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Western Blotting: An Introduction

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Summary

Western blotting is an important procedure for the immunodetection of proteins, particularly proteins that are of low abundance. This process involves the transfer of protein patterns from gel to microporous membrane. Electrophoretic as well as non-electrophoretic transfer of proteins to membranes was first described in 1979. Protein blotting has evolved greatly since the inception of this protocol, allowing protein transfer to be accomplished in a variety of ways.

Keywords

Western blotting; Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Nitrocellulose membrane; Polyvinylidene difluoride membrane

1. Introduction

The transfer of proteins or nucleic acids to microporous membranes is referred to as “blotting” and this term encompasses both “spotting” (manual sample deposition) and transfer from planar gels. Proteins that are resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gels are typically transferred to adsorbent membrane supports under the influence of an electric current in a procedure that is known as western blotting (WB) or protein blotting (1,2) (*see Chapters 2 and 3*). Nucleic acids are routinely transferred from agarose gels, to a membrane support, through capillary action (Southern blotting). Protein blotting evolved from DNA (Southern) blotting (3) (*see Chapter 1*) and RNA (Northern) blotting (4). The term ‘western blotting’ was coined to describe (5) this procedure to retain the ‘geographic’ naming tradition initiated by Southern’s paper (3) (*see Chapter 3*). The blotted proteins form an exact replica of the gel and have proved to be the starting step for a variety of experiments. The subsequent employment of antibody probes directed against the membrane bound proteins (immunoblotting) has revolutionized the field of immunology (Fig. 1). Dot blotting refers to

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the analysis of proteins applied directly to the membrane rather than after transfer from a gel.

The utility of the high resolving power of SDS PAGE (5) was limited in purpose, owing to the fact that the separated proteins in the gel matrix were difficult to access with molecular probes, until the advent of protein blotting. Protein transfer with subsequent immunodetection has found wide application in the fields of life sciences and biochemistry. This procedure (1,2) is a powerful tool to detect and characterize a multitude of proteins, especially those proteins that are of low abundance. It offers the following specific advantages; (a) wet membranes are pliable and are easy to handle compared to gels, (b) easy accessibility of the proteins immobilized on the membrane to different ligands, (c) only small amount of reagents are required for transfer analysis (d) multiple replicas of a gel are possible (e) prolonged storage of transferred patterns, prior to use, becomes possible and (f) the same protein transfer can be used for multiple successive analyses (7–9).

Protein blotting has been evolving constantly, since its inception, and now the scientific community is faced with a multitude of ways and means of transferring proteins (10). Nonetheless, western blot sensitivity is dependent on efficiency of blotting or transfer, retention of antigen during processing and the final detection/amplification system used. Results are compromised if there are deficiencies at any of these steps (11).

1.1. Blotting Efficiency

The efficient transfer of proteins from a gel to a solid membrane support depends greatly on the nature of the gel, the molecular mass of the proteins being transferred and the membrane used. Running the softest gel, in terms of acrylamide and cross-linker that yields the required resolution is the best option. Transfer becomes more complete and faster with the use of thinner gels. However, the use of ultra-thin gels may cause handling problems and a 0.4 mm thickness represents the lower practical limit (12). Proteins with a high molecular mass blot poorly following SDS PAGE, resulting in low levels of detection on immunoblots. However, the efficiency of transfer of such proteins has been facilitated with heat, special buffers and partial proteolytic digestion of the proteins prior to transfer (11,13–17).

2. Immobilizing Supports for Protein Transfer

A wide range of solid phases is available for immobilization, ranging from the truly solid such as glass and plastic to latex and cellulose that are porous. The most common phases used for blotting are comprised of microporous surfaces and membranes like cellulose, nitrocellulose (NC), polyvinylidene difluoride, cellulose acetate, polyethane sulfone and nylon. The unique properties of microporous surfaces that make them suitable for traditional assays such as “western blotting” are (a) large volume to surface area ratio (b) high binding capacity (c) short and long term storage of immobilized molecules (d) ease of processing by allowing a solution phase to interact with the immobilized molecule (e) lack of interference with the detection strategy and (f) reproducibility. These properties are useful for the high-throughput assays used in the post-genomic era as well (2,4,14,18,19).

