Chapter 1

Origins of Protein Blotting

Harry Towbin

Summary

The development of protein blotting in its early days is recounted as arising from the need to tackle a specific analytical problem. Combining diverse elements of common methods and simple lab equipment resulted in a procedure of general utility. The expansion of the idea of carrying out immunoassays on membranes as predecessors of microarrays is briefly touched upon.

Key words: Blotting, Western, History, Protein array

Easily persuaded by the editors' invitation to write about the origins of protein blotting, I begin the story in the laboratory of Julian Gordon at the Friedrich Miescher Institute in Basel. In need of a postdoctoral fellow, Julian had hired me in 1978 for the task of creating antibodies against ribosomal proteins. With some experience in ribosomes and none in immunology, I set out to purify our proteins from chicken liver. We believed that it was necessary to get really fresh samples. So, together with a courageous colleague, who later turned to running a motorbike shop, we ventured to a slaughterhouse in the countryside. While we watched their grisly machinery, the friendly workers were captivated by the fog running over the brim of the Dewars as the freshest ever chicken liver dropped into the liquid nitrogen.

Back in the new and spacious laboratory, we struggled with ultracentrifuges, ample amounts of urea, and countless column fractions. We wondered whether the animals we were going to immunize would raise the antibodies we so eagerly sought. How could we ever be sure that we would not get antibodies to some

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contaminants? We were lucky to be in contact with Theo Staehelin at Roche, a pioneer in the field of initiation factors for mammalian protein synthesis who had ample expertise in ribosomes. Theo also told us about the new hybridoma technique. The idea of immunizing with mixtures of proteins and still getting an eternal source of a specific antibody was irresistible. After learning the secrets of the trade from Theo, we happily switched to mice and spared the rabbits and goats we had already injected to familiarize ourselves with time-honored immunological techniques. I was fascinated by precipitation arcs of the Ouchterlony double diffusion test and by the sensitivity of solid phase immunoassays achieved with remarkably simple equipment. Still, the problem of assuring specificity of hybridoma antibodies remained.

A common way of characterizing ribosomal proteins was by electrophoresis on two-dimensional gels. Could one recover the proteins from the gel as the literature described and use them in these sensitive immunoassays? The extraction worked, in principle, but the bulk of homogenized polyacrylamide was deterring. Even today, few researchers take that approach for purifying proteins from gels. We also discarded the idea of letting the antibodies react with proteins in the gel because of the impeded diffusion within the polyacrylamide matrix.

As the three of us later realized, the idea of preparing a replica of a protein gel on a membrane, in close analogy to Southern's DNA blotting, was in many people's mind *(1)*. But how could we copy the proteins to a membrane? It was a lucky coincidence that Julian had an electrophoretic destainer in use. This apparatus, now rarely seen in the laboratory, served to remove excess stain from gels, simply by placing the gel between two grids and applying current at a right angle to the plane of the sheet. The electrically charged dye molecules quickly cleared off the gel. Well, proteins were also charged – would they behave like the dye? The basic setup was quickly put together. Meticulously cleaning the destainer from residual amido black I remember as being the most tedious part of the chore. Pipette tip holders, Scotch Brite scouring pads, and rubber strings were all what was needed to build the sandwich that is still popular for protein blotting. A series of straightforward experiments showed us that the nitrocellulose sheets reliably captured the proteins as they were leaving the gel. From my wife, Marion, I knew about the art of immunohistochemical staining. One could easily test staining procedures by placing little dots of proteins directly on the membrane and running series of dilutions. Developing the first blot from a gel with antibodies thrilled me with bands that darkened within seconds. I felt like a child who reveals secret messages written in invisible ink by holding a sheet of paper over a flame.

After publication of the method *(2)*, it dawned on us that the blotting procedure might have some commercial value, after all.

We learned from the lawyers that we could still claim protection in some countries. We also learned how hard it was to define what was really new in an invention and also that there needed to be an unforeseen element in it. In some way, almost everything in our procedure had some precedence! We stand on the shoulders of giants, as every Google Scholar user knows. Finally, the patent application was written; the fact that Theo Staehelin was at Roche and Julian and myself at Ciba-Geigy, though unusual, was no impediment.

