Chapter 56

A Brief Review of Other Notable Protein Detection Methods on Blots

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Summary

Several methods have been used for detecting proteins on membranes. These include the use of quantum dot luminescent labels, oxyblot immunochemical detection, polymer immunocomplexes, "coupled" probing approach, in situ renaturation of proteins for detecting enzyme activities in crude or purified preparations, immunochromatographic assay, western-phosphatase assay, and the use of Congo red dye, a cosmetic color named Alta, Pro-Q Emerald 488 dye, or amine-reactive dye in combination with alkaline phosphatase and horseradish peroxidase–antibody conjugates for the simultaneous trichromatic fluourescence detection of proteins. Several methods have been used to improve the detection of proteins on membranes, including glutaraldehyde treatment of nitrocellulose blots, elimination of keratin artifacts in immunoblots probed with polyclonal antibodies, and the washing of immunoblots with excessive water and manipulation of Tween-20 in wash buffer. These methods are briefly reviewed in this chapter.

Key words: Protein detection, Membranes, Background reduction, Dyes, Quantum dot luminescent labels

1. Two-Dimensional Oxyblot

Oxidative modification of proteins by free radicals has been implicated in several diseases including Alzheimer's disease (AD) *(1, 2)*. Protein carbonyl formation is considered to be a detectable marker of protein oxidation and it is increased in AD. The level of carbonyls is higher in areas where the histopathology of the disease is more pronounced. Formation

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of carbonyls is thought to be due to reactive oxygen species (ROS)-mediated oxidation of amino acid side chains or by covalent binding to lipid peroxidation products or glycoxidation *(3)*. Unless the oxidative modification process brings about protein aggregation resulting in deposition of proteolysis-resistant protein aggregates *(4)*, oxidized proteins are more susceptible to degradation by specific proteases *(5)*. Increased production of ROS and oxidative modification of proteins in the brain has been noted in AD pathology *(2)*, thus suggesting the involvement of protein oxidation in the neurogenerative processes peculiar to AD.

By coupling two-dimensional (2D) gel fingerprinting of oxidized proteins and immunochemical detection of protein carbonyls, the identification of protein targets of oxidative modification, which could help in establishing a relationship between oxidative modification and neuronal death in AD, has been achieved *(6)*. Since this procedure was laborious, resulting in identification of only a few oxidized proteins Castegna et al. *(2)* coupled 2D fingerprinting with immunological detection of carbonyls and mass spectrometric identification of proteins. Such an approach led them to identify specific protein targets of oxidative modification.

1.1. Protein Carbonyl Derivatization

To each brain sample (obtained at autopsy from AD patients), 2,4-dinitrophenylhydrazone (DNP)/HCl was added (for mass spectrometry analysis only HCl was used). Samples were precipitated with ice-cold trichloroacetic acid following a brief incubation. Samples were centrifuged and the precipitate was resolubilized in urea. DNPH-treated samples of brain proteins from AD and control subjects were used for one-dimensional (1D) and 2D immunoblotting analysis of protein carbonyls *(6)*.

1.2. Oxyblot Immunochemical Detection

The 1D and 2D gels were electrotransferred to nitrocellulose or PVDF. After blocking with bovine serum albumin, the membranes were incubated with anti-DNP polyclonal antibody. Following addition of appropriate alkaline phosphatase secondary antibody the blots were developed with NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrate. The blots were dried and scanned. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry of trypsin-digested spots from a Coomassie Blue-stained 2D gel was also carried out for protein identification *(2)*. Using this procedure the authors identified creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1 as the targets of oxidative modification in AD.