Typically, these microporous surfaces are used in the form of membranes or sheets with a thickness of 100 micrometers and possessing an average pore size that ranges from 0.05 to 10 micrometers in diameter. The interaction of biomolecules with each of these membranes is not completely understood, except for the fact that it is generally known to interact in a non-covalent fashion (20,21).

Regardless of the type of membrane used, it must be borne in mind that exceeding the protein binding capacity of the membrane used tends to reduce the signal obtained in immunoblotting. Excess protein, weakly associated with the membrane, is readily accessible to react with the primary antibody or any other ligand in solution (e.g.lectin). However, the resulting antibody-protein complexes will easily wash off during further processing of the membrane. Such a scenario would not have prevailed if the protein had initially made good contact with the membrane (18).

2.1. Nitrocellulose Membranes

Nitrocellulose (NC) is perhaps the most versatile of all the surfaces mentioned earlier for the immobilization of proteins, glycoproteins or nucleic acids (3,4,19). In addition to traditional blotting, NC is used in high-throughput array, immunodiagnostic and as well as mass-spectrometry coupled proteomic applications, filtration/concentration, ion exchange and amino acid sequencing in addition to traditional blotting procedures. It was Southern who first demonstrated (in 1975) the usefulness of NC to capture nucleic acids. Towbin in 1979 (4) and Burnette in 1981 (1) showed that they could also be used for proteins.

This unique polymer derived from cellulose, has been used as the most common immobilization surface in biological research for over 65 years. Since high-throughput methodologies for proteomics and genomics rely heavily on traditional concepts of molecular immobilization followed by hybridization binding or analysis, NC continues to be useful in the post-genomic era technology (19).

2.1.1. Synthesis of Nitrocellulose from Cellulose—Treatment of cellulose with nitric acid results in the hydroxyl moieties on each sugar unit of cellulose being substituted by nitrate groups, resulting in NC. Organic solvents readily dissolve dry NC resulting in the formation of a lacquer. When the solvents are evaporated the polymer is deposited as a thin film. By including a non-solvent such as water in the lacquer pores, can be introduced into the film to create a microporous membrane. Pore formation is a consequence of differential evaporation of the non-solvent and the solvent. Therefore pore size and porosity can be readily controlled by the amount of the non-solvent in the lacquer (2). The pore size of 0.45 μm refers to the average effective diameter of the irregular long and tortuous channels that traverse the membrane. The pores of 0.45 μm in NC membranes account for about 80% of the filters' volume reaching an average density of $450 \times 10^6/\text{cm}^2$ (18). In the blotting process, the membrane needs to be porous to allow it to be saturated with buffer and will permit the required flow of current or liquid for electro and convection blotting.

2.1.2. Immobilization Mechanism—Even though the exact mechanism by which biomolecules interact with NC is unknown, several lines of evidence suggest that the interaction is non-covalent and hydrophobic. One evidence favoring hydrophobic interaction

is the fact that since most proteins at *pH* values above 7 are negatively charged it is surprising that NC which is also negatively charged can bind proteins efficiently. An additional fact is that non-ionic detergents, like Triton X-100 are effective in removing bound antigens from NC (8).

High concentrations of salt and low concentrations of methanol increase immobilization efficiency (22). NC is unique, when compared to other microporous membranes, in its ability to distinguish between single and double stranded nucleic acids, small and large proteins, short and long nucleic acids and complexed versus uncomplexed molecules (22).

It can be stained with amido black (4), Coomassie brilliant blue (CBB) (1), aniline blue black, Ponceau S, fast green or toluidine blue. Amido black staining can detect a 25 ng dot of bovine serum albumin readily with acceptable background staining. The background staining tends to be higher with CBB while Ponceau S gives a very clean pattern but with slightly less sensitivity than Amido black.

2.1.3. Disadvantages of NC—One clear disadvantage of NC is the fact that it cannot be stripped and reprobed multiple times owing to its fragile nature. It also has a tendency to become brittle when dry. In addition, small proteins tend to move through NC membranes and only a small fraction of the total amount actually binds. Using membranes with smaller pores can obviate this (12). Gelatin coated NC have been used for quantitative retention (10). In supported NC (e.g. Hybond-C Extra), the mechanical strength of the membrane has been improved by incorporating a polyester support web, thereby making handling easier.

