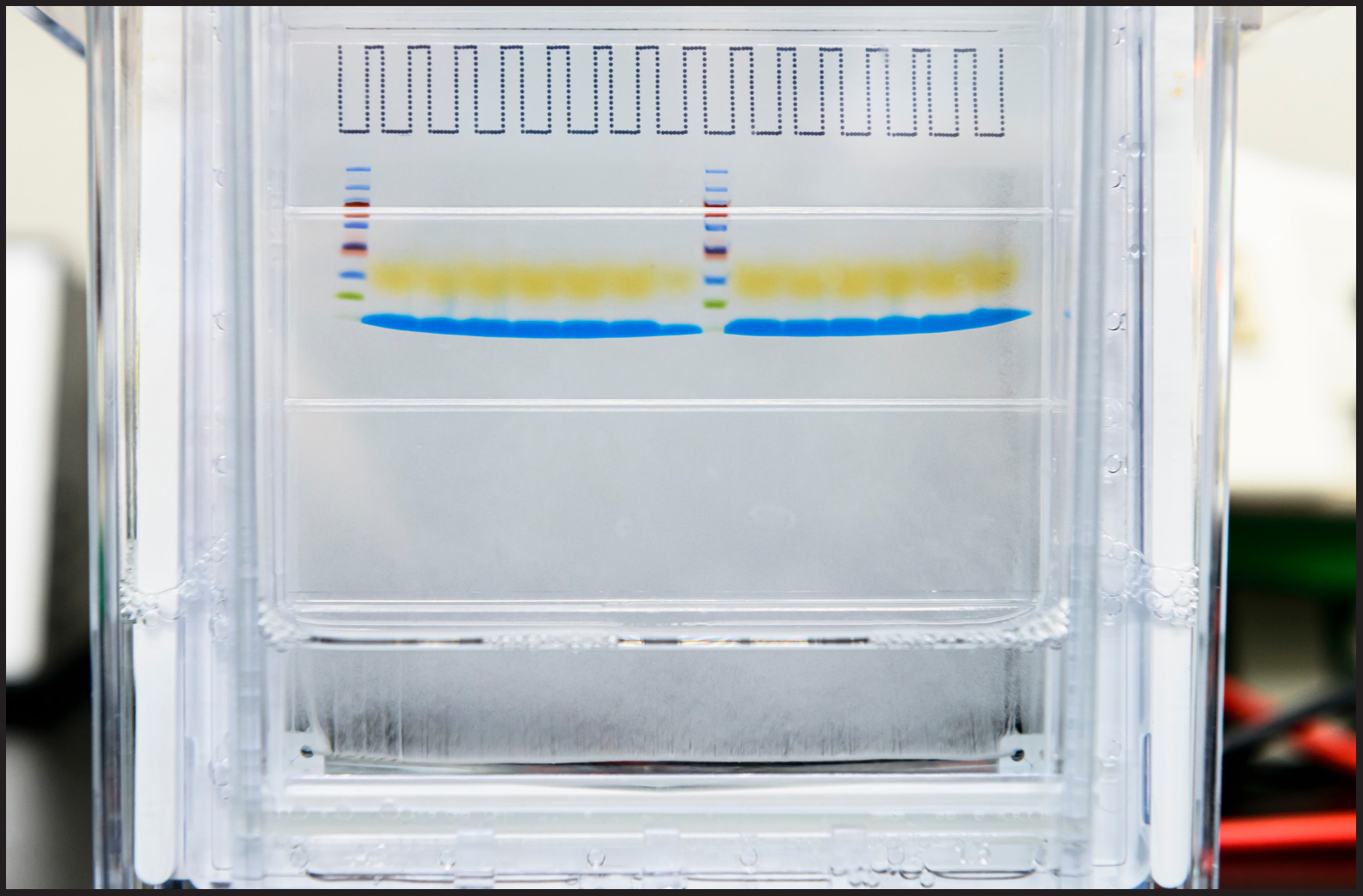

Foundational Laboratory Techniques: Western Blot

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Contents

Introduction

Western blotting, also known as immunoblotting, made its first appearance in 1979, developed at the Towbin Laboratory of the Friedrich Miescher Institute in Basel, Switzerland (Towbin et al. 1979). Since that time, the western blot has spread throughout life science and has become one of the foundational laboratory techniques to identify and quantify protein expression. Advances have been made in all aspects of the technique, from sample preparation to labelling and visualization of proteins, as well as automation.

In this article collection, we present a series of review and research articles that illustrate the power of western blotting and recent advances in the underlying techniques as well as new approaches. In addition to protein identification and quantitation western blotting can be used to understand how proteins interact with DNA, to measure protein modification such as phosphorylation or glycosylation and to evaluate different protein isoforms. Meftahi et al (2020) provides an overview of the many modalities of Western blot, and its applications beyond just basic science for clinical diagnostics. Western blotting begins with preparation of the sample, moves to gel separation, transfer of proteins onto the detection membrane and then visualization of proteins. Ni et al (2017) provides a clear and easy-to-follow protocol covering each step of the process. One of the critical steps in western blotting is the transfer of the samples to the membrane, which can be checked by examining total protein through a variety of stains. Gong (2017) introduces the use of a polyvinylidene difluoride (PVDF) membrane which allows for the direct optical detection of transferred protein without staining based on changes in its hydrophobic/hydrophilic properties. A wide variety of techniques can be used for visualization of labelled proteins and several innovations have been developed over the years. Ni et al (2016) discusses various ways to visualize the proteins, and provides a detailed protocol for implementation. One increasingly popular method involves imaging of multiple targets in a single gel using primary antibodies from multiple hosts and fluorescent secondaries. Berkelman (2020) provides a protocol for detecting and quantifying multiple protein targets without having to strip and re-probe the same membrane, greatly reducing potential issues with incomplete removal of primary and secondary antibodies.

Through this article collection, we hope to illustrate to the reader that western blotting is both a fundamental and constantly evolving technology. New techniques, from more effective sample preparation, to enhanced labelling, to automated processing, aim to elevate the quality and depth of western blot results.

By Jeremy Petravicz, PhD,
Senior Editor,
Current Protocols

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Received: 8 November 2020 | Accepted: 30 March 2021

DOI: 10.1002/bmb.21516

HISTORICAL REVIEW

Biochemistry and Molecular Biology Education WILEY

Applications of western blot technique: From bench to bedside

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Abstract

Western blot (WB) or immunoblot is a workhorse method. It is commonly used by biologists for study of different aspects of protein biomolecules. In addition, it has been widely used in disease diagnosis. Despite some limitations such as long time, different applications of WB have not been limited. In the present review, we have summarized scientific and clinical applications of WB. In addition, we described some new generation of WB techniques.

