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Other Notable Protein Blotting Methods: A Brief Review

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Abstract

Proteins have been transferred from the gel to the membrane by a variety of methods. These include vacuum blotting, centrifuge blotting, electroblotting of proteins to Teflon tape and membranes for N- and C-terminal sequence analysis, multiple tissue blotting, a two step transfer of low and high molecular weight proteins, blotting of Coomassie Brilliant Blue (CBB) stained proteins from polyacrylamide gels to transparencies, acid electroblotting onto activated glass, membrane-array method for the detection of human intestinal bacteria in fecal samples, protein microarray using a new black cellulose nitrate support, electrotransfer using square wave alternating voltage for enhanced protein recovery, polyethylene glycol mediated significant enhancement of the immunoblotting transfer, parallel protein chemical processing before and during western blot and the molecular scanner concept, electronic western blot of matrix-assisted laser desorption/ionization mass spectrometric-identified polypeptides from parallel processed gel-separated proteins, semi-dry electroblotting of peptides and proteins from acid-urea polyacrylamide gels, transfer of silver-stained proteins from polyacrylamide gels to polyvinylidene difluoride (PVDF) membranes and the display of K^+ channel proteins on a solid nitrocellulose support for assaying toxin binding. The quantification of proteins bound to PVDF membranes by elution of CBB, clarification of immunoblots on PVDF for transmission densitometry, gold coating of non-conductive membranes before matrix-assisted laser desorption/ionization tandem mass spectrometric analysis to prevent charging effect for analysis of peptides from PVDF membranes, a simple method for coating native polysaccharides onto nitrocellulose are some of the methods involving either the manipulation of membranes with transferred proteins or just a passive transfer of antigens to membranes. All these methods are briefly reviewed in this chapter.

Keywords

Centrifuge blotting; Black cellulose nitrate; Protein microarray; Square wave alternating voltage; Acid electroblotting; Electronic western blotting; Gold coating of membranes

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1. Vacuum Blotting

Renart *et al* first used a capillary blotting system to passively transfer proteins from polyacrylamide gels onto nitrocellulose membrane. However, the transfer was slow and inefficient using this method. Electroblotting, describe later by Towbin and Burnette, was much more efficient. However, Peferoen et al, owing to serious financial constraints in their department, developed another way of transferring proteins. They decided to speed up the transfer process (compared to that obtained by the capillary transfer method) by using suction force to draw proteins out of the gel. The authors used the suction power of a pump that was connected to a slab dryer systems to drive proteins from a gel (polyacrylamide or sodium dodecyl polyacrylamide gels) to a nitrocellulose membrane. Using this method, Peferoen et al efficiently blotted *Sarcophaga bullata* egg homogenate and *Leptinotarsa decemlineata* hemolymph proteins (1).

2. Centrifuge Blotting

Hermansen *et al* (2) describe a method to elute and transfer proteins, detected in the gel by visualization with 1*M* potassium chloride (KCl), to a polyvinylidene diflouride (PVDF) (Immobilon-P, BioRad Laboratories, Hercules, CA, USA) membrane by centrifugation instead of electroblotting. The SDS polyacrylamide gel was immersed in 1*M* KCl for 2 min. Protein bands that appear as a clear zone against an opaque background was cut out and immersed in distilled water to remove excess KCl (3–5 min). The gel pieces were then used for centrifuge blotting or stored moist in a microcentrifuge tube at −20°C.

PVDF and dialysis membrane (10 kD cut off) were excised into 12 mm disks. The PVDF disk was wetted in methanol for 5 min for activation. The gel bit was soaked in an eluant for 15 min at 37°C. The dialysis and PVDF membranes were soaked in the eluant (3–5 min) prior to centrifugation.

The centrifuge receptacle assembly consists of an outer chamber that supported the entire structure, an inner cylinder (serving as the reservoir for the eluant), a 12-mm sinter base support made of polyethane which holds the dialysis and PVDF membrane in place, a polystyrene tube for eluate collection and a flat O-ring gasket made of silicon to prevent leakage in the receptacle during centrifugation.