2.2. Polyvinylidene Difluoride (PVDF)

PVDF is a linear polymer with repeating $-(CF_2-CH_2)-$ units. The use of “di” in polyvinylidene difluoride is redundant (including its use here) and its use needs to be discouraged (2). Polyvinylidene fluoride or polyvinylidene difluoride refer to the same membrane first made available for protein blotting by Millipore in June of 1986. The product was renamed as Immobilon-P™ Transfer Membrane after being initially referred to as Immobilon™PVDF transfer membrane to differentiate it from other PVDF and non-PVDF-based blotting membranes referred to collectively as Immobilon family and marketed by Millipore. Immobilon-P^{SQ} membrane with a 0.2 μm pore size suitable for proteins with a molecular weight less than 20 kD (to prevent blow through) and immobilon-FL membrane optimized for all fluorescence applications also form part of the Immobilon family of PVDF membranes, added recently. Sequelon (24), a PVDF-based sequencing membrane, sold by Milligen/BioSearch, a Millipore subsidiary are advantageous because of high protein binding capacity, physical strength and chemical stability.

2.2.1. Immobilization Mechanism—Proteins transferred to the Immobilon-P membrane during western transfer are retained well on its surface throughout the immunodetection process via a combination of dipole and hydrophobic interactions. The antigen binding capacity of the membrane is 170 $\mu g/cm^2$ for bovine serum albumin and this is proportionate with that binding capacity of NC. In addition the immobilon-P membrane has very good mechanical strength and like Teflon™ (a related fluorocarbon polymer) is

compatible with a range of chemicals and organic solvents (acetonitrile, trifluoroacetic acid, hexane, ethylacetate and trimethylamine (2,25).

Blotting mechanics are not different from that seen with NC, except for the fact that it is necessary to pre-wet the membrane in either methanol or ethanol before using with aqueous buffers. This is because PVDF is highly hydrophobic and there is no added surfactant in PVDF.

2.2.2. Advantages of PVDF—One of the advantages of electroblotting proteins onto PVDF membranes is that replicate lanes from a single gel can be used for various purposes such as N-terminal sequencing, proteolysis/peptide separation/internal sequencing along with Western analysis. Proteins blotted to PVDF membranes can be stained with amido black, India ink and silver nitrate (26). These membranes are also amenable to staining with CBB, thus allowing excision of proteins for N-terminal protein sequencing, a procedure first demonstrated by Matsudaira in 1987 (25).

2.3. Activated Paper

Activated paper (diaz groups) binds proteins covalently, but is disadvantageous in that the coupling method is incompatible with many gel electrophoresis systems. Linkage is through primary amines and therefore systems that use gel buffers without free amino groups must be used with this paper. In addition, the paper is expensive and the reactive groups have a limited half-life once the paper is activated.

2.4. Nylon Membranes

Nylon based membranes are thin and smooth surfaced as NC, but with much better durability. Two kinds of membranes are available commercially, Gene Screen and Zetabind (ZB). ZB is a nylon matrix (polyhexamethylene adipamine or Nylon 66) modified by the addition of numerous tertiary amino groups during the manufacturing process (extensive cationization). It has excellent mechanical strength and also offers the potential of very significant (yet reversible) electrostatic interactions between the membrane and polyanions. Nylon shows a greater protein binding capacity compared to NC (480 μg versus 80 μg BSA bound/ cm^2). In addition, nylon offers the advantages of more consistent transfer results and a significantly increased sensitivity compared to other membranes (7,18). This effect is possible owing to the extra potential difference created by the positive charge of ZB.

2.4.1. Disadvantages of Nylon—The high binding capacity of these membranes however produces higher non-specific binding. Another problem with using nylon membranes is the fact that they bind strongly to the commonly used anionic dyes like Coomassie blue, amido black 10B (18), aniline blue black, Ponceau S, fast green or toluidine blue. SDS, dodecyl trimethylammonium bromide or Triton X-100 at low concentrations (0.1% in water) remove the dyes from the membrane while simultaneously destaining the transferred proteins, with SDS being the best. Destaining of this membrane is thus not possible, unlike NC, and therefore the background remains as high as the signal (8). On account of these problems, NC membranes have remained the best compromise for most

situations. However, an immunological stain and India ink have been used to detect proteins on ZB (27,28) and NC membranes.

Nylon membranes have been found very useful in binding the negatively charged DNA, especially the positively charged ZB membranes. As a consequence it has been used more for DNA blotting than for protein blotting.