KEYWORDS

antibody, clinical application, protein immunoblot, scientific application, western blot

1 | INTRODUCTION

Western blotting (also known as protein immunoblot) is a gold standard method for identifying and quantifying a specific protein in a complex mixture extracted from cells or tissue lysate.[1,2] In brief, native or denatured proteins are separated by gel electrophoresis, then transfer to a protein binding membrane, after that a target protein is detected by a specific antibody[3] (Figure 1). Such as the other immunoblotting technique, antibodies play a key role in WB which can guarantee the accuracy, reproducibility and specificity of the method. Considering the importance of antibodies role, the International Working Group for Antibody Validation (IWGAV) was convened to improve standards for antibody use and validation.[4] Antibody reagent portal Antibodypedia (http://www.antibodypedia.com) have been scored over 19,000 human protein targets base on validation principles outlined by IWGA.[4,5] Human Protein Atlas is also applying recommendations from the International Working Group on Antibody Validation.[6] Although western blotting is a semiquatitative and error-prone technique. However, using various factors including; proper statistical design, normalization method, valid reference proteins, and

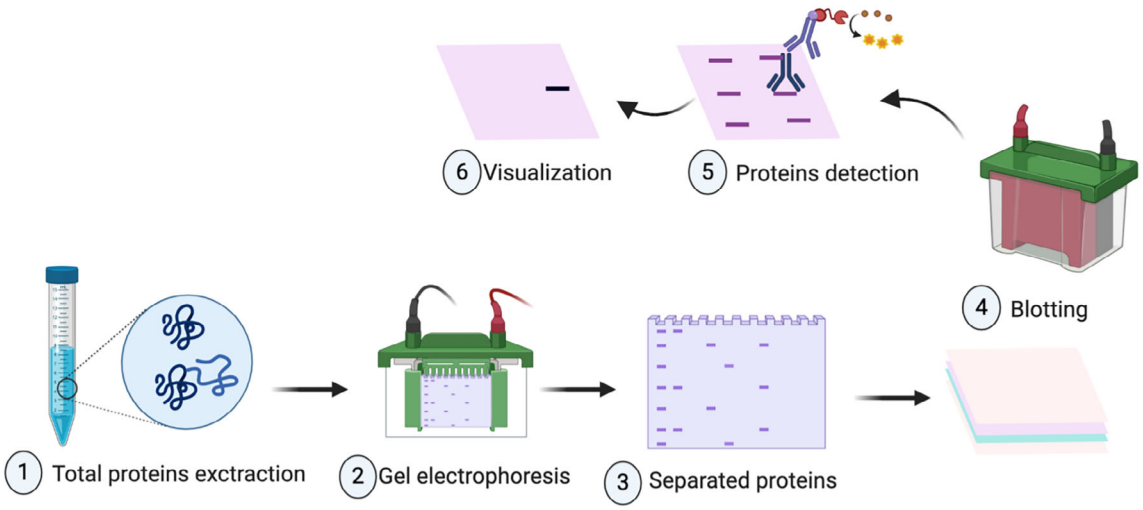

FIGURE 1 The workflow of western blot assay. Isolated proteins are first separated by size using gel electrophoresis (1 and 2). Separated proteins are then transferred to a nitrocellulose or PVDF blotting membrane (3). Blotted membrane is then incubated with primary and conjugated secondary antibodies (5). Finally, depending upon the used secondary antibody, the target protein is detected by colorimetric, fluorescent, or luminescent methods [Color figure can be viewed at wileyonlinelibrary.com]

selecting of valid antibodies, can decrease the systematic error which compromises the interpretation of results.[7,8] In addition, with the advancement in high technology, the next generation of WB techniques have been introduced which have been able to reduce the impact of bottlenecks of conventional WB and turn it into a powerful analytical tool in scientific and clinical laboratories worldwide.[9] In this review, we have described the clinical and scientific applications of WB.

2 | SCIENTIFIC APPLICATIONS OF WESTERN BLOT

Although the most common application of western blot is detecting the size and the amount of proteins in a given sample, WB has also appeared to be effective in study of other aspects of protein analyses. Here we discuss some the other applications of WB in scientific fields (Figure 2).

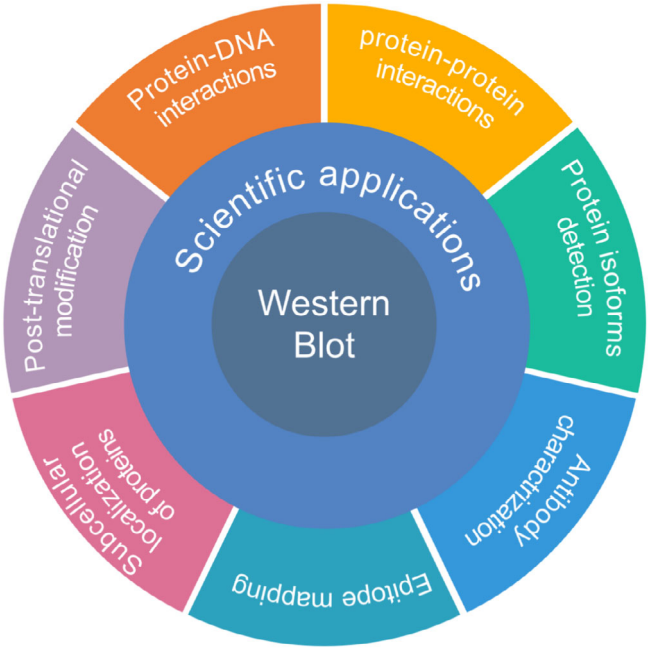

FIGURE 2 Different applications of western blot [Color figure can be viewed at wileyonlinelibrary.com]

2.1 | Detecting different isoforms of proteins

Alternative splicing is a posttranscriptional process in which a single coding gene produces multiple protein isoforms.[10] Due to having different functions in various biological and pathological processes, protein isoforms can be used as biomarkers and therapeutic targets.[11,12] Then, detection and quantification of specific protein isoforms can be a major in protein analyses field. Recently, advanced mass spectrometry (MS)-based method has been able to enhance accuracy and sensitivity of targeted protein isoforms analyses.[13] Regardless of which high-throughput technique is used, protein isoforms data must be confirmed and validated by a convectional antibody-based method, such as western blotting and ELISA.[14] Western blotting has enough potential to detect different isoforms of the same proteins with minimal difference in size.[15] Using monoclonal

antibodies for detecting the isoform specific epitopes could be a useful approach for detection of different protein isoforms.[16]

2.2 | Detecting protein–protein interactions

Proteins are functional and structural biomolecules which more than 80% of them do not operate alone but in complexes.[17] Protein–protein interactions (PPIs) are the basis of various critical cellular processes; including signal transduction, molecular transport, as well as various metabolic pathways.[18] Several in vivo and in vitro approaches have been developed to study of PPIs.[19] Far-western blotting is a molecular biological method which was derived from the standard WB method to detect direct PPIs in vitro.[20] In Far WB, nonantibody proteins are used to probe the protein(s) of interest on the blot. If blotted protein interacts with the probe, the spots are visualized in the membrane.[21,22]

2.3 | Detecting protein–DNA interactions

Proper regulation of gene expression plays an important role in human development and pathogenesis.[23] Transcription factors (TFs) are DNA-binding proteins which bind DNA in a sequence-specific manner and are considered as main components of gene expression regulation.[24] Then, identification of TFs can be a necessity in the study of gene regulation. Southwestern blotting (SWB) is a technique for rapid characterization of DNA-binding proteins and/or TFs. SWB assay is similar to conventional WB except for probing step in which blotted membrane is probed with radiolabeled predesigned DNA oligonucleotides. In final step, proteomics approaches are used to identify labeled proteins[25] (Figure 3).