The dialysis membrane was positioned on the base support of the receptacle followed by the O-ring. Eluant was added on the dialysis membrane prior to placing the PVDF membrane. This prevents formation of vacuum between the PVDF and dialysis membranes. The inner cylinder is screwed in the receptacle after the PVDF membrane is placed on top of the O-ring. Eluant was added into the receptacle after placing the gel bit on top of the PVDF membrane. Centrifugation was carried out at 3000 x g at 20°C for 1 h in a table top centrifuge. Following centrifugation the PVDF membrane with the transferred protein adhering to it was vacuum dried and stored at −20°C for *N*-terminal sequence analysis or other analyses. The dialysis membrane placed beneath the PVDF membranes helps retain the non-immobilized proteins (2).

3. Electroblotting of Proteins to Teflon tape and Membranes for N- and C-terminal Sequence Analysis

Several membranes have been used in electroblotting since the inception of the electrotransfer of proteins from polyacrylamide gels to membranes and the subsequent characterization of the blotted proteins (3). The chemical inertness of a membrane has been one of the critical properties that determined the usefulness of a particular membrane in electroblotting. Proteins eletrotransferred to nitrocellulose cannot be subjected to Edman sequencing but are amenable to *in situ* proteolytic digestion. PVDF (polyvinylidene diflouride) membranes have been widely used for N-terminal sequencing applications on account of the fact that it is inert to Edman degradation chemistry.

This study, to evaluate the electrotransfer of proteins to Teflon tape and membranes, was initiated on account of the fact that PVDF membranes were not inert to the chemistry used on the Hewlett-Packard G1009A C-terminal sequencer. Burkhart *et al*, (4) report the optimized conditions for electrotransfer of proteins from polyacrylamide gels to low-density Teflon tape and GORE-TEX expanded polytetrafluorethylene membranes that were discovered to provide performance similar to that obtained with PVDF. Teflon was found to be a suitable Edman sequencing support in an earlier study (5). Teflon blots were found to be amenable to amino acid analysis, *in situ* proteolytic digestion, and a combination of N-terminal sequencing followed by C-terminal sequencing in addition to being inert to the chemistry used on the Hewlett-Packard G1009A C-terminal sequencer.

In this procedure, electrophoretically separated proteins were electrotransferred onto Teflon membrane. The Teflon tape or GORE-TEX was moistened thoroughly with absolute ethanol prior to assembling the blotting sandwich. The proteins were stained with 0.005% sulforhodamine B in 30% methanol for 10 min following transfer. The blots were washed several times with distilled water, prior to drying, to remove excess stain (4).

Blotted protein bands were cut out from dry blots and placed directly into the reaction cartridge without additional washing for automated sequencing purposes. They were found to be amenable to both N-and C-terminal sequencing. As long as the described wetting procedures (it was important to moisten the membranes with absolute ethanol or isopropanol and not methanol and to keep the membrane wet with ethanol and not equilibrated with transfer buffer prior to being placed next to the gel on the blotting sandwich) were followed, the ease of preparing samples on Teflon blots was similar to PVDF membranes. However, Teflon tapes were not useful for western blotting owing to their low porosity.

4. Multiple Tissue Western Blot

An important part of protein blotting is the study of comparative protein levels in various tissues. Analysis of this kind permits characterization of tissue-specific protein isoforms, detection of immunologically related proteins and examination of posttranslational modifications leading to changes in molecular weight. Immunoblot analysis of this kind requires considerable expenditure of money, time and energy in terms of obtaining hard-to-get human tissues, protein content normalization, preparation of protein samples,

electrophoretic separation and transfer. The availability of pre-fabricated immunoblots would allow the investigator to proceed directly to the antigen detection phase of the experiment.

Human multiple tissue western (MTW) blots provide a new immunological tool for the investigation of tissue-specific protein expression. MTW blots are premade immunoblots (6) and are prepared utilizing proteins isolated from adult human tissue. The proteins are obtained from whole tissue under conditions of minimal proteolysis and ensuring maximal representation of tissue specific proteins. Proteins are solubilized in sodium dodecyl sulfate (SDS) and fractionated by SDS polyacrylamide gels. They are then electroblotted onto polyvinylidene fluoride membranes to generate blots ready for incubation with specific antibodies. Following detection of antigen, the blots can be reused several times using a stripping protocol that permits the selective removal of both primary and secondary antibodies in a single incubation. Multiple reprobings makes this protocol very useful to study human-tissue specific proteins.