3. Buffers Used in Transfer Protocols

Commonly used buffers for western blotting are (a) Towbin system buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v), none to 0.01% SDS (1) and (b) CAPS buffer system (CAPS: 10 mM 3-(cyclohexyl-amino)-1-propanesulfonic acid, 10 % methanol (v/v), pH 11) for transfer to PVDF popularized by Matsudaira (24) for use prior to *in situ* blot sequencing. Transfer buffers without SDS are better, in general, when using Immobilon-P, since proteins have been reported to pass through the plane of the membrane in the presence of SDS (29,30). However, for proteins that have a tendency to precipitate, it would be better to have SDS in the buffer (<0.01%) during the transfer and fine-tune transfer time, current etc. The Towbin system is used widely for applications that require immuno-development while the low ionic strength buffer system of Matsudaira (25) allows rapid transfer (ca 10 min) and prevents introduction of additional Tris and glycine that is detrimental to sequence analysis using PVDF membranes.

Methanol, introduced originally by Towbin, is typically present in the transfer buffer and aids in stripping SDS from proteins transferred from denaturing SDS-containing polyacrylamide gels. It stabilizes the geometry of the gel during the transfer process, and tends to increase the binding capacity of NC for protein as well as helps proteins to bind better to NC membrane (5,8,18,31). It is possible to eliminate methanol completely from transfer buffer when using immobilon-P membranes as well as NC. Ten to 15% methanol is suggested for general protein transfer (standard Towbin buffer used 20% methanol). Methanol shrinks the gel and therefore when transferring high molecular weight proteins (>150 kD) best results are obtained without added methanol. Non-methanolic transfer is also advised when enzyme activity needs to be preserved as well as when transferring conformation sensitive antibodies. PAGE gels tend to swell in low-ionic strength buffers in the absence of methanol. The “bands” may become distorted if this swelling is allowed to occur during protein transfer. Pre-swelling of the gel by incubating it in transfer buffer for 30 min to 1-hour prior to transfer has been shown to obviate this problem (5,8).

4. Settings (Current/Voltage) for Protein Transfer

Some of the issues to be considered before electrotransfer includes deciding on whether to use constant voltage or constant current and the use of tank of semidry electroblotting units. The use of constant voltage provides the best driving force (that is, potential difference) during transfer (2). The buffer composition changes as salts are eluted from the gels, resulting in an increase in current and a drop in resistance (8,18). However, joule heating can cause an accompanying rise in current. Ohm's law states that Voltage (V) = Current (I) × resistance (R). A transfer using constant voltage leads to an increase in current and a

decrease in resistance while a transfer using constant current there is decrease in voltage as well as resistance ($I = V/R$). When current reaches over 500 mA heating can be problematic in tank buffer systems and the use of cooling elements have been recommended in such a scenario. However, it has been shown recently that transfer can be efficiently carried out using heated buffer, from which methanol was omitted, to transfer high molecular weight proteins (17,32). Semi-dry blotters have been used to rapidly transfer proteins electrophoretically without excessive heat and using small volumes of buffer, short electrode distances and planar electrodes that also serve as heat sinks (33).

Low molecular weight proteins are preferentially eluted out of the gel into the plane of the blotting membrane when a planar gel having electrophoretically resolved protein is exposed to a current perpendicular to its surface. As a result, large molecular weight proteins will be under-transferred under conditions optimized for transfer of low molecular weight polypeptides. On the other hand, a prolonged transfer will help the movement of large molecular weight species with accompanying loss of smaller species consequent to “blow through.” A second sheet of membrane as a “backup” is useful to capture proteins that span a large molecular weight range. The use of gradient electric fields to reduce overall current use and allow the quantitative transfer of a wide range of proteins has been suggested (18). Another approach involves a two-step electrotransfer beginning with elution of low molecular weight proteins at low current (1 mA/cm²) for an hour followed by transfer at high current density (3.5–7.5 mA/cm²) which aids the elution of high molecular weight proteins (34). Recent work has shown the utility of heated buffer to transfer high molecular weight proteins rapidly (17,32).

5. Techniques to Transfer Proteins from Gel to Membrane

Transfer of proteins from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or native gels to nitrocellulose or PVDF membranes has been achieved by (a) simple diffusion; (b) vacuum-assisted solvent flow; and (c) “Western” blotting or electrophoretic elution (4,12,35–39).