The idea of placing proteins on nitrocellulose sheets by direct spotting, as trivial as it appeared, proved to be stimulating *(3)*. You could easily probe little dots of protein on nitrocellulose or create sandwich tests, for example, for determining antibody subtypes in hybridoma supernatants. The potential of carrying out assays on arrayed protein spots, a bit awkwardly named dot immunobinding, was most clearly recognized by Julian. With the advent of spotting devices, always in the footsteps of DNA technologies, protein arraying is only now gaining popularity. These efforts might all be viewed as aiming at eliminating the cumbersome gel electrophoresis step from western blotting. Also, for those weary of running gels and handling membranes, relief is in sight with a new system that automatically resolves proteins according to isoelectric point in a capillary *(4)*. Still, as the contributions to this volume attest, membranes remain attractive supports, giving room for countless variations, unforeseen applications, and an expanding nomenclature inspired from the compass set by E.M. Southern.

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Chapter 2

Western Blotting: Remembrance of Past Things

W. Neal Burnette

Summary

Western blotting sprung from the need to develop a sensitive visual assay for the antigen specificity of monoclonal antibodies. The technique employed SDS-PAGE of protein antigens, electrophoretic replica transfer of gel-resolved proteins to unmodified nitrocellulose sheets, probing the immobilized antigens with hybridomas, and detection of antibody–antigen complexes with radiolabeled staphylococcal protein A and autoradiography. The simplicity and relevance of the method has led to its expansive application as an immunodiagnostic and a ubiquitous research tool in biology and medicine.

Key words: Western blotting, SDS-PAGE, Electroblotting, Unmodified nitrocellulose, Immobilized replica, Antigen specificity, Antibody–antigen complex, Protein A

Paraphrasing Plato, Jonathan Swift once famously observed that "Necessity is the Mother of Invention" *(1)*. Such necessity was the antecedent of western blotting. The fact that similar techniques arose within the same time frame indicates the temporal pressure of an unfilled demand in biology and medicine – a common exigency to provide a tool by which to visualize specific antigens.

The requirement that impelled the development of western blotting *(2)* in my laboratory came to light in 1977, when I moved to Robert Nowinski's RNA tumor virus group at the Fred Hutchinson Cancer Research Center. This was just at the time when monoclonal antibodies were first described by Köhler and Milstein *(3)*, and Bob's group was developing monoclonal reagents as probes to assess the structural and immunologic nature of retrovirus proteins *(4)*. It quickly became clear that there was no simple, objectively visual way to easily screen the vast numbers of

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generated clones for their specificity toward individual structural polypeptides comprising the retrovirus envelope and core.

Although the main focus of my work at the time was in other areas of retroviral research, I had a methodological background in electrophoretic antigen assessment; therefore, I agreed to undertake the effort in the Nowinski group to develop new and streamlined techniques to facilitate screening of the hybridomas for antigen specificity. Having been trained as a postdoc in Tom August's lab at Albert Einstein College of Medicine in radioimmunoassays, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE), I attempted to conceive of ways in which these methods might be combined. RIAs had great sensitivity, but lacked the ability to give a simple picture of specificity, especially in complex protein mixtures. Conversely, immunoprecipitation required radiolabeling of diverse antigen species and, while it provided reasonable sensitivity and definition of specificity when linked to SDS-PAGE and autoradiography, it was plagued by significant background that led to substantial uncertainty and was not easily adaptable to high-throughput screening.

Launching into this project, essentially on my own and without benefit of knowledge of others who might be engaged in similar work, I attempted a wide array of techniques, hoping that I would stumble upon something useful or, at least, something that might light the pathway to proceed further. Here, I was trying through trial-and-error to fulfill another Swiftian dictum: "Discovery consists of seeing what everybody has seen and thinking what nobody else has thought" *(1)*. In retrospect, some of the things I tried verged on the laughable. Nevertheless, the early work furnished me with the recognition that purified, radiolabeled (in this case, radioiodinated) staphylococcal protein A *(5)* provided a more functionally stable and "universal" imaging agent for detection of antigen–antibody complexes than did "second antibody" reagents.