2.4 | Detecting post-translational modification

Post-translational (PTM) modifications of proteins account for proteome expansion and diversity. PTMs play a key role in protein folding, targeting and subcellular localization. Many cellular processes, such as differentiation, protein degradation, signaling pathway, and protein–protein interaction, are affected by PTMs. Aberrant PTMs have been linked to numerous human diseases, highlighting the importance of PTMs study in health and disease states. Several methods have been developed to study of PTMs. Immunoprecipitation, western blot, Mass spectrometry, biochemical assay, proximity ligation assay are the main methods which have been used for investigation of PTMs. For identification, validation, and mechanistic characterization of a PTM, the use of more than one assay is recommended. For low throughput PTM analyses, western blot is a suitable technique because it needs no specialized expertise and tools. This method provides investigators capturing the low-abundance, endogenous and novel PTMs in combination with IP. It should be noted that the selection of a PTM specific antibody is considered to be a challenge in PTM detection through this way.

2.5 | Detecting proteins' subcellular localization

Proteins can perform their native functions when they are at the appropriate subcellular compartment.[26] Protein localization and function is correlated to each other. It means that when proteins change their locations, they may acquire new or different function.[26,27] Understanding the physiological function of a protein is not possible without knowledge of its subcellular localization. The study of protein compartment is a major challenge in cell biology, especially about multicompartmental and dynamic proteins.[28] Various approaches can be used to

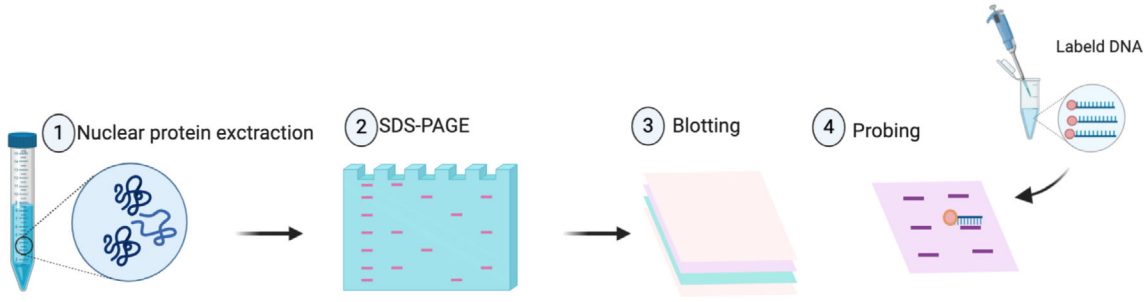

FIGURE 3 Southwestern blotting is a modified form of the WB for detection of transcription factors [Color figure can be viewed at wileyonlinelibrary.com]

identify the subcellular localization of a protein. Although flow cytometry and immunocytochemistry (ICC) via fluorescence microscopy are considered as a standard single cell immunoassays for subcellular localization studies, false positive signals due to antibody cross reactivity have remained as inevitable trouble.[29] The best methods are immunogold transmission electron microscopy (TEM) with specific antibodies and fluorescent fusion with the target protein. The most important advantage of these methods is visualization of protein co-localizations.[30] Due to the possibility of high-throughput analyses, MS-based approaches have also been at the center of attention for study of protein localization.[31] Considering the demand for specialized personnel and expensive equipment for the mentioned methods, western blotting is still major methods for protein localization. Western-blot is routinely used for this purpose alone or following the high-throughput technique for further confirmation.[3] Validation of antibody specificity and need to pre sub-cellular fractionation is the major bottlenecks of this WB application. In addition to conventional WB, single-cell western blotting (scWestern) is considered as a revolution in protein subcellular localization studies as well single-cell protein analyses. WB has enough potential to mitigate antibody probe cross-reactivity as major drawbacks of other single-cell assays such as ICC. In scWestern, specificity and selectivity of western blotting have been brought to single-cell-protein analyses.[32] The protein assay can be considered as a high-throughput technique in which an array of polyacrylamide (PA) microwell on a microdevice supports thousands of concurrent single-cell western blots.[32] Each run of scWestern consists of five steps (Figure 4). First, cells are seated in microwells and then, the cells are lysed chemically. After PAGE of each single-cell lysate, proteins are blotted to the gel by applying UV light. Finally, antibody probing is performed.[29,33,34] Zhang and coworkers showed the increased phosphorylation of MAP kinases (ERK1/2, p38) under hypertonic conditions on with the help of modified scWB.[35]

2.6 | Antibody development and characterization, epitope mapping

Antibodies are the workhorse molecules of the biomedical research, immunotherapy and disease diagnosis, and then high quality antibodies are essential for reliable, consistent, and reproducible results.[36] New produced antibodies must be confirmed, validated, and characterized. Validation of antibodies is considered as a main concern for research and clinical laboratory communities.[4,37] European Monoclonal Antibody Network (EuroMAbNet),[37] the International Working Group on Antibody Validation (IWGAV)[5] and the Global Biological Standards Institute (GBSI) is tree initiatives which have presented standard guidelines for antibody validation. "Alpbach recommendations" is also useful for improving the quality of new produced antibodies.[4] Epitope mapping is at the center of practice for antibodies characterization and validation. Epitope mapping is described as identifying the binding sites (Epitopes) of an antibody to its target protein. Understanding the binding epitope of an antibody is the key to the development and discovery of new vaccines, therapeutics and diagnostics.[38,39] Thus, a variety of techniques have been developed for antibody epitope mapping. X-ray co-crystallography has been considered as the gold standard for direct visualization of antibody: antigen complex. In spite of providing high-resolution maps of antibody: antigen complex, this

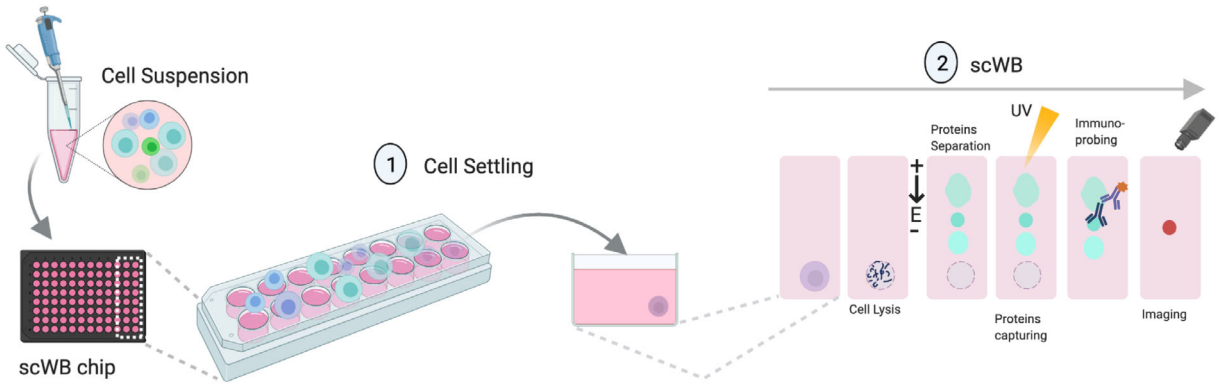

FIGURE 4 The workflow of scWB assay. Cell suspension is applied to the surface of scWestern array which consists of thousands of microwells. Cells are settled on microwells by gravity (1). Each microwells are coated with a thin photoactive polyacrylamide (PA) gel after cell trapping, a modified version of the western blot assay is performed in each microwell. this modified version has five steps, including: Cell lysis, electrophoresis and protein separation, the proteins capering, immuno-probing and imaging (2) [Color figure can be viewed at wileyonlinelibrary.com]

technique is not always feasible due to being time-consuming, expensive and not being applicable for all complexes. Other high-throughput and low-throughput methods are available for epitope mapping, such as phage display, site directed mutagenesis mapping, array-based oligo-peptide scanning, which have been strengthened by computational algorithm and bioinformatics sciences.[40–42] Specificity is the main characteristics of WB, which has turned it to a powerful method among the other low-throughput techniques for epitope mapping.[39] Generally, similar amounts of different-sized fragments of the desired antigen are blotted onto the membrane, and then specific antibody is added. The specific epitope(s) are detected by HRP-conjugated secondary antibody.[43–45]