2. Bioconjugation of Quantum Dot Luminescent Probes for Western Blot Analysis

Detection of multiple antigens is usually done by stripping and reprobing a blot with transferred protein. Krajewski et al. *(7)* showed that it is possible to detect multiple antigens on a single blot without stripping off antibodies that have been added first by employing sequential reactions. By employing multiple fluorescent probes made from small organic dye molecules it is also possible to detect multiple antigens on a single blot without stripping off antibodies *(8)* (*see* Chapters "'Rainbow' Western Blotting" and "Multiple Antigen Detection Western Blotting"). However, such probes have several limitations. Most of these problems can, however, be eliminated by the use of quantum dot (QD) luminescent labels *(9)*.

QDs are semiconductor nanoparticles (e.g., CdSe, Inp, InAs) (having diameters of 2–10 nm) whose fundamental physical properties are influenced by quantum confinement effects *(10)*. QDs display absorption and emission peaks that progressively change to longer wavelengths with increasing particle size. Compared with standard fluorescent dyes QDs have significant advantages. Dyes, for example, have narrow absorption bands, and therefore it is difficult to excite several colors with a single excitation source. In addition, the broad spectral overlap between emissions of dyes necessitates complex mathematical analysis of the data. In comparison, QDs possess a narrow, tunable, symmetric emission spectrum allowing a larger number of probes within a detectable spectral region. A single light source can be used to excite different size populations of QDs. This makes it possible to develop simpler and more cost-effective instrumentation for multiplex detection of biomolecules. Compared with organic dyes, QDs are considerably more stable against photobleaching *(11)*. This property is important, especially for imaging applications, where the high photostability of QDs permits real-time monitoring of intracellular processes over longer periods of time *(12)*. QDs show large Stokes shifts (the difference between the maximum absorbance and emission wavelengths). In contrast to the use of fluorescent proteins (e.g., green fluorescent protein), this property permits the target signals to be clearly separated from autofluorescence and thus enables the entire emission spectra to be collected.

Makrides et al. *(9)* show a novel method of conjugating antibodies (primary or secondary) to QD, allowing the easy generation of QD-based probes for the multiplex detection of proteins in western blots. They used the immunoglobulin G (IgG)-binding Z domain, which is based on the B domain of *Staphylococcus aureus* protein A. The Z-affinity tag (6.5 kDa) is highly specific for its ligand, IgG Fc, and can easily be purified by affinity

chromatography using IgG-sepharose. It has been shown earlier that the divalent ZZ domain showed ten times higher affinity for its IgG ligand compared with the monovalent Z domain. The authors designed a ZZ protein fused to a peptide that is biotinylated in vivo (by biotin protein ligase, the *birA* gene product), followed by a six-histidine tag. Bacteria were used to produce the biotinylated ZZ tag and was purified over a monomeric avidin or Ni^{2+}-NTA column and attached to streptavidin-coated QDs. Such a technology enables the biospecific coupling of any antibody to the functionalized QDs *(9)*.

Proteins electrotransferred to PVDF membranes were washed with TBST (Tris-buffered saline containing 0.1% Tween-20) and then blocked. The membranes were then incubated with the diluted primary antibody in blocking buffer and washed. The membrane was then incubated with QD_{565}-ZZ or QD_{655}-ZZ nanoparticles conjugated to secondary antibody. Following washing the protein bands were visualized using long-wavelength ultraviolet irradiation *(9)*.

The authors detected two different proteins simultaneously on the same blot by probing first with primary antibodies, followed by incubation with QD_{565}-ZZ or QD_{655}-ZZ nanoparticles or both, conjugated to secondary antibodies *(9)*.

3. Simultaneous Trichromatic Fluourescence Detection of Proteins on Western Blots Using an Amine-Reactive Dye in Combination with Alkaline Phosphatase and Horseradish Peroxidase–Antibody Conjugates

Duplicate gels are often required, for general protein staining and the other for immunoblotting, for concurrently visualizing total protein profile and a specific protein by immunoblotting. It is also possible to immunodetect two antigens by stripping the antibody complexes from the original blot and reprobing with another antibody. However, changes in gel size relative in the blot occur as a result of gel shrinkage, swelling, or other artifacts of electrophoresis, making definitive identification of protein bands/spots unreliable.