5.1. Simple diffusion

Diffusion blotting was originally developed for transferring proteins separated by iso-electric focusing on thin gels to membranes and this was later expanded to other gel systems (32,40–46). In this procedure a membrane is placed on the gel surface with a stack of dry filter papers on top of the membrane. A glass plate and an object with a certain weight are usually placed on this assembly to enable the diffusion process. However, since there was no quantitative transfer of protein this protocol had not gained widespread acceptance. A waning interest in diffusion transfer was resuscitated when it was demonstrated that it was possible to obtain up to 12 blots from a single gel by sandwiching it between two membranes sequentially (*see Chapter 11*) (31) (Fig. 2). Non-electrophoretic membrane lifts from SDS-PAGE gels for immunoblotting, obtained by using this method, are very useful for identification of proteins by mass spectrometry (47,48). The gel can be stained with Coomassie following diffusion blotting. The antigens on the blot are detected by immunostaining and the immunoblotted target band can be compared with the Coomassie

stained gel by superimposing the blot and the stained gel, allowing the identification of the band to be excised for tryptic digestion and subsequent matrix assisted laser desorption time of flight mass spectrometric analysis. The main advantage of diffusion blotting compared to electroblotting is that several transfers or imprints can be obtained from the same gel and different antisera can be tested on identical imprints.

Subsequently, quantitative information regarding protein transfer during diffusion blotting was obtained using ^{14}C labeled proteins. A three-minute diffusion blotting was shown to allow a transfer of 10% compared to electroblotting. Diffusion blotting of the same gels carried out multiple times for prolonged periods at 37°C causes the gel to shrink. This can be overcome by using gels cast on plastic supports (44,45).

Zymography or activity gel electrophoresis has also been studied with regard to the utility of diffusion. This involves the electrophoresis of enzymes (either nucleases or proteases) through discontinuous polyacrylamide gels containing enzyme substrate (either type III gelatin or β -casein). Following electrophoresis, SDS is removed from the gel by washing in 2.5% Triton X-100. This allows the enzyme to renature, and the substrate to be degraded. Staining of the gel with CBB (in the case of proteins) allows the bands of enzyme activity to be detected as clear bands of lysis against a blue background (49). An additional immunoblotting analysis using another gel is often required in this procedure to examine a particular band that is involved. Diffusion blotting has been used to circumvent the use of a second gel for this purpose (45). The activity gel was blotted onto PVDF for immunostaining and the remaining gel after blotting was used for routine "activity staining". Since the blot and the activity staining are derived from the same gel, the signal localization in the gel and the replica can be easily aligned for comparison.

Diffusion blotting transfers 25–50% of the (45) proteins to the membrane compared to electroblotting. However, the advantage of obtaining multiple blots from the same gel could outweigh the loss in transfer and actually could be compensated for by using sensitive detection techniques. The gel remains on its plastic support, which prevents stretching and compression; this ensures identical imprints and facilitates more reliable molecular mass determination. If only a few imprints are made, sufficient protein remains within the gel for general protein staining. These advantages make diffusion blotting the method of choice when quantitative protein transfer is not required.

5.2. Vacuum Blotting

This method was developed (50) as an alternative to diffusion blotting and electroblotting. The suction power of a pump connected to a slab gel dryer system was used to drive the separated polypeptides from the gel to the nitrocellulose membrane. Both low and high molecular weight proteins could be transferred using this method. Since small molecular weight proteins ($\pm 14,000$ Daltons) are not well adsorbed by the $0.45\text{ }\mu\text{m}$ membrane nitrocellulose, membranes with a small pore size (0.2 or $0.1\text{ }\mu\text{m}$) should be used when using low molecular weight proteins.

The gel can dry out if the procedure is carried out over 45 min and in such a scenario enough buffer should be used. In some instances low concentration polyacrylamide gels stuck to the

membrane following transfer. Rehydrating the gel helps detaching the nitrocellulose membrane from the gel remnants in such a scenario.

5.3. Electrophoretic blotting

Electrophoretic blotting is the most commonly used procedure to transfer proteins from a gel to a membrane. The main advantages are the speed and the completeness of transfer compared to diffusion or vacuum blotting. Electroelution can be achieved either by (a) complete immersion of a gel-membrane sandwich (Fig. 3) in a buffer (wet transfer) or by (b) placing the gel-membrane sandwich between absorbent paper soaked in transfer buffer (semi-dry transfer).