3 | CLINICAL APPLICATIONS OF WB

Due to direct protein detection, western blot is considered as *a powerful diagnostic technique* which is routinely used in the clinic. Given that WB is not a quantitative method, the clinical application aids in confirmatory diagnosis of diseases.[9] In this section, we describe some of the most important clinical applications of WB.

3.1 | Diagnosis of infectious diseases

Based on the Febig staging system, there are six distinct stages for initial HIV infection detection. Full western blot reactivity is considered as stage 6 of this traditional system.[46] Commercial western blot kits that has been designed based on this system detected the antibodies against HIV proteins.[47] The package of each kit contains some nitrocellulose membrane strips on which viral proteins have been transferred and immobilized. Each strip is incubated with HIV antibodies present in the patient serum or other body fluids such as saliva and urine based on kit protocol. Based on published Centers for Disease Control and Prevention (CDC) and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) criteria, a positive Western blot indicates the presence of at least two of the following bands: p24, gp41, and gp120/160 and a negative Western blot is defined by the absence of any bands .[48] Because of WB indeterminate profile frequency, interpretation of reports has been problematic. Although choice of commercial kit is a paramount important, Western blot is going to be eliminated from the new testing sequence algorithm.[48] In addition, there are studies that still indicate the performance of WB for HIV diagnosis and confirmation.[49,50] Herpes simplex virus and parvovirus are also diagnosed with the aid of Western blot assay kits. Application of WB assay as a confirmatory test for Hepatitis B has also been reported.[51] Western blotting is also used for the clinical diagnosis of nonviral infectious disease including, bacterial, parasitic and fungal diseases which have been summarized in the Table 1.

3.2 | Diagnosis of noninfectious diseases

It is obvious that specific protein or specific antibody as disease biomarker(s) can be detected by protein detection assays and western blot is considered an available technique which used for detection of specific antibodies and/or proteins during diagnosis of disease, such as autoimmune disease, cancer and prion diseases.[52] Western blot can be used for detection of autoantibodies in some autoimmune diseases which the most important of them have been listed in Table 2. Antibodies concentration and cross-reacting antibodies are among the main problematic issues which can limit the use of WB as a routine technique for autoantibodies detection. Further purification of antigens, using of recombinant antigen and G protein-coupled HRP can be solutions for some of these problems.[47] Despite these solutions, WB has been not able to replace ELISA methods in detecting autoantibodies in patient sera. Not recognizing of denatured and immobilized antigens presented on immunoblots by all antibodies can be considered as the main limitation of WB.[53–55] High abundant protein in sera can be a limitation.[56] Recently, Walpurgis and coworkers developed complementary assays for the detection of myostatin-neutralizing antibody Domagrozumab in doping control serum samples of supporters by using ammonium sulfate precipitation and immunoaffinity purification either in combination with tryptic digestion and LC-HRMS or Western blotting.[57] Due to the ability to detect different isoforms of proteins, WB can be a suitable choice for diagnosis of prion and protein isoforms related diseases such as different cancers and the other diseases.[58,59] For example, Creutzfeld–Jacob disease can be diagnosed by assess of isoform pattern of 14-3-3 proteins in the cerebrospinal fluid, using Western blot analysis.[60] Duncombe et al. used a modified form of single cell western blot for analysis of oncoprotein-related signaling in human breast biopsy.[61] Andersen and coworkers used Differential-In-Gel-Electrophoresis (DIGE) coupled with Western blotting to quantify and validate the increase of medium and low abundance proteins in ovarian cancer sera.[62] Farmer's lung disease (FLD) is considered a pulmonary disorder caused by inhalation of antigenic particles which some studies have been confirmed that WB can be a suitable choice for detection of FLD-associated immunoreactive proteins.[63]

TABLE 1 The list of nonviral infectious disease for which western blot is used for diagnosis

Infectious group	Infectious agent	Disease	Comment
Bacteria	Borrelia burgdorferi	Lyme disease	Western blot can be a supplementary and/or confirmatory test for confirming the ELISA results.[64] WB with Penalized linear discriminant analysis (PLDA) have potential to differentiate patients with early and late Lyme disease.[65]
	Ehrlichia. Phagocytoph,E. ewingii	Ehrlichiosis	Western immunoblot analysis using rP44 and rP30 may be useful in discriminating dually human monocytic and granulocytic ehrlichiosis.[66]
	Mycobacterium tuberculosis (MTB)	Tuberculous meningitis	Western blot showed more specificity than ELISA fore detection of antibodies.[67,68]
	Yersinia enterocolitica	Yersiniosis	Western blot could be an acceptable replacement of the complement fixation (CF) assay.[69]
	Helicobacter pylori	Peptic ulcers	Immunoblot have shown higher high sensitivity, specificity and accuracy compared to the PCR.[70]
	Treponema pallidum	Congenital Syphilis	The comparative IgG WB test can be an addition to the conventional laboratory methods used for CS diagnosis.[71] WB can be useful additional confirmatory test or alternative to the fluorescent treponemal antibody absorption (FTA-ABS).[72]
Parasite & fungi	Toxoplasma	Toxoplasmosis	Diagnose of congenital toxoplasmosis in cases in which the infection had not been detected by classical serology techniques has been possible.[73] The IgG-WB with high sensitivity can be used for the diagnosis of congenital toxoplasmosis in association with other congenital infection markers.[74]
	Aspergillus	Aspergillosis	Western blot detection enhanced standardization and a higher sensitivity than with Immunoprecipitin detection (IPD).[75]
	Taenia solium	Cysticercosis	The superiority of the method base on immunoelectrotransfer blot over ELISA for the diagnosis of Cysticercosis in human has been shown.[76]
	Babesia microti	Babesiosis	Biomarkers for disease progression were identified using western blot analyses.[77,78]
	Echinococcus granulosus	Cystic echinocccosis (CE)/ Hydatid disease	Western blot might be useful in the diagnosis, efficiacy of medical treatmen and post-surgical monitoring of CE patients.[79,80]
	Toxocara canis	Toxocariasis visceral larva migrans (VLM)	Obligatory verification of all ELISA IgG positive and questionable results by WB was suggested.[81]
	Baylisascaris procyonis	larva migrans	In combination with ELISA, WB could be an efficient tool for diagnosis and differentiation of larva migrans caused by Baylisascaris procyonis and Toxocara species in human.[82]
	Paracoccidioides brasiliensis	Paracoccidioidomycosis (PCM)	Western blot has enough sensitivity to be included among routine laboratory assays as a safe method for PCM diagnosis.[83,84]