Martin et al. *(13)* report the use of an improved trichromatic detection procedure using 2-methoxy-2,4-diphenyl-2(2*H*)-furanone (MDFF) for the detection of total protein profiles, a red-fluorogenic substrate 9*H*-(1,3-dichloro-9.9-dimethylacridin-2-one-7-yl) phosphate (DDAO phosphate) to detect alkaline phosphatase conjugates, and Amplex gold reagent to detect horseradish peroxidase conjugates. The authors refer to this method as the "DyeChrome Double western blot stain." Using this procedure two different targets can be identified, when using conventional enzyme conjugates or secondary antibodies, as long as primary antibodies from two different species are employed in

the analysis. For instance, one primary antibody could be raised in mouse while the other in rabbit. However, by using Zenon antibody labeling technology *(13)* two antibodies from the same species could be utilized on the same blot.

Following protein blotting, membranes were equilibrated in sodium borate buffer. MDPF in sodium borate buffer was added to the blot after removing the buffer. Following several washing steps the membrane was blocked. Both primary antibodies were prepared and added to the membrane after the blocking step. Secondary antibodies were added after washing the membrane. DDAO phosphate and Amplex gold dye were added and signals were visualized using ultraviolet epi-illumination and photography.

4. A New Immunoblotting Method Using Polymer Immunocomplexes: Detection of Tsc1 and Tsc2 Expression in Various Cultured Cell Lines

In biological research the detection of protein expression is important for functional analysis of gene products. One of the most useful conventional methods for this purpose is western blotting. It is, however, sometimes difficult to get antibodies with sufficiently high titer, especially when effective antigenic regions are unknown. High concentrations of secondary antibodies and/or long exposure times are essential when using low titer antibodies. In such a scenario secondary antibodies sometimes have a tendency to bind to membranes directly, leading to nonspecific bands.

Fukuda et al. *(14)* use a polymer immunocomplex method to effectively reduce the background in immunostaining of tissue sections and also to improve the specificity and sensitivity of western blotting. The authors obtained low titer rabbit antibodies against tuberous sclerosis proteins (Tsc1 and Tsc 2). While they obtained high background binding at first using these antibodies, they have used the polymer immunocomplex method to reduce background binding by these antibodies.

In this method, polyclonal primary or antigen preabsorbed antibodies were diluted and mixed with Envision polymer (DAKO) to generate immunocomplexes of primary and secondary antibodies. This polymer is an immunological reagent in which secondary antibodies are conjugated with several horseradish peroxidase molecules (HRP) via dextran polymer. Following this, normal rabbit serum was added and mixed with antibody solutions in order to mask the "free" antigen binding sites of Fab domains of secondary antibodies. The solution was applied to blocked membranes. The authors used biotinylated species-specific secondary antibodies and HRP-conjugated streptavidin for traditional biotin–streptavidin detection following washing of the membranes.

5. Washing of Immunoblots with Excessive Water and Manipulation of Tween-20 in Wash Buffer for Reducing Background in Western Blotting

There are some important steps that need to be taken for avoiding high background staining and unacceptable results. Wu et al. *(15)* show that of all other factors that can cause high background, inefficient washing of the membrane is the main cause. In addition, they use a lower Tween-20 concentration (0.02–0.005%) in the buffer for the reaction with antibody and a higher concentration (0.05%) in the phosphate buffered saline-Tween-20 (PBST) wash buffer.

Following standard electrophoresis and blotting to presoaked Immobilon-P membrane (Millipore, Bedford, MA, USA) the membrane was blocked and incubated with primary antibody. The major changes to standard immunoblotting occur from this stage onward, mainly in membrane washing and in the composition of buffer used for diluting antibody. The membrane was rinsed five times with distilled or deionized water followed with one 5-min wash with PBST. The authors have found that five rinses are enough to remove unbound antibody and the 5-min wash with the buffer was sufficient to make the pH and ionic strength appropriate for interaction with antibody. The wash after secondary antibody incubation was also repeated similarly.