5. A Two Step Transfer of Low and High Molecular Weight Proteins

Transfer of high molecular weight has been the bane of investigators everywhere (7). When higher percentage gels are used this problem is accentuated. Interestingly investigators have sought to increase the transfer of large proteins by enhancing the degree of protein migration out of the gel during the transfer. Researchers have used prolonged electrotransfer (16–21 h) at high current density along with inclusion of sodium dodecyl sulfate (SDS) in the transfer buffer, to enhance protein elution and efficiently transfer high-molecular weight proteins (8,9).

Adding SDS to a final concentration of 0.1% in the transfer buffer and transferring for 21 h have helped to quantitatively transfer proteins (8). Some investigators have used novel gels and blotting buffers (7) to efficiently transfer high molecular weight proteins. These investigators made gels using a 50:1 ratio of acrylamide: bisacrylamide in all experiments, since they were mainly interested in transferring high-molecular weight proteins. In addition, they also used a different blotting buffer. Others (10) have cleaved high molecular weight proteins with periodate or alkali, before transfer, to facilitate transfer of proteins. Partial proteolytic digestion of high-molecular-weight proteins prior to transfer (11) has also been attempted. Bigger pore sizes provided by agarose gels have been used to advantage in (12) a composite agarose-polyacrylamide gel containing SDS and urea for this same purpose.

Otter *et al* (9,13) used a two-step procedure to electrotransfer both high-molecular-weight (greater than 400,000) and low-molecular-weight (less than 20,000) proteins from polyacrylamide gels to nitrocellulose sheets. The salient features of this method included a two-step electrotransfer. The low-molecular-weight polypeptides were eluted at a low current density (approximately 1 mA/cm^2) for 1 h. This was followed by prolonged electrotransfer (16–20 h) at high current density (approximately 3.5–7.5 mA/cm^2) under conditions that favored the transfer of high-molecular-weight proteins. SDS (0.01%) was added to the transfer buffer to enhance protein transfer. The nitrocellulose was air-dried

following the transfer to eliminate protein loss during subsequent processing. This transfer procedure was found to work well with all polyacrylamide gel systems tested and with proteins prepared from many different cell types.

6. Toxin Binding to Chimeric K^+ Channels Immobilized on a Solid Nitrocellulose Support

Chip technology is playing an important role in pharmaceutical and biological research. Microarrays that are used currently involve DNA. Peptides are also used to some extent. Large proteins, however, have not been used extensively mainly owing to difficulties associated with purification and structural integrity (14,15). It is even more problematic in the case of membrane proteins that are often stabilized by the lipid moiety. There have been reports of the functional immobilization on gold/glass (16) or sensor surface of BIAcore chip (17) of a ligand-activated G-protein-coupled receptor. K^+ channels are membrane proteins making up the largest and most ubiquitous family of ion channels that control excitability in a number of cell types. Several neurological diseases have been thought to be involved owing to the dysfunction of these channels and as a result are potential targets for therapeutic drugs (15). Therefore, displaying K^+ channel proteins on a solid surface is valuable, potentially leading to clinical applications. Such an approach also has the potential for drug screening methodologies.

7. Development of a Membrane-array Method for the Detection of Human Intestinal Bacteria in Fecal Samples

All mammals, including human beings, are adapted to life in a world of microbes. For every gram weight of human intestinal contents there are 10^{10}-10^{12} colony forming units of bacteria (this is 10–20 times the total number of tissue cells in the whole body) (18). At least 400 different species of bacteria (of which 30–40 species account for 99% of the total microflora) colonize the human gastrointestinal tract (GIT) (19,20). The indigenous intestinal bacterial play important roles in food digestion, production of vitamins and other essential nutrients, metabolism of endogenous and exogenous compounds and in preventing pathogenic microflora from colonizing the GIT. Therefore the knowledge relating to the numbers and bacterial species found in the human GIT is important (21). The safety implications of antibiotic resistant bacteria in foods, contamination of foods by fecal material, the effect of diet, food additives, veterinary drug residues on the ecosystem of the intestine and the use of probiotics in the prevention and treatment of GI disorders have stimulated the interest in intestinal microflora (20).

The population of anaerobic bacteria in the human GIT has been characterized traditionally by biochemical, microscopic, physiological, and selective culture plating methods of fecal samples from human beings. A number of molecular techniques have been used in recent years to analyze the bacterial flora in human faecal samples. Such analysis can detect changes in the human GIT flora rapidly and precisely (19,20).