TABLE 2 Autoantibodies and related autoimmune disease which can be detected by western blot

Disease	Autoantibodies
systemic lupus erythematosus (SLE)	Anti-double- strand DNA (*Anti-dsDNA)* antibodies, anti-SmD1 antibodies.[85]
mixed connective tissue disease (MCTD)	Anti-Ui-nuclear riboprobtein antibodies, anti-SS-A/Ro and anti-SS-B/RoLa antibodies.[85]
Sjogren's (SHOW-grins) syndrome	Anti-SSA autoantibodies (anti–Sjögren's-syndrome-related antigen a autoantibodies.[85]
Systemic sclerosis (SSc)	Antifibrillarin antibodies.[86]
Paraneoplastic neurological syndromes (PNS)	Anti-nuclear antibody test (ANA), Anti-Purkinje cell antibody, Hu antibody.[87,88]
Myositis	Anti-nuclear antibody test (ANA), ENA (extractable nuclear antigens).[89]

4 | CONCLUSION

Since western blot has been developed, it has been able to adopt by biology labs and turns to one of the most important techniques in research and clinical applications. Despite some technical limitation related to performance and sensitivity, further advancements can improve sensitivity and reproducibility of the WB. Although WB is more powerful technique in research, application rather than clinical application, with the aim of other tools and new advancement, clinical applications of the western blotting technique will continue to progress.

ACKNOWLEDGMENT

This work was partially supported by Biochemistry Department of Baqiyatallah University of Medical Sciences.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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Immunoblotting and Immunodetection

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Immunoblotting (western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. This unit provides protocols for all steps, starting with solubilization of the protein samples, usually by means of SDS and reducing agents. Following solubilization, the material is separated by SDS-PAGE and the antigens are electrophoretically transferred to a membrane, a process that can be monitored by reversible staining with Ponceau S. The transferred proteins are bound to the surface of the membrane, providing access to immunodetection reagents. After nonspecific binding sites are blocked, the membrane is probed with the primary antibody and washed. The antibody-antigen complexes are tagged with fluorophores, horseradish peroxidase, or alkaline phosphatase coupled to a secondary anti-IgG antibody, and detected using appropriate fluorescent imaging technologies or with chromogenic or luminescent substrates. Finally, membranes may be stripped and reprobed.

Keywords: immunoblot • western blot • horseradish peroxidase • alkaline phosphatase • antibodies

INTRODUCTION

Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). Following solubilization, the material is separated by SDS-PAGE [*UNIT 10.1* (Gallagher, 2012)], The antigens are then electrophoretically transferred to a nitrocellulose, PVDF, or nylon membrane in a tank (see Basic Protocol 1) or semidry (see Alternate Protocol 1) transfer apparatus, or a dot or slot blotting apparatus (see Alternate Protocol 2). The transfer of proteins to the membrane can be monitored qualitatively or quantitatively by staining with Ponceau S (see Support Protocols 1 and 2). Proteins may also be transferred from previously stained gels (see Alternate Protocol 3).

The transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining nonspecific binding sites are blocked by immersing the membrane in a solution containing a protein or detergent blocking agent. After probing with the primary antibody, the membrane is washed and the antibody-antigen complexes are identified with a secondary anti-IgG antibody coupled to a horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzyme. The enzymes can be attached directly (see Basic Protocol 2) or via an avidin-biotin bridge (see Alternate

Current Protocols in Protein Science 10.10.1-10.10.37, April 2017
Published online April 2017 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpps.32

Protocol 4) for increased sensitivity. Chromogenic or luminescent substrates (see Basic Protocol 3 and Alternate Protocols 5 and 6) are then used to visualize the enzyme activity. Finally, membranes may be stripped and reprobed (see Support Protocol 3).

NOTE: A video demonstration of this technique can be found at *http://www.jove.com/index/Details.stp?ID=759*.

BASIC PROTOCOL 1

PROTEIN BLOTTING WITH TANK TRANSFER SYSTEMS

In this procedure, blotting is performed in a tank of buffer with the gel in a vertical orientation, completely submerged between two large electrode panels. In some systems, up to four gels can be transferred at one time. For difficult-to-transfer proteins (>100 kDa or hydrophobic; e.g., myosin), tank blotting is preferable to semidry systems because prolonged transfers are possible without buffer depletion. However, transfers >1 hr at high power require cooling using a heat exchanger and a circulating water bath that can maintain a constant transfer temperature of 10° to 20°C.

Materials

Samples for analysis
Protein molecular weight standards (Table 10.10.1; *UNIT 10.1*; Gallagher, 2012): prestained (Sigma or Bio-Rad), biotinylated (Vector Labs or Sigma), fluorescent (e.g., BenchMark Fluorescent Protein Standards; Invitrogen), or compatible with other colorimetric and fluorescent detection methods (e.g., MagicMark and MagicMark XP Western Protein Standards; Invitrogen)
Transfer buffer (see recipe)

Powder-free gloves
Scotch-Brite pads (3M) or equivalent sponge
Whatman 3MM filter paper or equivalent
Transfer membrane: 0.45-μm nitrocellulose (Millipore or Schleicher & Schuell), PVDF (Millipore Immobilon P), neutral nylon (Pall Biodyne A), *or* positively charged nylon (Pall Biodyne B; BioRad Zetabind)
Electroblotting apparatus (BioRad, Invitrogen, Amersham, or Hoefer; e.g., Figs 10.10.1 and 10.10.2)
Indelible pen (e.g., Paper-Mate) or soft lead pencil

Additional reagents and equipment for one- and two-dimensional gel electrophoresis [*UNIT 10.1* (Gallagher, 2012) and *UNIT 10.4* (Harper et al., 1998)] and staining proteins in gels [*UNIT 10.5* (Echan and Speicher, 2002) and *UNIT 10.6* (Joo and Speicher, 2007)] and on membranes [see Support Protocol 1 and *UNIT 10.8* (Goldman et al., 2016)]

Table 10.10.1 Protein Standards for Immunoblotting

Protein standard	Application
Unstained	Molecular weight calibration and transfer efficiency; can be visualized with total protein stains
Tagged	Molecular weight calibration and transfer efficiency; visualized during immunodetection steps; a variety of potential tags, including biotinylated and antibody-specific amino acid sequence engineered into standard proteins
Prestained	Excellent for checking transfer efficiency and visually inspecting the blot; typically does not produce as sharp a band as other standards, making precise molecular weight calculations difficult. Both colored and fluorescent tags are available.