The authors report better background reduction using the chemiluminiscence technique with their method of immunodetection compared with that obtained with classical immunodetection with chemiluminiscence. There are two advantages by washing with water coupled with a single buffer wash compared with the conventional washing with buffer alone. First of all, it is possible to use large volumes of water without significantly increasing the cost or labor involved. Second, water has a lower ionic strength and therefore removes excess antibodies and other molecular contaminants more efficiently (owing to its lower ionic strength it can absorb both solute and solvent molecules). The authors have found no membrane damage or stripping of blotted proteins on account of this excessive water washing. The authors also report obtaining high-quality, reproducible protein blots with significantly lower background using this procedure. This method was found to be applicable to a variety of proteins under different experimental conditions *(15)*.

6. Elimination of Keratin Artifacts in Immunoblots Probed with Polyclonal Antibodies

Ever since silver staining of protein in sodium dodecyl sulfate (SDS) polyacrylamide gels *(16)* had become common it became clear that contaminating protein bands are often present in protein patterns. It was first thought that these bands were artifacts owing

to the use of β-mercaptoethanol, since they appeared only under reducing conditions *(17)*. Skin keratins were suspected to be responsible for these undesirable bands resulting from the contamination of protein samples or the buffers used for SDS-PAGE *(18)*. This observation was consistent with the fact that, under nonreducing conditions, interchain disulfide linkages occurring between keratin molecules may prevent entry into polyacrylamide gels *(19)*. Also, these artifacts were not present when monoclonal antibodies were used. To eliminate these artifacts, the only way, until Berube et al. described the adsorption of rabbit polyclonal antiserum on skin keratins, was to take great care to avoid keratins during sample preparation or performing SDS PAGE *(19)*.

The authors prepared the keratins, used in adsorbing the sera, from human skin. They first obtained the skin specimens from the hospital they worked in and later obtained large quantities of human squamous keratin obtained from the feet from local estheticians. They froze the human keratin in liquid nitrogen and then pulverized it to a powder using a mortar and pestle. The powder was washed by centrifugation and incubated with shaking in Tris-buffered saline (TBS) containing Nonidet P40. The detergent was removed by six successive centrifugations of the keratin in TBS. Following the final spin, the keratin was frozen at –80°C until use. Preimmune or immune sera (3 volumes) were adsorbed on keratin (1 volume) for a minimum of 2 h under agitation. After this the sera were spun and recovered and used in western blotting. Thus, it was shown that the quality of immunological detection could be improved by adsorption of the rabbit polyclonal antiserum on skin keratins *(19)*.

7. A Simplified "Coupled" Probing Approach for the Detection of Proteins on Dot and Western Blots

Sundaram *(20)* describes a novel "coupled blotting" approach that used simultaneous probing of antigens on dot and western blots with primary and secondary antibodies. The author used the highly sensitive enhanced chemiluminescence (ECL) detection system, purified E7 protein of cottontail rabbit papillomavirus (CRPV), and E7-specific antibodies. The abilities of sequential primary antibody followed by secondary reagent and coupled treatments to detect E7 protein on blots were compared and it was found that there was no reduction in signal strength after coupled probing. This coupled probing procedure, involving the addition of the primary and secondary antibodies together in one incubation, has the advantage of saving hands-on time and buffer solutions compared with the standard procedure. The coupled blotting has been shown to be useful for the rapid detection of proteins without compromising quality.