As incongruous as it might seem in the hindsight of nearly 30 years, I struggled with how to apply the monoclonal antibodies (as well as monospecific antisera) to gel-separated antigens. The "Eureka" moment occurred while I was concomitantly performing other experiments that employed "Northern" blots *(6)*, an effulgent clarity of vision that an immobilized "replica" of the PAGE-resolved proteins was to be an intrinsic element. Initially, I attempted passive transfer by placing gels in direct contact with derivatized, and later unmodified, nitrocellulose sheets. After overcoming problems associated with nonspecific binding of immunoglobulin and protein A reagents to the nitrocellulose by the use of a blocking agent (I employed immunoglobulin-depleted, purified bovine serum albumin), it became apparent that capillary transfer was slow, inefficient, and resulted in unacceptable diffusional band-spreading of the gel-resolved antigens.

A second Archimedean moment occurred at this point, when I came across an old electrophoretic gel destainer that I had not used for years. Perhaps, I reasoned, if I could work fast enough or keep temperatures low enough to minimize band diffusion *within* the parent gel, and find electrophoretic conditions and nitrocellulose pore size to prevent driving the proteins out of the gel and *through* the paper, I might be able to make better "replicas" of the gel-resolved antigens.

It only took about a week from this point to work out the "final" parameters of the basic electroblotting technique, and another few weeks to work on adaptations that could increase resolution and sensitivity in complex mixtures (e.g., cell culture, blood, tissue, and other clinical samples) using isotachophoresis in a first dimension, then applying such cylindrical gels to the SDS-PAGE slab gels. During this period, a manuscript was prepared and a discussion with Bob Nowinski ensued wherein the name "western blotting" was conceived. It was just at this time that the publication of Towbin et al. *(7)* appeared. Although the basic technique described by these investigators was similar, I believed that many of the simplifying and "universalizing" aspects of western blotting (e.g., unmodified nitrocellulose, radiolabeled protein A detection, 2-D separations, etc.) were sufficiently important to warrant submission of my manuscript. I also became aware at this time of the publication by Renart et al. *(8)*; however, the technique described in their paper employed conditions with which I had experimented (e.g., derivatized paper, passive capillary transfer, second antibody, etc.) and found wanting from the perspectives of simplicity, ease of use, resolution, sensitivity, and specificity.

The manuscript was submitted to *Analytical Biochemistry* and was rejected without, it seemed, any recourse to resubmission. It was interesting to note that the rejection appeared to me to be based not on any technical criticisms or its ostensible similarity to the methods of Towbin et al. *(7)*, but rather on the reviewers' sentiment of the pedestrian nature of the contribution and, particularly, to the flippant and frivolous whimsy in the name "western blotting."

As previously documented *(9)*, preprints of the rejected manuscript had been sent to colleagues, who subsequently provided them to others, and they to others until, eventually (even in this preelectronic era of written communications), it seemed as though this unpublished article had received wider distribution than many published ones. I only became aware of this subsequent to my move to the Salk Institute at the end of 1979. It was there that I was tracked down and spent a good part of every work day fielding telephonic questions about the technique and providing readable copies of the preprint – the original I had sent to a few colleagues had undergone many cycles of photocopy replication as it wended its way from lab to lab, the later generations being difficult to read. After about a half year

of operating this private "journal club," I called the editor-in-chief of *Analytical Biochemistry*; he agreed that the situation was untenable, that the general immunoblotting technique (as well as the name "western blotting") was becoming widely accepted, and that the initial rejection of my manuscript was probably unfortunate. Therefore, I resubmitted the paper (with only very minor changes); it was accepted immediately, and finally published a few months later *(2)*.

For those who have felt the sting of journal rejection, it is worth noting that this paper has entered a small pantheon of the most highly cited scientific articles, all of which were initially rejected for publication *(10)*. Humility is an oft-reinforced virtue in science; it is humbling to realize that this little paper on western blotting far transcended the sum of journal citations for all of my other published research efforts. Nevertheless, it is a source of immense satisfaction to have made – along with Towbin et al. *(7)* – a lasting contribution to the methodological armamentarium of biological and medical scientists.