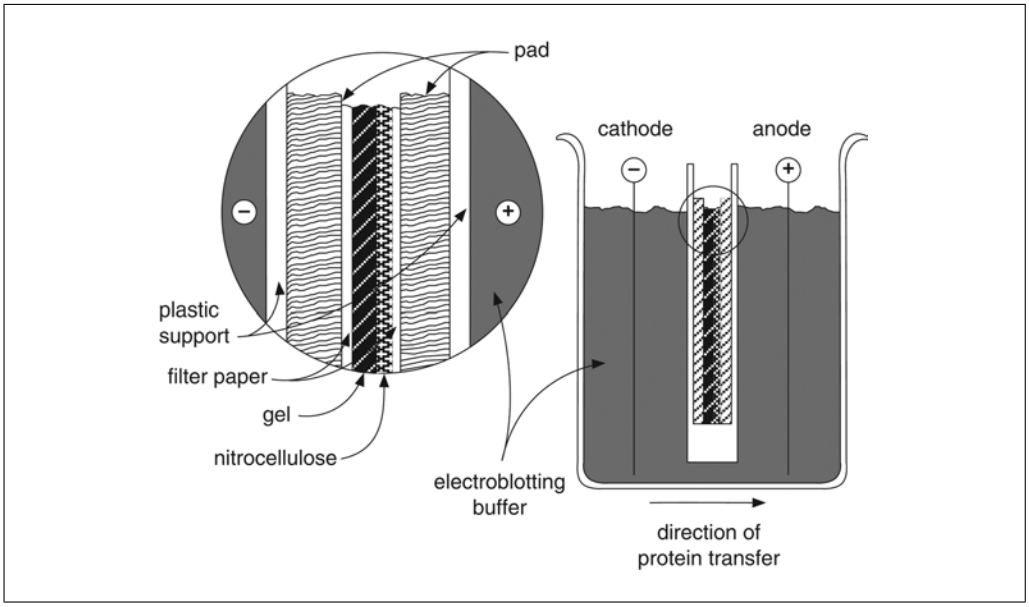

Figure 10.10.1 Immunoblotting with a tank blotting unit. The polyacrylamide gel containing the protein is laid on a sheet of filter paper. The uncovered side of the gel is overlaid with a sheet of membrane precut to the size of the gel plus 1 to 2 mm on each edge, then this membrane is overlaid with another sheet of filter paper. The filter paper containing the gel and membrane is sandwiched between Scotch-Brite pads. This sandwich is placed in a plastic support, and the entire assembly is placed in a tank containing transfer buffer. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane. Transfer is achieved by applying a voltage of 100 V for 1 to 2 hr (with cooling) or 14 V overnight.

NOTE: Deionized, distilled water should be used throughout this protocol.

Electrophorese the protein sample

1. Prepare antigenic samples and separate proteins using small or standard-sized one- or two-dimensional gels [*UNIT 10.1* (Gallagher, 2012), *UNIT 10.4* (Harper et al., 1998)]. Include prestained or biotinylated protein molecular weight standards in one or more gel lanes.

   The protein markers will transfer to the membrane and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

   MagicMark Western Protein Standards (Invitrogen) allow direct visualization of protein size standards on membrane blots without the need for protein modification or special detection reagents. The standard proteins are derived from E. coli cells containing a construct with repetitive units of a fusion protein forming the size variation and an IgG binding site. The proteins can be visualized with colorimetric, chemiluminescent, or fluorescent detection simply by probing the membrane for the specific protein. The IgG binding site will allow all the standards to react with the specific primary and secondary antibodies. The standards are in a ready-to-use format and do not have to be heated or reduced. Alternatively, BenchMark Fluorescent Protein Standards (Invitrogen) are visualized directly via UV transillumination on a UV transilluminator (UVP, LLC) when wet, or via overhead UV illumination (apparatus also available from UVP, LLC) when dry.

   A variety of gel sizes and percentages of acrylamide can be used (UNIT 10.1; Gallagher, 2012). Most routinely used are either 14 cm × 14 cm × 0.75–mm gels or 8 cm × 10 cm × 0.75–mm minigels. Acrylamide concentrations vary from 5% to 20%, but are usually in the 10% to 15% range.

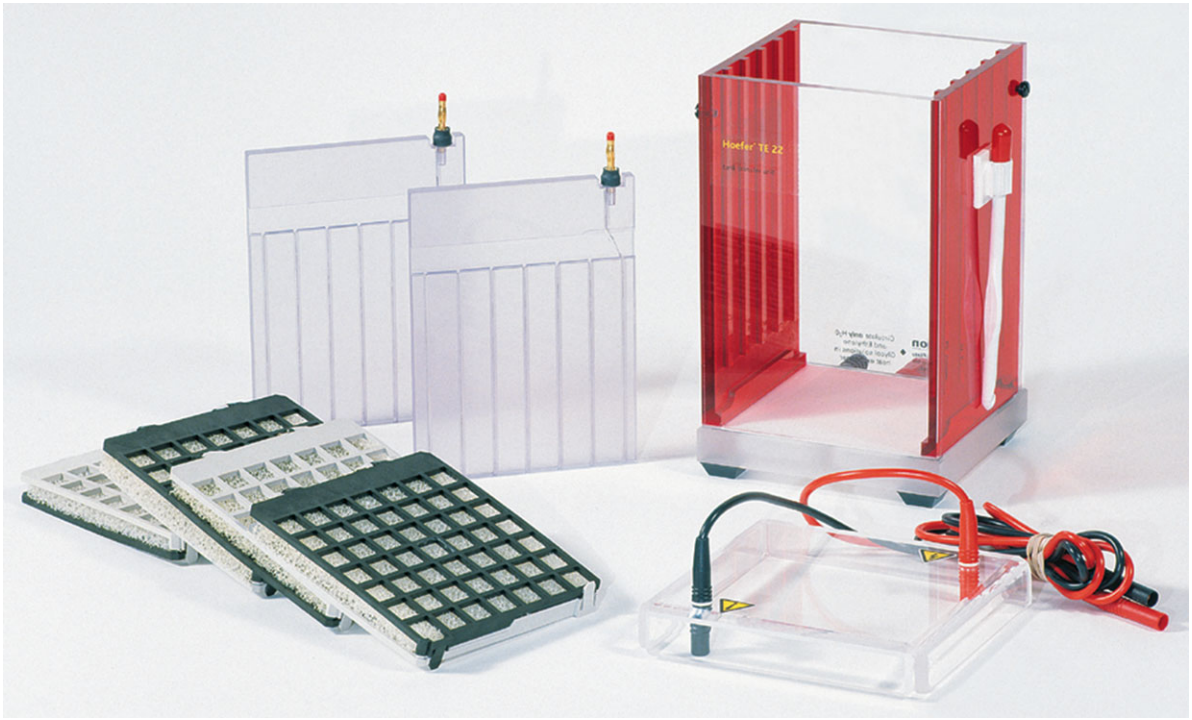

Figure 10.10.2 Minigel tank electrotransfer unit. Designed for smaller (8 × 10–cm) gels, these units will process four gels at a time. Note that the two outside panels hold the electrode grid.

Assemble the immunoblot sandwich

2. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Equilibrate gel 30 min at room temperature in transfer buffer.

   IMPORTANT NOTE: *Use gloves when manipulating filter papers, gels, and membranes. Oil from hands blocks the transfer.*

   Match the appropriate transfer buffer to the membrane (see Reagents and Solutions).

   Gel equilibration is required to prevent a change in the size of the gel during transfer. Any shift in gel dimension will result in a blurred transfer pattern.

3. Assemble transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with transfer buffer so that cassette is covered.

   The transfer cassette should be assembled under buffer to minimize trapping of air bubbles. Use Figure 10.10.1 as a guide to assembly.