8. Staining Proteins on Nitrocellulose with Congo Red Dye

Protein visualization on nitrocellulose is a procedure that is routinely carried out in enzymology, molecular biology, and protein chemistry. Staining with Ponceau S dye and amido black has been popular (*see* Chapter "Protein Stains to Detect Antigen on Membranes"). Congo red (an anionic dye) binds with carboxymethyl cellulose (CMC) but not with degraded CMC, a property that has been exploited for detection of endoglucanase activity in agar *(21)*. The observation of a blue or violet band while detecting the enzyme activity in gel, showing that it interacted with protein, encouraged Mehta and Rajput *(22)* to develop a staining method on nitrocellulose membrane using Congo red dye.

A stock solution of Congo red dye (Reidel, Germany) was made in distilled water and stored at room temperature. A working solution of this was made by diluting 1 mL of the stock solution with 9 mL of 0.2 M acetate buffer (pH 3.5; while diluting, some dye was found to become insoluble without affecting the results obtained).

The nitrocellulose membrane was immersed for 5 min at room temperature in the diluted Congo red dye immediately following transfer of proteins from sodium dodecyl sulfate polyacrylamide gel (10%). The staining was stopped by washing the membrane with distilled water. The membrane was destained with water until brown bands against a light pink background became visible. The membrane was shaken mildly during both staining and destaining. The dye was found to stain widely different proteins. The method was found to detect up to 500 ng within 10 min. The sensitivity was found to be higher than that obtained with Ponceau S. Staining of blots with India ink is capable of detecting as little as 80 ng of protein, but the staining procedure takes several hours. Therefore, for qualitative purposes Congo red dye staining of nitrocellulose offers a quick method to detect proteins. If sensitivity is a prime concern, then alternative staining procedures such as colloidal gold staining could be the method of choice *(22)*.

9. Staining of Proteins on SDS Polyacrylamide Gels and on Nitrocellulose Membranes by Alta, a Color Used as a Cosmetic

Pal et al. *(23)* describe the use of Alta, a preexisting scarlet-red stain used for cosmetic purposes, for staining gels and nitrocellulose membranes during western blot analysis. Alta is made of 0.8% Crocein scarlet (brilliant Crocein) and 0.2% Rhodamine B.

It is inexpensive and easy to use, while being almost as sensitive as Coomassie Blue R-250. This stain has been used in some parts of India by women as a cosmetic to decorate their feet. For western blot analysis, Alta (purchased from local market in Pune, India) is added in the upper tank buffer to a final concentration of 5% (v/v; 0.4% Crocein scarlet and 0.1% Rhodamine B) prior to electrophoresis. The gel was viewed on a UV-transilluminator, following SDS-PAGE and the protein profile was recorded using a gel documentation system. The gel was electrotransferred and following transfer the membrane was viewed on a UV-transilluminator as before. In addition, this membrane can be processed further for immunodetection without any interference by the background stain. The protein profile can be monitored as the gel runs and can be seen on the nitrocellulose membrane following electrotransfer. This eliminates the need to run individual gels for protein staining on the gel and for western blot analysis.

10. Renaturation of Calcium/Calmodulin-Dependent Protein Kinase Activity After Electrophoretic Transfer from Sodium Dodecyl Sulfate-Polyacrylamide Gels to Membranes

Detecting enzyme activities in crude or purified preparations has been performed by in situ renaturation of proteins following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Removal of SDS from the gel by extensive washing has been used to allow renaturation of the proteins and detection of enzyme activity in activity gels or gel overlay protocols. Electroblotting of proteins separated by SDS-PAGE to nitrocellulose or PVDF membranes prior to denaturation, renaturation, and phosphorylation in situ has been a further refinement to this approach.

Shackelford and Zivin *(24)* have used a protocol to renature calcium/calmodulin-dependent protein kinase following transfer to PVDF membranes. Following transfer to PVDF, the proteins bound to the membrane were denatured first in a denaturing buffer. The membrane is rinsed and incubated in a renaturation buffer for around 16 h (with gentle rocking). This was followed by a brief incubation with Tris–HCl. Functional assays were carried out following this. The authors have used this system to identify autophosphorylation of a subset of bound kinases. They also find that the membrane can also be used for immunoblotting, phosphoamino acid analysis, or peptide mapping following the in situ renaturation and phosphorylation procedure.