Wang *et al* (20) have used a nitrocellulose membrane-array method to detect human GIT bacteria in fecal samples without the use of expensive microarray-arrayer and laser scanner. Three 40-mer oligonucleotides specific for each of 20 important human GIT bacterial species (total 60 probes) were designed and synthesized, based on comparison of the 16S rDNA sequences in the GenBank data library.

The oligos were diluted and xylene cyanol was added as an indicator. The diluted oligos were heated in a boiling water bath for 2 min and then cooled immediately in ice-water for 1 min. The cooled oligos were applied to the nitrocellulose membrane with a micropipetman in a 6 × 10 array. The membranes were heated at 80°C for 2 h after air drying and UV-crosslinking. Digoxigenin (DIG)-labeled 16S rDNAs were amplified by polymerase chain reaction from human fecal samples or pure cultured bacteria with the help of two universal primers and hybridized to the membrane-array. Hybridization signals were read by NBT (nitro blue tetrazolium) / BCIP (5-bromo-4-chloro-3-indolyl phosphate) color development. The two universal primers were found to amplify full size 16 S rDNA from all the 20 bacterial species that were tested. The membrane-array method was thus found to be a reliable method for the detection of important human intestinal bacteria in the fecal samples (20).

8. A New Black Cellulose Nitrate Support for Protein Microarray

Protein microarray is still in an early stage of development compared to the well established DNA microarray technology. This is due to the different physical and chemical properties of nucleic acids and proteins. There is no comparable amplification method for proteins like PCR used for nucleic acids and also proteins are much more complicated to purify. The irreversible denaturation of proteins during the process of immobilization and the insufficient stability of purified proteins is still problematic (22,23). An optimal surface for all proteins has not been discovered yet owing to the varying properties of different proteins. Reck *et al*. (24) have used a modified nitrocellulose membrane for the optimization of a microarray sandwich-enzyme-linked immunosorbant assay (ELISA) against hINF (human interferon)-γ. This membrane was found to provide an excellent signal-to-noise ratio (SNR) and very little autofluorescence. The novel microarray slide used by these investigators was a self-made prototype coated with a black microporous cellulose nitrate substrate. The porous nitrocellulose substrate was produced by Sartorius Stedim Biotech (GmbH, Gottingen, Germany) using an evaporation technology. Porous nitrocellulose substrates that are available commercially tend to produce a high background fluorescence through their overall thickness even though they provide high protein binding capacity. Such background fluorescence may be caused by the substrate itself or by the adhesives used to attach the cellulose nitrate to glass. Sartorius developed a novel recipe and adhesive-free manufacturing procedure to overcome this problem. The white substrate made by Sartorius was already optimized for high binding capacity and low autofluorescence (made possible by the choice of various cellulose nitrate grades). The black substrate was made to provide an additional benefit by adsorbing background fluorescence originating in the depth of the structure. The added coloring material is essential for the low autofluorescence of the black substrate.

Reck *et al.* (24) used this self-made black nitrocellulose membrane with a high SNR and low autofluorescence as a microarray substrate. For spotting automation, an affymetrix 417 contact printer was used. Probes were spotted using a spotting buffer containing phosphate buffered saline and 0.5% trehalose to which 40% glycerol was added to prevent denaturation and improving the stability of protein probes. Using this black nitrocellulose membrane the authors have optimized a microarray sandwich-ELISA against hINF-γ (24).

9. Quantification of Proteins Bound to PVDF Membranes by Elution of Coomassie Brilliant Blue

Proteins transferred to PVDF membranes can be stained with a variety of stains including Coomassie Brilliant Blue (CBB), amido black, colloidal gold (25). It becomes essential to determine the amount of protein present on the membrane in order to accurately determine the sensitivity of immunostaining, initial yields from protein microsequencing and the specific activity of enzymes bound to PVDF. Since the extent and rate of electroblotting is likely to change between samples, such values cannot be obtained from the amount of loaded proteins. Therefore, Kain and Henry, 1990 (26) developed a method to quantify proteins bound to PVDF membrane by elution of CBB, in order to quantify proteins for microsequencing. Proteins were resolved on a 13% gel and transferred to PVDF.