4. On bottom half of plastic transfer cassette, place Scotch-Brite pad or sponge, followed by a sheet of filter paper cut to same size as gel, and prewet with transfer buffer.

5. Place gel on top of filter paper; the side of the gel touching the paper arbitrarily becomes the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank). Remove any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over surface of gel.

   Any bubbles between the filter paper, gel, and membrane will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

Proteins are usually negatively charged in transfer buffer and move toward the positive anode. However, some proteins may be positively charged. An additional membrane placed on the cathode side of the gel will bind these proteins.

6. Prepare transfer membrane. Cut membrane to same size as gel plus 1 to 2 mm on each edge. Place into distilled water slowly, with one edge at a 45° angle, so that the water wicks up into the membrane, wetting the entire surface. Equilibrate 10 to 15 min in transfer buffer.

   If the membrane is placed into the water too quickly, air will be trapped and will appear as white blotches in the membrane. Protein will not transfer onto these areas.

   This wetting procedure works for nitrocellulose and nylon membranes only. PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer. For these membranes, first immerse 1 to 2 sec in 100% methanol, then equilibrate 10 to 15 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet once again with methanol and transfer buffer as described above.

7. Moisten surface of gel with transfer buffer. Place prewetted membrane directly on top side of gel (i.e., anode side). To avoid bubbles, start by placing one corner of the membrane on the gel and slowly lower the rest of the membrane onto the gel. Remove all air bubbles as in step 5.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

   The use of 0.2-μm membranes may improve retention of smaller-molecular-weight proteins.

8. Wet another piece of Whatman 3MM filter paper, place on anode side of membrane, and remove all air bubbles. Place another Scotch-Brite pad or sponge on top of this filter paper.

9. Complete assembly by locking the top half of the transfer cassette into place (Fig. 10.10.1).

   It is important to orient the sandwich so that the membrane faces the anode (positively charged) side of the tank.

Transfer proteins from gel to membrane

10. Fill tank with transfer buffer and place transfer cassette containing sandwich into electroblotting apparatus in correct orientation. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus.

    Transfer buffer should cover the electrode panels but should not touch the base of the banana plug.

11. Electrophoretically transfer proteins from gel to membrane for 30 min to 1 hr at 100 V with cooling or overnight at 14 V (constant voltage) in a cold room.

    Transfer time is dependent on the thickness and the percent acrylamide of the gel, as well as the size of the protein being transferred. In general, proteins are transferred within 1 to 6 hr, but high-molecular-weight molecules may take longer. Overnight transfer at low voltage is reliable and convenient. Cooling (at 10° to 20°C) is required for transfers >1 hr at high power. Heat exchanger cooling cores using a circulating water bath are placed into the transfer unit for cooling.

12. Turn off the power supply and disassemble the apparatus. Remove membrane from blotting apparatus and note orientation by cutting a corner or marking with a soft lead pencil or Paper-Mate pen.

Many ballpoint inks come off, but Paper-Mate stays on the membrane.

Membranes can be dried and stored in resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol.

13. Stain gel for total protein with Coomassie blue (*UNIT 10.5*; Echan and Speicher, 2002) to verify transfer efficiency. If desired, visualize transferred proteins on nitrocellulose or PVDF membranes by staining reversibly with Ponceau S (see Support Protocol 1), or irreversibly with Coomassie blue, India ink, naphthol blue, or colloidal gold (*UNIT 10.8*; Goldman et al., 2016).

    These staining procedures are incompatible with nylon membranes.

    If membrane shows significant staining on the back side, either the gel was heavily overloaded or the membrane has poor protein-binding capacity (see Troubleshooting). In either case, protein-binding sites on the side facing the gel are saturated, allowing protein to migrate to the other side of the membrane. Nitrocellulose, in particular, will show diminished binding capacity with age or poor storage conditions (e.g., high temperature and humidity). In addition, some proteins simply do not bind well to a particular matrix. By using several membrane sheets in place of one, the protein can be detected as it passes through each consecutive sheet. This will give an indication of how efficiently the membrane binds to a particular protein.

14. Proceed with immunoprobing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 4 and 6).

ALTERNATE PROTOCOL 1

PROTEIN BLOTTING WITH SEMIDRY SYSTEMS

Even and efficient transfer of most proteins is also possible with semidry blotting, a convenient alternative to tank transfer systems. Instead of being placed vertically into a tank filled with transfer buffer, the gel is held horizontally between buffer-saturated blotting paper that is in contact with the electrodes (Fig. 10.10.3), greatly reducing the amount of buffer required. The electrodes are close together, giving high field strengths and rapid transfer with a standard electrophoresis power supply. Prolonged transfers (>1 hr) are not recommended; tank blotting (see Basic Protocol 1) should be used for proteins that require long blotting times for efficient transfer.

Additional Materials *(also see Basic Protocol 1)*

Six sheets of Whatman 3MM filter paper or equivalent, cut to size of gel and saturated with transfer buffer
Semidry transfer unit (Hoefer, Bio-Rad, or Invitrogen)

Assemble the immunoblot sandwich

1. Prepare samples and separate proteins using small or standard-sized one- or two-dimensional gels [*UNIT 10.1* (Gallagher, 2012), *UNIT 10.4* (Harper et al., 1998)].

    Because transfer efficiency depends on many factors (e.g., gel concentration and thickness, protein size, shape, and net charge), results may vary. Below is a guideline for 0.75-mm-thick SDS-PAGE gels transferred by semidry blotting.

Percent acrylamide (resolving gel)	Size range transferred (~100% efficiency)
5-7	29–150 kD
8-10	14–66 kD
13-15	<36 kD
18-20	<20 kD