The transfer conditions as such are dependent on gel type, the immobilization membrane, the transfer apparatus used as well as the protein themselves. SDS gels, urea gels (4), lithium dodecyl sulfate-containing gels, nondenaturing gels, two-dimensional gels and agarose gels have been used for protein blotting (electrophoretic) (18). The electric charge of the protein should be determined and the membrane should be placed on the appropriate side of the gel. When using urea gels the membrane should be placed on the cathode side of the gel (4). Proteins from SDS PAGE gels are eluted as anions and therefore the filter should be placed on the anode side of the gel.

5.3.1. Wet transfer—In this procedure, the sandwich is placed in a buffer tank with platinum wire electrodes. A large number of different apparatuses are available to efficiently transfer proteins (or other macromolecules) transversely from gel to membrane. Most of these, however, are based on the design of Towbin *et al.* (1): i.e., they have vertical stainless steel / platinum electrodes in a large tank.

5.3.2. “Semi-dry” transfer—In “Semi-dry” transfer, the gel-membrane sandwich is placed between carbon plate electrodes. “Semi-dry” or “horizontal” blotting uses two plate electrodes (stainless steel or graphite / carbon) for uniform electrical field over a short distance, and sandwiches between these up to six gel / membrane / filter paper assemblies, all well soaked in transfer buffer. The assembly is clamped or otherwise secured on its side, and electrophoretic transfer effected in this position, using as transfer buffer only the liquid contained in the gel and filter papers or other pads in the assembly.

The advantages to this procedure over the conventional upright protocol are (a) gels can be blotted simultaneously; (b) electrodes can be cheap carbon blocks and (c) less power is required for transfer (and therefore a simpler power pack).

As will be seen in the following chapters, protein blotting has been evolving constantly and now the scientific community is faced with a plethora of ways and means of transferring and detecting proteins.

References

1. Towbin H, Staehelin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to NC sheets: procedure and applications. *Proc Natl Acad Sci USA* 76, 4350–4354. [PubMed: 388439]

2. LeGendre N (1990). Immobilon-P transfer membrane: applications and utility in protein biochemical analysis. *Biotechniques* 9 (6 Suppl): 788–805. Review. [PubMed: 2271178]
3. Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.* 98, 503–517. [PubMed: 1195397]
4. Alwine JC, and Kemp DJ (1977) Stark GR Method for detection of specific RNAs in agar gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci USA* 74, 5350–5354. [PubMed: 414220]
5. Burnette WN (1981) “Western Blotting”: electrophoretic transfer of proteins from sodium dodecyl sulfate—polyacrylamide gels to unmodified NC and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem.* 112, 195–203. [PubMed: 6266278]
6. Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227, 680–685. [PubMed: 5432063]
7. Kost J, Liu L-S, Ferreira J, and Langer R (1994) Enhanced protein blotting from PhastGel media to membranes by irradiation of low-intensity. *Anal Biochem.* 216, 27–32. [PubMed: 8135362]
8. Gershoni JM, and Palade GE (1982) Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter. *Anal Biochem.* 124, 396–405. [PubMed: 7149237]
9. Gershoni JM (1988) Protein blotting: a manual. *Methods Biochem Anal.* 33, 1–58. Review. [PubMed: 2451778]
10. Kurien BT, and Scofield RH (2006) Western blotting. *Methods* 38, 283–293. [PubMed: 16483794]
11. Karey KP, and Sirbasku DA (1989) Glutaraldehyde fixation increases retention of low molecular weight proteins (growth factors) transferred to nylon membranes for Western blot analysis. *Anal Biochem.* 178, 255–259. [PubMed: 2502042]
12. Harlow E, and Lane D (1988) *Immunoblotting In: Antibodies. A laboratory manual.* Cold Spring Harbor Laboratory, p. 485.
13. Renart J, Reiser J and Stark GR (1979) Transfer of proteins from gels to diazobenzyloxymethyl paper and detection with anti-sera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci USA*, 76, 3116–3120. [PubMed: 91164]
14. Elkon KB, Jankowski PW and Chu JL (1984) Blotting intact immunoglobulins and other high-molecular-weight proteins after composite agarose-polyacrylamide gel electrophoresis. *Anal Biochem.* 140, 208–213. [PubMed: 6486407]
15. Gibson W (1981). Protease-facilitated transfer of high-molecular-weight proteins during electrotransfer to NC. *Anal Biochem.* 118, 1–3. [PubMed: 7039405]
16. Bolt MW, and Mahoney PA (1997) High efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem.* 247, 185–192. [PubMed: 9177676]
17. Kurien BT, and Scofield RH (2002) Heat mediated, ultra-rapid electrophoretic transfer of high and low molecular weight proteins to NC membranes. *J Immunol Methods* 266,127–133. [PubMed: 12133629]
18. Gershoni JM, and Palade GE (1983) Protein blotting: principles and applications. *Anal Biochem.* 131, 1–15. [PubMed: 6193725]
19. Thornton DJ, Carlstedt I, and Sheehan JK (1996) Identification of glycoproteins on nitrocellulose membranes and gels. *Mol Biotechnol.* 5, 171–176. [PubMed: 8734429]
20. Tonkinson JL, and Stillman B (2002) NC: a tried and true polymer finds utility as a post-genomic substrate. *Front Biosci.* 7:c1–12. Review. [PubMed: 11779718]
21. Lauritzen E, Masson M, Rubin I, Bjerrum OJ, and Holm A (1993) Peptide dot immunoassay and immunoblotting: electroblotting from aluminum thin-layer chromatography plates and isoelectric focusing gels to activated NC. *Electrophoresis* 14, 852–859. [PubMed: 8223393]
22. Masson M, Lauritzen E, and Holm A (1993) Chemical activation of NC membranes for peptide antigen-antibody binding studies: direct substitution of the nitrate group with diaminoalkane. *Electrophoresis* 14, 860–865. [PubMed: 8223394]
23. Too CK, Murphy PR, and Croll RP (1994) Western blotting of formaldehyde-fixed neuropeptides as small as 400 daltons on gelatin-coated NC paper. *Anal Biochem.* 219, 341–348. [PubMed: 7915887]

