVDF membranes are highly hydrophobic and lack surfactant; hence they must be moistened with methanol or ethanol before use. Background noise has recently been reduced by using low auto-fluorescence membranes (28).

IMMUNODETECTION

Proteins maintained in the solid phase block non-specific and unoccupied membrane locations. Because they can bind to the membrane's vacant spots, blocking agents that contain proteins are most frequently used. Background noise is lessened since the antibody can only attach to the locations of the proteins of interest (29). If there is a lot of background noise tween 20 and horse or fetal calf serum or two protein-containing blocking agents (a detergent) can be employed. Conversely, non-ionic detergents need to be handled carefully since they could solubilize proteins that have been transported to the membrane. Non-fat milk and bovine serum albumin are the most widely used. Because

non-fat milk contains casein, a phosphor-protein that may compete for the antibody target, lowering the intensity of a non-specific signal, it performs poorly in the analysis of phosphorylated proteins.
The primary specific antibody of the target protein is treated with the previously blocked membranes. The antibodies used in western blot might be monoclonal antibodies that only recognize a single antigen region and polyclonal antibodies that recognize a variety of antigen regions. Most of the time, the main antibody is never coupled to any developing agent. A secondary antibody must then be incubated as a result. Secondary antibodies are chosen based on the species from which the primary antibody was derived and bind to primary antibodies that are not linked to antigens (29).

Immuno-detection of Proteins - Chemiluminescent Immunodetection

Utilizing secondary antibodies and peroxidase or phosphatase enzymes, immunodetection by chemiluminescence emits energy in the form of light as a result of the reaction of luminous substances (luminol). This makes it possible to identify antigen-antibody binding. To identify the target protein, the light reaction is generated on auto-radiographic film and digitally recorded using a digital camera (29).
The linearity of the detection, which is typically present in weights smaller than 5 g, determines the sensitivity of the chemiluminescence immunodetection approach. The signal saturation is directly related to protein expression. This method is semiquantitative because the signal saturation point is a crucial factor in determining the differences in expression levels of the samples and leads to inaccurate measurements. Utilizing the analysis software included in the digital systems, the acquired image pixels of each band were obtained through densitometric studies (29).

Immunodetection with fluorescence

In a recently proposed fluorescent immunodetection approach, secondary antibodies link to a fluorescent marker or fluorophore and produce light when excited, producing a lineal-detection signal. The light released is detected by a digital camera that has filters of various wavelengths (30). The collected images are examined using a similar theory to the one disclosed by the chemiluminescence approach. Nevertheless, the result is quantitative as opposed to the chemiluminescence immunodetection approach because of the signal stability.

APPLICATIONS OF WESTERN BLOTTING

Western blot is an analytical technique utilized in biochemistry and drug discovery for protein study (31-32). This technique can be used to evaluate the size and amount of protein expression. It can be used in the diagnosis of diseases. It can also be used in the identification of the specific protein. The western blot is used to demonstrate the specific antibodies in the serum for the diagnosis of diseases.

CONCLUSION

The western blot technique has been used as a suitable tool in the fields of drug discovery, biomedical research, protein expression, purification and analysis, biomarker study and diagnosing & detecting several diseases for more than a decade. This is due to the utilization of different samples. The main disadvantage of this method is that it requires a primary antibody to target a protein. Research needs to be envisaged to utilize the full benefits of the western blot method.

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