To complete the analogy hinted in the title of this review, I wish to thank the editors of this volume for providing me, like the proffered "madeleine" in Proust's *À la recherche du temps perdu (11)*, the occasion for this reminiscence.

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Chapter 3

Introduction to Protein Blotting

Biji T. Kurien and R. Hal Scofield

Summary

Protein blotting is a powerful and important procedure for the immunodetection of proteins following 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.

Key words: Western blotting, Sodium dodecyl sulfate polyacrylamide gel electrophoresis, Nitrocellulose membrane, Polyvinylidene difluoride membrane

1. Introduction

The transfer of macromolecules (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)*. 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)* 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)*. The blotted

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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 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 *(6)* 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 with gels, (b) easy accessibility of the proteins immobilized on the membrane to different ligands, (c) only small amount of

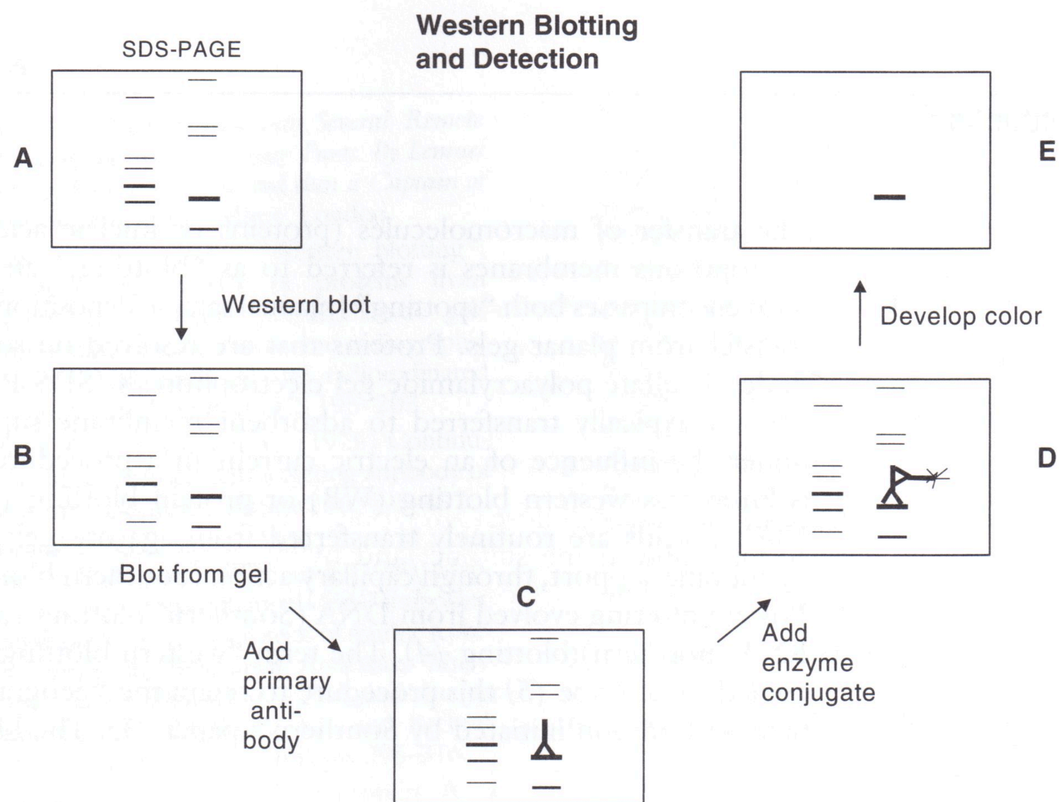

Fig. 1. Schematic of western blotting and detection procedure: (**A**) Unstained SDS PAGE gel prior to western blot. The bands shown are hypothetical. (**B**) Exact replica of SDS PAGE gel obtained as a blot following western transfer. (**C**) Primary antibody binding to a specific band on the blot. (**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 *(10)* with permission from Elsevier).