24. Coull JM, Dixon JD, Laursen RA, Koester H, and Pappin DJC (1989) Development of membrane supports for the solid-phase sequence analysis of proteins and peptides, 69–78 In Witmann-Liebold B (Ed.) *Methods in Protein Sequence Analysis*. Springer-Berlag, Berlin.
25. Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem*. 262,10035–10038. [PubMed: 3611052]
26. Pluskal MF, Przekop MB, Kavonian MR, Vecoli C, and Hick DA (1986) *BioTechniques* 4, 272–282.
27. Kittler JM, Meisler NT, Viceps-Madore D, Cidlowski JA, Thanassi JW (1984) A general immunochemical method for detecting proteins on blots. *Anal Biochem*. 137, 210–216. [PubMed: 6375450]
28. Hughes JH, and Mack K, and Hamparian VV (1988) India ink staining of proteins on nylon and hydrophobic membranes. *Anal Biochem*. 173, 18–25. [PubMed: 2461118]
29. Tovey ER, and Baldo BA (1989) Protein binding to NC, nylon and PVDF membranes in immunoassays and electroblotting. *J Biochem Biophys Methods* 19, 169–183. [PubMed: 2584606]
30. Xu QY, and Shively JE (1988) Microsequence analysis of peptides and proteins. VIII. Improved electroblotting of proteins onto membranes and derivatized glass-fiber sheets. *Anal Biochem*. 170, 19–30. [PubMed: 2455458]
31. Nielsen PJ (1982) The phosphorylation of ribosomal protein S6 in rat tissues following cycloheximide injection, in diabetes, and after denervation of diaphragm. A simple immunological determination of the extent of S6 phosphorylation on protein blots. *J Biol Chem*. 257, 12316–12321. [PubMed: 6749858]
32. Kurien BT, and Scofield RH (1997) Multiple immunoblots after non-electrophoretic bidirectional transfer of a single SDS-PAGE gel with multiple antigens. *J Immunol Methods* 205, 91–94. [PubMed: 9236919]
33. Kyhse-Andersen J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 10, 203–209. [PubMed: 6530509]
34. Otter T, King SM, and Witman GB (1987) A two-step procedure for efficient electro transfer of both high-molecular weight (greater than 400,000) and low-molecular weight (less than 20,000) proteins. *Anal Biochem*. 162, 370–377. [PubMed: 2440344]
35. Harper DR, Kit ML and Kangro HO (1990) Protein blotting: ten years on. *J Virol Methods* 30, 25–39. Review. [PubMed: 2086595]
36. Egger D, and Bienz K (1994) Protein (western) blotting. *Mol Biotechnol*. 1, 289–305. [PubMed: 7532104]
37. Wisdom GB (1994) Protein blotting. *Methods Mol Biol*. 32, 207–213. [PubMed: 7524941]
38. Kurien BT, and Scofield RH (2003) Protein blotting: a review. *J Immunol Methods* 274 1–15. Review. [PubMed: 12609528]
39. Blotting techniques, Kurien BT and Scofield RH, p 425, in *Encyclopedia of Analytical Science*, Second Edition (Worsfold Paul J., Townshend Alan and Poole Colin F, eds.), Elsevier, Oxford, Second edition. x, pp. x–xx, 2005
40. Reinhart MP and Malamud D (1982). Protein transfer from isoelectric focusing Gels: the native blot. *Anal Biochem*. 123, 229–235. [PubMed: 6181706]
41. Jagersten C, Edstrom A, Olsson B, and Jacobson G (1988) Blotting from PhastGel media after horizontal sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Electrophoresis* 9, 662–665. [PubMed: 3243230]
42. Kazemi M, and Finkelstein RA (1990) Checkerboard immunoblotting (CBIB): an efficient, rapid, and sensitive method of assaying multiple antigen/antibody cross-reactivities. *J Immunol Methods* 128, 143–146. [PubMed: 2324502]
43. Heukeshoven J, and Dernick R (1995). Effective blotting of ultrathin polyacrylamide gels anchored to a solid matrix. *Electrophoresis* 16, 748–756. [PubMed: 7588557]
44. Olsen I, and Wiker HG (1998) Diffusion blotting for rapid production of multiple identical imprints from sodium dodecyl sulfate polyacrylamide gel electrophoresis on a solid support. *J Immunol Methods* 220, 77–84. [PubMed: 9839928]