The membrane was stained with CBB and rapidly destained. The stained membrane was air-dried and individual protein bands were excised with a scalpel. The protein from each PVDF piece was eluted with 0.1% SDS/50% isopropanol. The colored liquid was removed and read at 595 nm using a Beckman Model DU-40 spectrophotometer after calibrating with CBB eluted from a PVDF fragment derived from a region of the blot that did not have a protein band. The proteins are not eluted along with the dye. The authors eluted CBB from ^{14}C labeled proteins electrotransferred to PVDF membranes and subjected the eluted solution and the PVDF membrane containing the radiolabeled protein to liquid scintillation counting. Virtually all of the protein was found to be retained on the PVDF membrane fragment during CBB elution. The authors also found that each of the protein that they tested gave a linear response with respect to the change in absorbance in the protein range of 500 ng to 10 μg. The slope of each curve was found to differ considerably, confirming the well known fact regarding the variance of CBB bound by different proteins. Thus, in any assay of protein content a standard curve with the protein of interest needs to be obtained to get the most accurate quantitative results. This protocol is useful for the analysis of multiple samples since the absorbance readings obtained from the eluted dye stays stable for up to 1 h. This procedure is not dependent on bandshape since, it could be useful in the quantitation of proteins separated by two-dimensional gel electrophoresis. Densitometric scanning of 2-D gels is complicated by the lack of uniformity in the shape of protein spots. On account of the fact that the proteins are not extracted from the membrane, this procedure is useful for protein quantitation before protein microsequencing, for analysis of amino acid composition and for immunological procedures.

10. Enhanced Protein Recovery After Electrotransfer Using Square Wave Alternating Voltage

Bienvenut *et al* 2002 (27) studied the efficiency of protein transfer using the conventional continuous current (as in Towbin transfer system) and the use of an unusual square wave alternative voltage (SWAV).

In this procedure, immediately following SDS PAGE, the gels were soaked in deionized water for 5 min, and then equilibrated two times (five min each time) in the cathodic blotting buffer. Trans-blot PVDF membranes were equilibrated with the anodic buffer for 5 min. The standard blotting technique used a continuous current (1 mA/cm^2) using 3-(cyclohexylamino)-1-propane-sulfonique acid (CAPS) buffer. The voltage used in the SWAV method of transfer was an asymmetrical alternating voltage also using CAPS buffer. This method delivered +12 V for 125 ms followed by −5V for 125 ms repetitively. This corresponded to a 4 Hz frequency signal and an average tension of 3.5V. Following transfer the gels were stained with Coomassie blue and the membranes were stained with amido black and destained by repeatedly washing with deionized water. An average 65% increase of protein recovery was observed using the SWAV technology in combination with CAPS buffer compared to that obtained with standard immunoblotting conditions (27).

11. Polyethylene Glycol Mediated Significant Enhancement of the Immunoblotting Transfer

Zeng *et al* (28) have studied the effects of polyethylene glycol (PEG) on protein fixation, electrotransfer from SDS-polyacrylamide gels onto PVDF membranes and immunoblotting. Serum proteins were resolved by SDS-PAGE and the gel was then immersed in a 30% PEG 2000 buffer for 2 h for reversibly fixing the proteins (unlike trichloroacetic acid-sulfosalicylic acid or acetic acid-methanol systems that irreversibly fixes proteins). PEG 1000, 1500 or 2000 (at 30% level) were found to have almost equal ability to preserve the protein bands in the gels very well. PEG less than 1000 was not found to have significant effect. The proteins in the gel was electrotransferred to PVDF for 24–48 h (in a −20°C freezer at 200 mA/120 V) using the same buffer and visualized finally by the indirect HRP-antibody method. The total protein detected (lowest level) per lane was 25 pg. It was found that when the transfer was carried out in the absence of PEG, a similar immunostain revealed a significantly lower sensitivity and the bands obtained were found to be slightly blurred. PEG 1000–2000 was capable of increasing the sensitivity of immunoblotting by 10–100-fold. This increase is thought to be brought about by its non-denaturing hydrophobicity with (a) self-association of protein molecules that could diminish protein blow-through (through the PVDF membrane) (b) miniaturization caused by PEG, possibly increasing the intraband protein density and (c) possible enhancement of the interaction between free antigen and antibody by PEG treatment.