reagents is 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 in 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 ultrathin 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 are available for immobilization, ranging from the truly solid phase such as glass or plastic to latex and cellulose that are porous. The most common phases used for blotting comprise microporous surfaces and membranes such as cellulose, nitrocellulose (NC), polyvinylidine diflouride, 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-thoroughput assays used in the postgenomic 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 μm and possessing an average pore size that ranges from 0.05 to 10 μm 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 be noncovalent *(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 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 *(1)* and Burnette in 1981 *(5)* showed that NC 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 postgenomic 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 nonsolvent such as water in the lacquer pores, nonsolvent can be introduced into the film to create a microporous membrane. Pore formation is a consequence of differential evaporation of the nonsolvent and the solvent. Therefore, pore size and porosity can be readily controlled by the amount of the nonsolvent 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 filter's 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 noncovalent 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 nonionic detergents (such as 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 with 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 has been used for quantitative retention *(10, 23)*. 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

Polyvinylidene difluoride (PVDF) is a linear polymer with repeating $-(CF_2\text{-}CH_2)-$ units. The use of "di" in polyvinylidene *di*fluoride is redundant (including its use here) and its use needs to be discouraged *(2)*. Polyvinylidene fluoride or polyvinylidene difluoride refers 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 20,000 (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 is 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 the membrane surface throughout the immunodetection process via a combination of dipole and hydrophobic interactions. The antigen binding capacity of the membrane is 170 μg/cm^2 for bovine serum albumin and this is proportionate with the binding capacity of NC. In addition, the Immobilon-P membrane has very good mechanical strength and like Teflon™ (a related fluorocarbon polymer) it 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 those seen with NC, except that it is necessary to prewet 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, or 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 (diazo 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 with NC (480 μg vs. 80-μg BSA bound/cm^2). In addition, nylon

offers the advantages of more consistent transfer results and a significantly increased sensitivity compared with 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 nonspecific binding. Another problem with using nylon membranes is that they bind strongly to the commonly used anionic dyes such as CBB, 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, especially the positively charged ZB membranes, have been found very useful in binding the negatively charged DNA. 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, SDS should be in the buffer (<0.01%) during the transfer, and then one must fine-tune transfer time, current, etc. The Towbin system is used widely for applications that require immunodevelopment 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)*. Methanol can be eliminated completely from transfer buffer when using Immobilon-P membranes as well as NC. Ten to fifteen percent 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 (>150,000) best results are obtained without added methanol. Nonmethanolic 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. Preswelling of the gel by incubating it in transfer buffer for 30 min to 1 h prior to transfer has been shown to prevent this problem *(5, 8)*.

4. Settings (Current/Voltage) for Protein Transfer

Some of the issues to be considered before electrotransfer include 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 leads to decrease in voltage as well as resistance ($I = V/R$). When current reaches over 500 mA in a constant voltage setting, heating can be a problem in tank buffer systems and the use of cooling elements has been recommended in such a scenario. However, constant voltage transfer can be efficiently carried out using heated buffer, from which methanol was omitted, to transfer high molecular weight proteins *(17, 32)*. Semidry blotters have been used to rapidly transfer proteins electrophoretically without excessive heat, 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 from 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 undertransferred 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/cm^2) for an hour followed by transfer at high current density (3.5–7.5 mA/cm^2), 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 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 isoelectric 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 is no quantitative transfer of protein this protocol has not gained widespread acceptance. A waning interest in diffusion transfer was resuscitated when it was demonstrated that up to 12 blots can be obtained from a single gel by sandwiching it between two membranes sequentially (*see* Chapter "Non-electrophoretic bi-directional transfer of a single SDS-PAGE gel with multiple antigens to obtain twelve immunoblots") (**Fig. 2**) *(31)*.

