24

Protein Blotting

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1. Introduction

Protein blotting, also known as Western blotting refers to the transfer of electrophoresed proteins from the polyacrylamide gel electrophoresis (SDS-PAGE or 2-dimensional PAGE [2D-PAGE]) to an adsorbent membrane that binds the eluted macromolecules ("the blot"). This method was first described in 1979 by Towbin et al. ***(1)***.

Electrophoretic transfer uses the driving force of an electric field to elute proteins from gels. This method is fast, efficient and maintains the high resolution of the protein pattern. Different probes can be used to react with the transferred proteins on the blot:

- antibody for the identification of the corresponding antigen,
- lectin for the detection of glycoproteins,
- ligand for the detection of blotted receptor components, etc.

Western transfer with subsequent immunodetection has found wide application in the fields of life sciences and biochemistry. The blotted proteins can efficiently be detected and characterized, especially those that are of low abundance.

The blot is also widely used with various techniques of protein identification, from which the measurement of protein mass (mass spectrometry) or determination of the protein sequence (*N*-terminal Edman degradation, *C*-terminal sequence or amino acid analysis).

The power of protein blotting lies in its ability to provide simultaneous resolution of multiple immunogenic proteins within a sample. The blot analysis generally requires small amount of reagents, the transferred proteins on membrane can be stored for many weeks before their use and the same blot can be used for multiple successive analyses.

This chapter summarizes the different methods (1) to transfer proteins from gel to membrane (Section 2.1) and (2) to detect proteins on blots (Section 2.2). Several applications of the protein blotting technique are presented in Section 3 of this chapter.

From: *Molecular Biomethods Handbook, 2nd Edition.*
Edited by: J. M. Walker and R. Rapley © Humana Press, Totowa, NJ

2. Methods

2.1. Efficiency of Protein Blotting

Two principal factors affect the efficiency of protein blotting: (1) the elution efficiency of proteins out of the gel matrix and (2) the efficiency of binding by the membrane.

2.1.1. Elution Efficiency of Proteins and Techniques Used

The efficient transfer of proteins from the gel to a solid membrane support depends greatly on the acrylamide concentration of the gel, the ionic strength and the pH of the buffer, the additional constituents of the transfer buffer such as sodium-dodecyl-sulfate (SDS) and methanol ***(2)***, and the molecular mass of the proteins transferred. In a general way, the lower the percentage of acrylamide and cross-linker, the easier the transfer will be. The use of thinner gels allows a faster and more complete transfer. Methanol increases the binding capacity of matrix presumably by exposing hydrophobic protein domains, so that they can interact with the matrix. When there is SDS in transfer buffer (up to 0.1% w/v), the proteins are negatively charged and elute efficiently from the gel. High molecular-weight (MW) proteins blot poorly from SDS-PAGE or 2D-PAGE, which leads to low levels of detection on immunoblots. However, the transfer of high MW proteins can been facilitated with heat, special buffers and partial proteolytic digestion before transfer ***(3,4)***.

Protein transfer from gel electrophoresis to membrane has been achieved in three different ways: simple diffusion, vacuum-assisted solvent flow and electrophoretic elution ***(5)***. This later method is the most common and efficient and will be summarized here below. There are currently two main configurations of electroblotting apparatus ***(6)***:

1. tanks of buffer with vertically placed wire or plate electrodes (wet transfer) and
2. semi-dry transfer with flat-plate electrodes (see **Fig. 24.1**). The name semi-dry transfer refers to the limited amount of buffer that is confined to the stacks of filter paper.

Semi-dry blotting requires considerably less buffer than the tank method, the transfer from single gels is simpler to set up, it allows the use of multiple transfer buffers and it is reserved for rapid transfers because the use of external cooling system is not possible. Nevertheless, both techniques have a

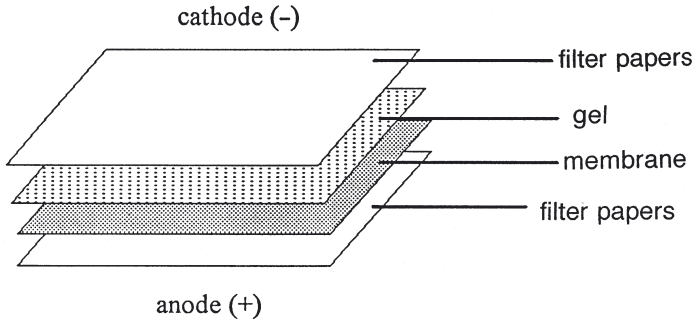

Fig. 24.1. Assembly of a horizontal electroblotting apparatus for a semi-dry transfer with flat-plate electrodes

high efficacy and the choice between the two types of transfer is a matter or preference.