12. Acid Electroblotting Onto Activated Glass

Data obtained from partial amino acid sequencing has been used frequently in assisting in isolation of a gene coding for a specific protein and also for confirming that the right gene has been isolated. In order to establish the structure of the mature gene translation product and to cross-check sequences determined at the nucleic acid level more extensive amino acid sequence data can be employed. Aeberold *et al* (29) have used a procedure to electroblot proteins to activated glass to isolate subpicomolar levels of proteins for microsequencing. They considered nitrocellulose and nylon membranes for this purpose. However, nitrocellulose was found to dissolve during the sequencing chemistry while the charge modified nylon membranes were found to collapse into a solid pellet during the procedure. The glass fiber paper support was found to be completely stable to the sequencing conditions. However, untreated glass fiber sheets possess a very limited ability to adsorb proteins. However, the authors found that the glass fiber sheets developed considerable capacity to adsorb proteins (7–10 microgram/cm^2) following treatment with triflouroacetic acid (TFA). The mechanism of protein adsorption was a result of ionic interaction of the net positively charged proteins (owing to the acidic transfer buffer) to the negative charges on the glass fiber sheet. The TFA treatment of the glass sheet apparently unveiled these negative charges on the sheet.

1. Activation of Glass Fiber Sheets (acid etching)

Whatman GF/C or GF/F circles or sheets were placed inside neat TFA in a covered Petri dish and incubated for 1 h at room temperature. Extreme care was taken to avoid air bubbles if more than one sheet was used. The glass fiber sheets were dried completely until there was no trace of TFA.

2. Acid Blotting

The SDS from the proteins, following SDS-PAGE, was displaced by immersing the gels in 0.5% (v/v) acetic acid containing 0.5% Nonidet P-40 for 10 min at room temperature. Before assembling the sandwich for blotting, each of the blotting component was incubated with the blotting buffer. However, in order to minimize acid-catalyzed protein cleavage, acid concentration was kept as low as possible. Blotting was carried out. At this low pH of protein transfer, the proteins have a net positive charge and migrate toward the cathode onto the glass fiber paper.

Protein bands, following detection with Coomassie or fluourescent staining, were excised out of the glass fiber sheets and placed in the cartridge of a gas-phase sequenator without further treatment (29).

13. Clarification of Immunoblots on PVDF for Transmission Densitometry

For protein quantitation, methods such as radioimmunoassay or enzyme-linked immunosorbent assay have been used. However, these assays cannot provide much information regarding the characteristics of the protein, nor can they distinguish between cross-reactive species including different protein components that react with the probe. Consequently, SDS PAGE followed by immunoblotting is required to quantify events such

as cleavage of polypeptide fragments utilizing polyclonal sera or the determination of enzyme activation status consequent to a loss of pro-enzyme domains (30).

While results are compromised by the limited linearity of photographic methods, immunoblot quantification has been done indirectly by densitometry carried out on the autoradiograph of the blot (31) as well as by making a transparent copy of the blot (30). Tagami *et al* (32) has performed densitometry of a dry, color-stained blot with a laser on account of the intensity and collimation of the light source. However, it was found that the absorbance of even the unstained parts of the membrane was approximately 2 and also leading to variable baselines since they were found to be very sensitive to warping (33). However, it was found that such densitometry was possible if the membranes could be rendered transparent like a polyacrylamide gel. Nitrocellulose could be made transparent by treating with 3-in-one lubricating oil (33), thus allowing the blot to be quantitated using a conventional densitometer, analogous to the manner in which gels are scanned. However, PVDF (on account of its more hydrophilic character) was not made transparent by treatment with oil.

Tarlton and Knight tried several solvents differing in refractive index to make PVDF transparent (30). Their idea was that if PVDF could be made transparent like nitrocellulose they could be used for several experiments that were not possible with nitrocellulose, such as adsorbing lipids for the detection of anti-cholesterol antibodies, to be used in conjunction with transmission densitometry. They found that ethylene glycol was the most effective. It was found that ethylene glycol/glycerol mixture (9:1; v/v; refractive index of 1.433) was found to bring about the most optimal clarification of PVDF membranes. For this procedure dry PVDF membrane was first moistened in methanol and then immersed in solvent mixture with a minimum of one change and optical absorbance of PVDF was measured with a laser densitometer. Immunoblots were taken from storage in water and immersed in the clarification mixture with two changes.

The authors found that the PVDF immunoblots could be examined over long periods of time without loss of transparency, owing to the low volatility of both ethylene glycol and glycerol. The authors found no fading of bands after repeated clarification of dried PVDF blots by ethylene glycol/glycerol over a period of two months. However, clarification of nitrocellulose for the purpose of quantitative densitometry was found to produce some fading when oil was used.