Nonelectrophoretic 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,

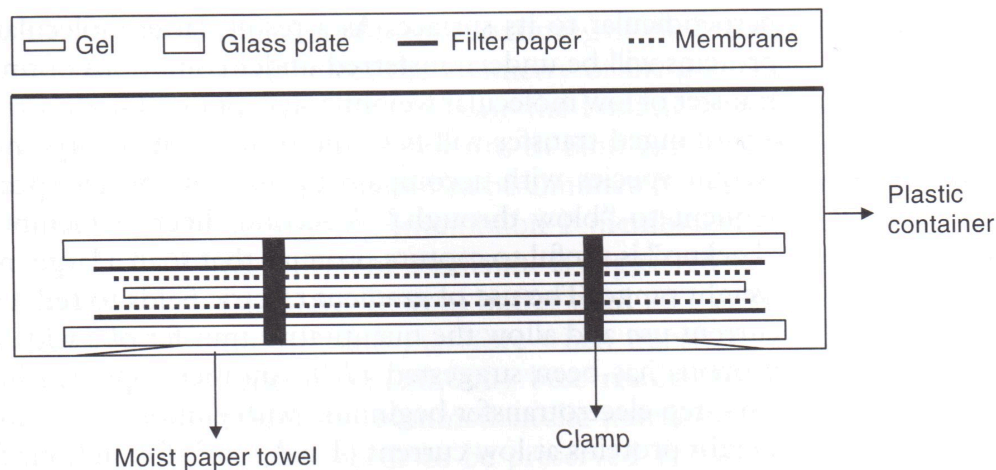

Fig. 2. Bidirectional, nonelectrophoretic transfer of proteins from SDS-PAGE gels to NC membranes to obtain up to 12 blots. The PAGE gel is sandwiched between two membranes, filter paper, and glass plates and incubated at 37°C for varying periods of time to obtain up to 12 blots (reproduced from *(10)* with permission from Elsevier).

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 with 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 3-min diffusion blotting was shown to allow a transfer of 10% compared with 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 proteins to the membrane compared with electroblotting *(45)*. However, the advantage of obtaining multiple blots from the same gel could outweigh the loss in transfer and actually 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 (±14,000) are not well adsorbed by the 0.45-μm membrane nitrocellulose, membranes with a small pore size (0.2 or 0.1 μm) 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 stick to the membrane following transfer. Rehydrating the gel helps detaching the nitrocellulose membrane from the gel remnants.

5.3. Electroblotting

Electroblotting 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 with diffusion or vacuum blotting. Electroelution can be achieved either by (a) complete immersion of a gel-membrane sandwich (**Fig. 3**) in a buffer

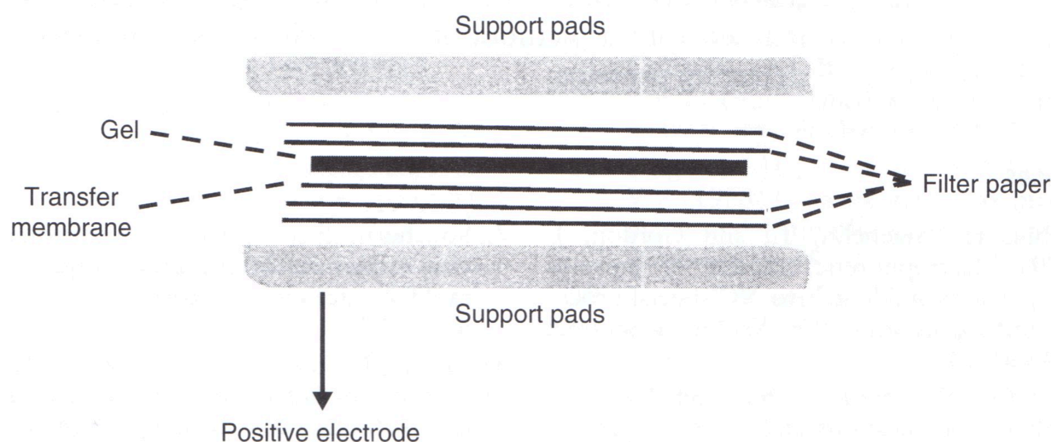

Fig. 3. The western blot transfer assembly (reproduced from *(10)* with permission from Elsevier).

(wet transfer) or by (b) placing the gel-membrane sandwich between absorbent papers soaked in transfer buffer (semidry 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)*, that is, they have vertical stainless steel/platinum electrodes in a large tank.

5.3.2. "Semidry" Transfer

In semidry transfer, the gel-membrane sandwich is placed between carbon plate electrodes. *Semidry* 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 the transfer buffer. The assembly is clamped or otherwise secured on its side, and electrophoretic transfer is 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 that (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.

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