11. Detection of Glycoproteins in Polyacrylamide Gels and on Electroblots Using Pro-Q Emerald 488 Dye, a Fluorescent Periodate Schiff-Base Stain

Oligosaccharides are co- or posttranslationally attached to proteins, commonly by a number of glycosidases and glycosyltransferases. Cell surface proteins and extracellular matrix proteins are especially rich in sugar moieties. Glycosylation of proteins is vital to growth control, cell adhesiveness, cell migration, tissue differentiation, and inflammatory reaction cascades. Changes in the profiles of glycosylation are often useful indicators for the assessment of disease states. There have been only relatively few methods available for direct analysis of glycoproteins transferred to membranes.

Reacting carbohydrate groups by a periodate/Schiff's base (PAS) mechanism and noncovalent binding of specific carbohydrate epitopes using lectin-based detection systems have been the two most widely utilized methods for the detection of glycoproteins on blots. In the PAS method the carbohydrate groups are oxidized followed by conjugating with a chromogenic substrate (Alcian Blue, acid fuchsin), a fluorescent substrate Pro-Q Emerald 300 dye, dansyl hydrazine, 8-aminonapthalene-1,3,6-trisulfonate, or a biotin hydrazide or digoxigenin hydrazide tag. In the case of the chromogenic and/or fluorescent conjugates the signal is detected directly and in the case of the tags it is detected indirectly using enzyme conjugates of streptavidin or antibodies to the tags *(25)*.

Pro-Q Emerald 300 dye is a fluorescent hydrazide excitable at 300 nm that was reported by Steinberg et al. *(26)* not too long ago. This dye is suitable for the sensitive direct fluorescence detection of glycoproteins in gels or on electroblots without using enzyme amplification systems. However, this dye does not have a visible excitation peak and therefore the glycoproteins cannot be visualized using laser-based gel scanners. Therefore, a new fluorescent hydrazide dye named Pro-Q Emerald 488 with an excitation maximum of 510 nm and emission maximum of 525 nm was developed by this group. This dye is also linked to glycoproteins through the standard PAS conjugation mechanism. The glycols in glycoproteins are oxidized initially to aldehydes with the use of periodic acid. A fluorescent conjugate is generated as the dye reacts with the aldehydes on the glycoproteins. In this procedure, there is no requirement for a reduction step with sodium metabisulfite or sodium borohydride to stabilize the resulting conjugate. Differential display maps of protein glycosylation and expression levels are easily generated using computer-assisted overlay techniques *(25)*.

12. Development of Rapid One-Step Immunochromatographic Assay

In the past, most immunoassays have been performed in laboratories possessing tools and devices for analysis and this has been customarily performed by skilled personnel. Instantaneous examination of alterations in one's own physical symptoms or health status is preferred increasingly. Future health care systems may have self-tests done at home as an integral part. The pregnancy test, based on the rapid detection of human chorionic gonadotropin in urine by just adding urine to the test kit, was the first successful commercial kit.

The novel concept of immunochromatographic assay that depends on the transport of a reactant to its binding partner immobilized on the surfaces of the membrane has been used to achieve the speed and convenience of the test. The transport is brought about by the capillary action of aqueous medium through membrane pores, and therefore, this transport also separates the unbound reactant from the binding complex formed at the liquid–solid surface. The immunochromatography assay provides a way for carrying out the test without the handling of reagents (that is, permitting a one-step assay) in addition to speeding up the analytical procedure.

Consequently, the assay may be carried out at places where the specimen is collected rather than at a specialized location.