14. Parallel Protein Chemical Processing Before and During Western Blot and the Molecular Scanner Concept

Following gel electrophoresis, the proteins are stained with either Coomassie Brilliant Blue or silver and spots containing the protein of interest are excised either manually or using robotic methods. Following several rounds of washing, the gels slices are subjected to in-gel tryptic digestion. The tryptic peptides are extracted from each gel slice and concentrated, purified and subjected to mass spectrometry. The robots that carry out the initial sample preparation are expensive. Furthermore, the investigator has to keep track of how each sample was related to the original gel during all of these steps, thus introducing

opportunities for confusion. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry directly on a polymeric surface has been performed as early as 1990 (34). Specifically, proteins transferred to a capture membrane can be analyzed by scanning the surface with the laser beam of a MALDI machine. The concept of molecular scanning (35) involves the use of enzymatic digestion during the blotting from an SDS-PAGE gel or two-dimensional gel to a PVDF capture membrane (a trypsin coated membrane being placed between gel and the PVDF membrane). This procedure does not require the use of gel staining, spot excision or extraction These authors found that a combination of in-gel digestion (prior to western blotting) together with the positioning of a trypsin coated membrane between the gel and the PVDF capture membrane (during western blotting) resulted in obtaining the best digestion efficiency (digestion of high molecular weight and basic proteins without losses of low molecular weight polypeptides due to diffusion) compared to either in-gel digestion or the use of trypsin coated-membrane alone during transfer. The capture membrane absorbs the peptide fragments from the digested proteins, following which the membrane is treated with a MALDI ionizing matrix and mass spectrometry analysis is carried out directly on the membrane. All the proteins that were originally present in the gel are processed at the same time using methods compatible with mass spectrometry. In order to obtain maximum digestion and transfer to the capture membrane the authors used a pulsed or alternating electric current (an unusual square wave alternative voltage-*see* Ref. 36). The time of contact between the proteins migrating out of the gel, with the trypsin immobilized on the membrane was maximized by this oscillating current. This present study highlighted a positive influence of the “shaking” effect of the asymmetric alternative voltage on gel protein extraction.

15. Electronic Western Blot of Matrix-assisted Laser Desorption/ionization Mass Spectrometric-identified Polypeptides From Parallel Processed Gel-separated Proteins

As described above, proteins separated by one-dimensional or two-dimensional gel electrophoresis are transferred directly through a trypsin coated membrane onto a membrane ready for matrix-assisted laser desorption/ionization (MALDI) (37). The authors found that the protein transfer and efficiency of digestion was more than 95%. They identified 110 unique proteins obtained from an *Escherichia coli* extract and 149 proteins from a mouse liver homogenate resolved by one-dimensional (1-D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, they used a Visual Basic Program to plot the identified proteins according to where they were found on the gel. Thus, the presence and distribution of any of the identified proteins could be visualized as in a western blot without the use of an antibody.

Nadler *et al* (37) used a special membrane and procedure to covalently couple trypsin to obtain a high activity, compared to that used by Bienvenut *et al* (38). Thus, they could avoid starting the digestion process in the separating gel before electroblotting and also the use of the special oscillating electroblotting apparatus as done earlier (38). The aldehyde-activated polyethersulfone membrane, Gelman US450, reacts with nucleophiles (such as primary amines) to form nucleophiles that are then reduced with sodium cyanaborohydride. The

authors determined that the amount of trypsin covalently attached to this membrane ranged from 14–22 μg/cm^2 of membrane. This amount of trypsin had sufficiently high activity to bring about a quick digestion of proteins in a single pass (37).

16. Gold Coating of Non-conductive Membranes Before Matrix-assisted Laser Desorption/ionization Tandem Mass Spectrometric Analysis Prevents Charging Effect

As seen earlier, it is possible to perform matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry directly on PVDF (polyvinylidene difluoride) membrane. However, it is not possible to carry out tandem mass spectrometry (MS/MS) directly on this polymeric surface. The reason is that the isolating material is not able to dissipate the charge made by the MALDI process (39,40). This charging effect has been postulated to create local perturbations in the electric field between the acceleration plate and the sample. To overcome this problem and allow MS/MS analysis on tandem TOF instruments, the sample has to be conductive to dissipate the charges. By depositing a thin gold layer on the surface of a non-conductive membrane like PVDF Scherl *et al* (40) describe a positive effect. The thin gold layer is applied by anodic vaporization following matrix deposition. The conductive material permits the dissipation of the charges, resulting in the first successful MS/MS analysis of peptides from PVDF membranes using a MALDI-TOF/TOF instrument under normal operating conditions (40).