2.1.2. Binding to the Membrane

The binding capacity is mainly determined by the character of the membrane but also by the transfer buffer composition *(7,8)*.

Nitrocellulose, PVDF (polyvinylidene difluoride), activated paper, or activated nylon have been used successfully to bind transferred proteins. Nitrocellulose was the first matrix used in electroblotting and is still the support used for most experiments. It has a high binding capacity, it is not expensive and the nonspecific protein binding sites are easily and rapidly blocked. However, the proteins are not covalently bound and small proteins tend to move through nitrocellulose membranes and only a small fraction of the total amount actually binds.

PVDF membranes are advantageous because of high protein binding capacity, physical strength and chemical stability. Most commonly used protein stains and immunochemical detection systems are compatible with PVDF membranes. In addition, replicate lanes from a single gel can be obtained and used for different purposes, along with Western analysis (*N*-terminal sequencing, proteolysis-peptide separation-internal sequencing, etc.). PVDF membranes can be stained with Coomassie brilliant blue (CBB), allowing excision of proteins for *N*-terminal sequencing.

The efficacy of the Western blot using semi-dry method that uses a simple buffer system is illustrated in **Fig. 24.2** *(6)*. Human plasma proteins (120 µg) were separated by 2-D PAGE, transferred on PVDF membrane and stained with Coomassie blue (**Fig. 24.2B**). The blot pattern is compared to the Coomassie blue staining of the same protein sample before transfer from 2-D PAGE (**Fig. 24.2A**). The resolution, shape and abundance of protein spots on membrane are comparable to the 2-D polyacrylamide gel pattern.

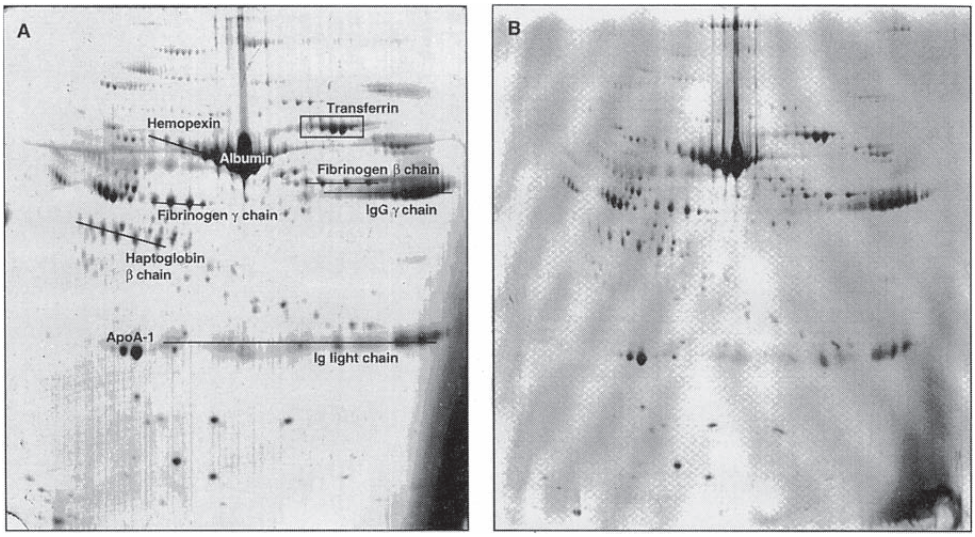

Fig. 24.2. Plasma proteins separated by 2-dimensional polyacrylamide gel electrophoresis and **(A)** stained with Coomasie Brilliant Blue R250 or **(B)** transferred to PVDF membrane using the semi-dry system (2 h, 15 V) with Towbin buffer diluted 1:2 in water and stained with Coomasie Blue

Several protocols have been developed from the basic electroblotting procedure to improve the amount of protein transferred and retained on the membrane. A review has been published recently *(9)*.

Whatever the membrane used, exceeding its binding capacity tends to reduce the signal eventually obtained on blots. For 2-D PAGE, the best recovery and resolution of proteins are obtained when loading 120 ug of human plasma or platelet proteins *(10)*.

2.2. Protein Detection

After blotting, the proteins are present in an accessible state, bound to the solid matrix of the membrane. They may be assayed for enzymatic function *(11)*, chemical reactivity *(12)* or amino acid sequence *(13)*. However, protein blotting is most often followed by reaction of the bound proteins with antibodies, before detection with antibody-specific labeled probes (immunoblotting). Another interesting probe is lectin, a class of carbohydrate-binding proteins, to discriminate and analyze the glycan structure of glycoproteins transferred to membranes (lectin blotting) *(10)*.