Immunochromatography can be used for both qualitative and quantitative analyses. Since the analytical system was first developed for on-site determination of pregnancy, it has been made as an on/off format without adopting a signal detector. In this current model, two antibodies binding distinct epitopes on an analyte are utilized. One antibody (detection antibody) is labeled with a signal generator (e.g., latex beads), gold colloids, and the other antibody (capture antibody) is immobilized onto solid surfaces. The labeled antibody is kept in a dehydrated state within a glass-fiber membrane in such a way that it can be dissolved immediately upon contact with an aqueous medium containing the substance to be measured (analyte). The antibody then takes part in the binding reaction to form a complex with the analyte in the liquid phase. This antibody–analyte complex moves forward in a continuous fashion until it is ultimately captured by the antibody immobilized on the surface of the nitrocellulose membrane. The membrane with its uniform pores provides a liquid–solid interface for reproducible antigen–antibody binding. The two membranes (with immunoreagents) are excised into strips and are attached in length contiguously and a cellulose membrane is present at the top

to bring about a contiguous wicking that permits the immune complex to be pulled to the immobilized antibody. Within 10 min a color signal can be read, the intensity determining the amount of analyte *(27)*.

13. Unmasking of Phosphorylation-Sensitive Epitopes on p53 and Mdm2 by a Simple Western-Phosphatase Procedure

Several cellular proteins become phosphorylated either constitutively or in response to a number of regulatory signals. Sometimes, this phosphorylation can happen on a part of an epitope recognized by a monoclonal antibody, leading to decreased immunoreactivity or total lack of binding by the antibody. To solve this, the extract or the immunoprecipitated sample has been treated with phosphatase prior to SDS PAGE.

Maya and Oren *(28)* describe a simple procedure in which the phosphatase treatment is carried out on the nitrocellulose membrane following western blotting. This procedure can be used before the use of any antibody whose epitope is known to be altered as a result of phosphorylation.

The nitrocellulose membrane, following protein transfer, was rinsed briefly with double-distilled water and incubated with phosphatase buffer containing calf intestinal alkaline phosphatase. The membrane was then washed and blocked prior to normal immunodetection. The authors found better detection of specific epitopes using this procedure.

14. Glutaraldehyde Fixation of Calmodulin to Nitrocellulose to Improve Immunodetection

Low molecular weight proteins such as calmodulin can be detected more efficiently by cross-linking to nitrocellulose using glutaraldehyde. Samples are dot blotted onto nitrocellulose membrane. The membrane is then incubated with 0.2% glutaraldehyde for 15–20 min at room temperature. Following fixation the membrane is rinsed briefly with Tris-buffered saline and subjected to immunodetection *(29)*.

15. Concentrating Protein Samples for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Isoelectric Focusing Using Protein-Blotting Membranes

A crucial limiting factor for successfully analyzing a biological sample electrophoretically is the protein concentration. Increasing protein concentration is not easy and several methodologies have been used for this purpose. However, they are often not satisfactory, require special equipment or the use of hazardous chemicals. Liang et al. *(30)* report a simple method for concentrating dilute protein samples. Their method consists of absorbing proteins onto protein-blotting membrane strips. They incubate blotting membrane strips in dilute protein solutions to capture proteins. Then they loaded the protein-absorbed membrane strips directly into the sample wells containing a strong protein elution buffer (for either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing (IEF), carried out according to standard techniques). In this manner the authors were able to successfully concentrate protein samples for SDS-PAGE or IEF.

16. Treatment of Nitrocellulose Blots to Improve Detection

Numerous proteins bind strongly to nitrocellulose under different experimental conditions. However, milk *(31)*, Triton X-100 *(32)*, Nonidet P-40 *(33)*, and Tween 20 *(34)* have all been shown to remove bound proteins. Milk, however, has been generally used to block unoccupied sites on the nitrocellulose membrane and thus prevent unspecific binding. Hoffman et al. *(34)* have shown that the sensitivity of nondenaturing blots can be increased by soaking the membrane in acidic buffer after the transfer. The authors showed that following exposure to acidic buffer, milk-stripping of antibody from membrane was completely eliminated.

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