17. Semi-dry Electroblotting of Peptides and Proteins From Acid-urea Polyacrylamide Gels

Electroblotting protocols have been mostly designed for protein transfer from SDS-containing gels, using tank-type apparatus typically requiring 10–18 h for transfer. Semi-dry transfer methods have been developed for SDS-PAGE not long ago (41). Owing to its speed and convenience this procedure has been adopted for DNA and RNA electroblotting.

Polyacrylamide gels without SDS provide an important avenue for separating proteins under partially denaturing or native conditions. Separation of proteins and peptides as a function of their combined size and charge has been made possible with the use of low *p*H PAGE systems. The acidic, urea-containing (AU)-PAGE system (42) enables excellent resolution of several proteins and peptides that cannot be resolved by SDS-PAGE.

Model polypeptides like calf thymus histone (21. 5 kD), ribonuclease A (13.7 kD), human lysozyme (14 kD), pancreatic trypsin inhibitor (6.5 kD) and others with molecular weight ranging from 6.5 to 3.3 kD were used to test transfer parameters. Using a power setting of 115 mA and 5 V, a transfer solution of 5% acetic acid (the same solution was used for electrophoresis), and a transfer time of 15 min it was possible to transfer the polypeptides almost completely. PVDF (0.2 μm) was found to be a superior membrane, compared to nitrocellulose (0.2 μm) for efficient transfer.

The authors studied the effect of adding methanol to the transfer buffer. Methanol addition has been recommended for increasing protein binding capacity of nitrocellulose membrane (43). The authors (44) found that the addition of methanol (10%) brought about precipitation of protein on the gel, impeding the electrophoretic transfer. Also, they found no increase in protein binding to either PVDF or nitrocellulose membranes upon addition of methanol to the buffer.

18. Transfer of Silver-stained Proteins from Polyacrylamide Gels to Polyvinylidene Difluoride Membranes

Wise *et al* (45) showed that they could transfer almost all the silver stained proteins from a polyacrylamide gel to PVDF by rinsing the gel in 2x SDS Laemmli sample before transfer. Some silver stained proteins were also found to be directly transferred without a rinse with the Laemmli buffer. The antigenicity of the transferred proteins was found to be retained when transferred either way.

19. A Simple Method for Coating Native Polysaccharides Onto Nitrocellulose

Lipid-free or protein-free polysaccharides (PS) have been reported to have very low affinity for plastic and polystyrene. Carbohydrate molecules have been modified in an effort to increase their binding affinity. These modifications have involved the covalent attachment of poly-L-lysine biotin or tyramine to the PS. The modification has the possibilities of being limited by high backgrounds, loss of specificity, loss of antigenicity and lack of reproducibility.

Feng *et al* describe a method to immobilize PS to nitrocellulose without using any modification and permitting antibody analysis by enzyme-linked immunoassays. Bacterial levan (a β(2→6)-polyfructosan and dextran (a polymer of α(1→6) glucose), both neutral PS and a highly charged PS (*Neisseria meningitides* group C polysaccharide; a polymer of α(2→9) sialic acid have been coated onto nitrocellulose through filtration devices. Various blotting assays can be used with the PS-coated membrane to investigate specific antibodies (46,47).

20. Fabrication of electrospun PVDF nanofiber membrane for western blot with high sensitivity

The microphase separation-based porous PVDF membrane normally used in western blotting methods is extremely hydrophobic. Unless pre-wetted with methanol, the membrane does not wet in aqueous solutions. In addition, the membrane's protein binding capacity and mechanical strength deteriorates following long-term immersion of the PVDF membrane in methanol. Cho *et al*. (48) studied a mechanically strong PVDF membrane with uniform and opened pores. This membrane, prepared by electrospinning and subsequent calendering, contained long nanofibers. Cho *et al*. showed that this membrane did not require a methanol

pre-wet step and displayed high sensitivity, low background and high protein binding capacity.

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