The following section described the protein stains, the immunodetection of antigens and the glycoprotein analysis using lectin blotting.

2.2.1. General Proteins Stains

It is often necessary to visualize the transferred proteins to allow exact alignment of bands (if the blot is obtain following SDS-PAGE) or spots (if 2-D PAGE is used) and to control the quality of the transfer. Staining refers to the reversible or irreversible binding by the proteins of a colored organic or inorganic chemical.

The proteins may be stained before or after the electrotransfer. Initially, the stains used for visualization of proteins in gels were also used for nitrocellulose membrane, typically Coomassie blue or amido black *(14)*, although these stains cannot be used with nylon membranes because the charged nature of the membrane results in very high levels of background staining *(15)*.

One problem with the staining before Western blotting is the longer staining time required with proteins contained in the gel matrix.

Staining on blots (after the Western blotting) often represents the preliminary step for specific detection/characterization procedures. *N*-terminal sequencing requires permanent staining stains; Coomassie blue has been routinely used with nitrocellulose membrane and amido black with PVDF membrane. Conversely, immunological and affinity reagents require reversible stains (colorimetric dyes such as direct blue 71, copper iodide, metal chelates or fluorescent dye).

The common limitation of protein staining, before or after the electrotransfer, is the reduction in immunoreactivity of the membrane-bound proteins. This may be caused by the blocking of antibody-binding by the protein-bound stain proteins or to the denaturing effects of solvent during the staining process *(16)*.

Proteins have also been detected after immunoblotting onto membrane supports directly by use of fluorescent labels (fluorescamine, coumarin), various silver staining methods *(17)* and colloidal particles such as gold, silver, copper, iron, or India ink *(18)*.

2.2.2. Specific Immunodetection of Antigens

After transfer, unused macromolecular binding sites of the membrane must be blocked to prevent nonspecific adsorption of probe molecules. The selection

of a blocking solution from the wide range available, together with the temperature and duration of the blocking incubation, may affect the level of background staining ***(19)***. The majority of blocking solutions are protein-based. Buffered solutions of skimmed milk or casein are widely used and effective.

Two methods are commonly used for detecting proteins, after the addition of primary antibody to protein blots that have been blocked: radioactive and enzyme-linked reagents.

With Western blotting, the proteins transferred are in a highly denatured state, which may prevent the reaction of the majority of antibodies, except those that react with conformation-insensitive epitopes to bind ***(20)***. This illustrates the importance of using polyclonal antibodies that contain multiple epitopes of a protein, some of which are likely to be denaturation-resistant. It is commonly found that monoclonal antibodies fail to react in Western blotting.

Different modifications of the electrophoresis technique have also been developed to retain more of the native protein structure and therefore increase the immunoreactivity, for example avoid the use of sulphydryl reagent, reduction or omission of SDS ***(21,22)***.

2.2.3. Specific Stains of Glycoproteins Using Lectin Blotting

Glycoproteins result from the covalent association of carbohydrate moieties (glycans) with proteins. The enzymatic glycosylation of proteins is a common and complex form of post-translational modification. It has been established that glycans perform important biological roles including: stabilization of the protein structure, protection from degradation, control of protein solubility, of protein transport in cells and of protein half-life in blood. They also mediate the interactions with other macromolecules and the recognition and association with viruses, enzymes and lectins ***(23,24)***.

Carbohydrate moieties are known to play a part in several pathological processes. Alterations in protein glycosylation have been observed, for example, with the membrane glycoproteins of cancer cells, with the plasma glycoproteins of alcoholic patients and patients with liver disease, with the glycoproteins in human brains from patients with Alzheimer disease, inflammation and infection. These changes provide the basis for more sensitive and more discriminative clinical tests ***(25–29)***.