45. Chen H, and Chang GD (2001) Simultaneous immunoblotting analysis with activity gel electrophoresis in a single polyacrylamide gel. *Electrophoresis* 22, 1894–1899. [PubMed: 11465485]
46. Bowen B, Steinberg J, Laemmli UK, and Weintraub H (1980) The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Res.* 8, 1–20. [PubMed: 6243775]
47. Kurien BT, and Scofield RH (2000) Association of neutropenia in systemic lupus erythematosus with anti-Ro and binding of an immunologically cross-reactive neutrophil membrane antigen. *Clin Exp Immunol.* 120, 209–217. [PubMed: 10759785]
48. Kurien BT, Matsumoto H, and Scofield RH (2001) Purification of tryptic peptides for mass spectrometry using polyvinylidene fluoride membrane. *Indian J Biochem Biophys.* 38, 274–276. [PubMed: 11811625]
49. Bischoff KM, Shi L, and Kennelly PJ (1998) The detection of enzyme activity following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem.* 260, 1–17. Review. [PubMed: 9648646]
50. Peferoen M, Huybrechts R, and De Loof A (1982) Vacuum-blotting: a new simple and efficient transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to NC. *FEBS. Lett* 145, 369–372.
51. Garribba F, Turi S, Corpetti G, Khiabani A, la Torre XD, Botrè F (2014) A modified procedure based on a vacuum-driven blotting system for the detection of erythropoietin and its analogs. *Bioanalysis.* 6, 1605–15. [PubMed: 25077622]

Figure 1:

Schematic representation of Western blotting and detection procedure

(A) Unstained SDS PAGE gel prior to Western blot. The bands shown are hypothetical (B) Exact replica of SDS PAGE gel obtained as a blot following Western transfer. (C) Primary antibody binding to a specific band on the blot (D) Secondary antibody conjugated to an enzyme (alkaline phosphatase or horse radish peroxidase) binding to primary antibody (E) color development of specific band (Reproduced from Ref 10 with permission from Elsevier).

Figure 2:

Bi-directional, non-electrophoretic transfer of proteins from SDS-PAGE gels to NC membranes to obtain up to 12 blots. The PAGE gel is sandwiched between two membranes, filter paper and glass plates and incubated at 37°C for varying periods of time to obtain up to 12 blots. (Reproduced from Ref 10 with permission from Elsevier).

Figure 3:
The Western blot transfer assembly. (Reproduced from Ref 10 with permission from Elsevier).