Lectins are carbohydrate-binding proteins that can bind specifically and noncovalently to a certain sugar sequence in oligosaccharides and glycoconjugates. Their restricted binding capacity is the basis not only for recognition of glycoproteins but also an indirect way for accessing the composition of their glycan moieties. In most cases, they bind more strongly to oligosaccharides (di, tri, and tetra saccharides) than to monosaccharides ***(30,31)***. Many lectins recognize terminal nonreducing saccharides, whereas others also recognize internal sugar sequences. For example, concanavalin A binds to internal and nonreducing terminal α-mannosyl groups, whereas lectins from *Sambucus nigra* and from *Maackia amunrensis* show affinity for specific types of sialic acid linkages. Together, these later lectins can detect most glycoproteins of animal origin because all antennae of the glycans from these sources ending with sialic acid residues. See Gravel et al. (2002) ***(31)*** for a review of the specificity of lectins for glycans and for the description of a protocol for the detection of glycoproteins on nitrocellulose membrane using biotinylated lectins and avidin conjugated with horseradish peroxidase or with alkaline phosphatase. **Figure 24.3A** below shows

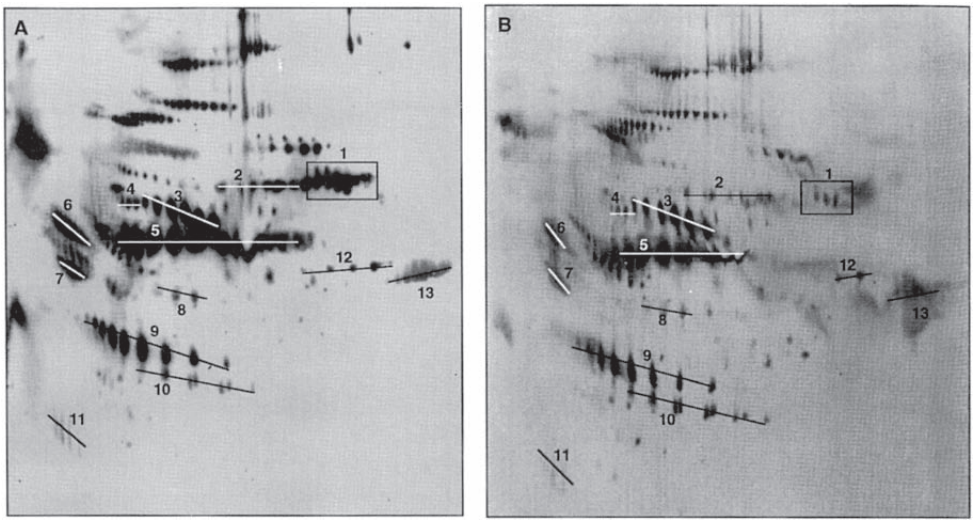

Fig. 24.3. Glycoprotein blot pattern of 2-D PAGE separation of plasma proteins (120 µg) probed with WGA (specific for *N*-acetylglucosamine and neuraminic acid) and (**A**) revealed with chemiluminesence (15 s film exposure) and (**B**) with NBT/BCIP (20 min for the development of the color reaction). Because most of the glycoproteins in plasma contain one or more N-linked glycans with at least two *N*-acetylglucosamine residues, the use of WGA allows a general staining of N-linked glycoproteins. (1) transferrin, (2) IgM µ-chain, (3) hemopexin, (4) α1-β-glycoprotein, (5) IgA α-chain, (6) α1-antichymotrypsin, (7) α2-HS-glycoprotein, (8) fibrinogen γ-chain, (9) haptoglobin β-chain, (10) haptoglobin cleaved β-chain, (11) apolipoprotein D, (12) fibrinogen β-chain, (13) IgG γ-chain

the plasma glycoprotein signals (using the above method, i.e., high-resolution 2-D PAGE followed by lectin blotting method on nitrocellulose membrane) detected with wheat germ agglutinin (WGA, specific for *N*-acetylglucosamine and neuraminic acid) and generated on a film after chemiluminescence detection. **Figure 24.3B** shows an identical blot stained with nitro blue tetrazolium/bromochloro-indolyl phosphate (NBT/BCIP). The same pattern of glycoprotein subunits are revealed by both methods but the chemiluminescent detection system shows higher sensitivity (about 10-fold) than NBT/BCIP staining. Albumin, which does not contain any carbohydrate moiety, represents a negative protein control in all blots (blank area below the IgM µ–chain [protein identified by number 2 in **Fig. 24.3**]).

Lectins do not have an absolute specificity and therefore can bind with different affinities to a number of similar carbohydrate groups. Despite this limitation, lectin probes do provide some information as to the nature and composition of oligosaccharide substituents on glycoproteins. Their use together with blotting technique provides a convenient method of screening complex protein samples for abnormalities in the glycosylation of the component proteins. See Section 3 for the application of this technique to identify carbohydrate-deficient transferrin from plasma sample of alcoholic patients.

3. Applications

Presently, there is an intense interest in applying proteomics (a new science that focuses on the study of proteins: their roles, their structures, their localization, their interactions, and other factors) to marker identification of different disease.