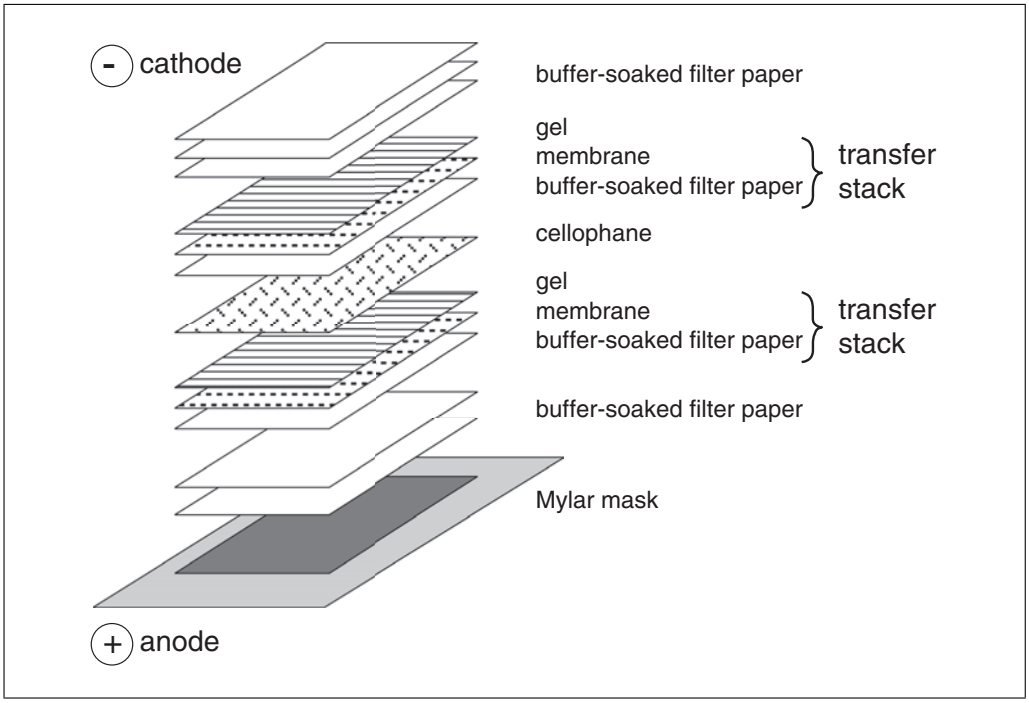

Figure 10.10.3 Immunoblotting with a semidry transfer unit. Generally, the lower electrode is the anode, and one gel is transferred at a time. A Mylar mask (optional in some units) is put in place on the anode. This is followed by three sheets of transfer buffer–soaked filter paper, the membrane, the gel, and finally, three more sheets of buffer-soaked filter paper. To transfer multiple gels, construct transfer stacks as illustrated, and separate with a sheet of porous cellophane. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Transfer is achieved by applying a maximum current of 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm), this means 60 and 200 mA, respectively.

2. Prepare transfer membrane (see Basic Protocol 1, step 6).
3. Disassemble gel sandwich. Remove and discard stacking gel.

   Equilibration of the separating gel with transfer buffer is not normally required for semidry blotting, but it may improve transfer in some cases.

4. Place three sheets of filter paper saturated with transfer buffer on the anode (Fig. 10.10.3).

   Most transfer units are designed so that negatively charged proteins move downward toward either a platinum or graphite positive electrode (anode).

   CAPS transfer buffer, pH 10.5 (see recipe for transfer buffer) can be used in place of the Tris/glycine/methanol transfer buffer of Basic Protocol 1. CAPS buffer should be used if the protein is to be sequenced right on the membrane as glycine will interfere with this procedure.

   The filter paper should be cut to the exact size of the gel. This forces the current to flow only through the gel and not through overlapping filter paper. Some manufacturers (e.g., Hoefer) recommend placing a Mylar mask on the lower platinum anode. With an opening that is slightly less than the size of the gel, the mask forces the current to flow through the gel and not the surrounding electrode area during transfer.

5. Place equilibrated transfer membrane on top of filter paper stack. Remove all bubbles between membrane and filter paper by rolling a test tube over surface of membrane.

Any bubbles in the filter paper stack or between the filter paper, membrane, and gel will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

6. Place gel on top of membrane. Gently roll a test tube over surface of gel to insure intimate contact between gel and membrane and to remove any interfering bubbles.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

7. Complete the transfer stack by putting the three remaining sheets of filter paper on top of gel. Roll out bubbles as described above.

   Multiple gels can be transferred at the same time by simply placing a sheet of porous cellophane (Hoefer) or dialysis membrane (BioRad) equilibrated with transfer buffer between the transfer stacks (Fig. 10.10.3). Transfer efficiency is dependent on the position of the transfer stack in the blotting unit, with the most efficient transfer occurring in the stack closest to the anode. For critical applications, it is best to transfer one gel at a time.

Transfer proteins from gel to membrane

8. Place top electrode onto transfer stack.

   Most units have safety-interlock features and can only be assembled one way. Consult manufacturer's instructions for details.

   Once the transfer stack has been assembled with both electrodes, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane, distorting the transfer pattern.

9. Carefully connect high-voltage leads to the power supply [see *UNIT 10.1* (Gallagher, 2012) for safety precautions]. Apply constant current to initiate protein transfer.

   Transfers of 1 hr are generally sufficient.

   In general, do not exceed 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm), this means ~60 and 200 mA, respectively.

   Monitor the temperature of the transfer unit directly above the gel by touch. The unit should not exceed 45°C. If the outside of the unit is warm, too much current is being applied. Note that units with graphite electrodes are more prone to heating, because graphite has much more resistance to current flow than platinum or steel electrodes.

10. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack, marking orientation as in step 12 of Basic Protocol 1. Proceed with staining and immunoprobing (see Basic Protocol 1, steps 13 and 14).

ALTERNATE PROTOCOL 2

RAPID WESTERN TRANSFER WITH iBlot DRY BLOTTING SYSTEM

An extension of the semidry concept presented in Alternate Protocol 1 is the new generation of fast semi-dry blotters. Using specialized buffers and integrated programmable power supplies, several dedicated dry/semi-dry transfer systems can transfer 10- to 220-kDa proteins from polyacrylamide gels to nitrocellulose or PVDF membranes in 3 to 12 min (Table 10.10.2). Among these rapid transfer systems, the iBlot Dry Blotting system from Thermo Fisher Scientific/Life Technologies is a dry transfer system that only supports rapid transfer processes; all others are semi-dry systems that are effective for both rapid (3- to 12-min) transfers or standard transfer protocols (30 to 60 min). Most of these systems require special kits (blotting paper, nitrocellulose, or PVDF membranes are included) from the same company to perform the rapid process. An exception is the

Table 10.10.2 Representative Rapid Transfer Systems

Name	Manufacturer	Protein mol. wt. range (kDa)	Transfer time (min)	Web site
Trans-blot Turbo system	Bio-Rad	5-300	3-10	*http://www.bio-rad.com/en-us/product/semi-dry-rapid-blotting-systems/trans-blot-turbo-transfer-system*
Pierce Power Blotter	Thermo Fisher Scientific	10-300	<10	*http://www.piercenet.com/product/pierce-power-blotter*
iBlot Dry Blotting system	Thermo Fisher Scientific	11-220	7-10	*https://www.thermofisher.com/order/catalog/product/IB21001?ICID=cvc-Western-Blot-Transfer-c4t1*

Figure 10.10.4 Top view of the iBlot Gel Transfer Device.

Pierce Power Blotter system, which only requires the Pierce 1-Step Transfer Buffer from the company.

Here we use iBlot Dry Blotting system and one minigel as an example to introduce the rapid transfer process for western blot.

Refer to the user manual for optimization conditions before proceeding. Pre-treatment of the gel after electrophoresis is generally not required, but transfer is improved for proteins >150 kDa by equilibration of the gel in 20% ethanol for 5 to 10 min prior to the transfer.

Materials

Pre-run gel containing protein samples and protein standard
iBlot transfer device (Thermo Fisher; see Fig. 10.10.4)
Blotting roller (included in the iBlot transfer device package)
Gel Transfer Stacks Mini (including both bottom stack, top stack and sponge) for blotting one minigel

Forceps

Additional reagents and equipment for visualization with chromogenic (Basic Protocol 3), chemiluminescent (Alternate Protocol 6), or fluorescent substrates (Alternate Protocol 8), and signal amplification with avidin-biotin visualization reagents (Alternate Protocol 7)

1. Place the iBlot Gel Transfer device on a stable flat surface, plug in the power cord, and turn on the power switch.
2. Open the lid of the iBlot Gel Transfer device (Fig. 10.10.4).
3. Remove the sealing from the disposable sponge and place it on the inside of the lid. Make sure the metal contact is in the upper right corner of the lid.
4. Remove the sealing from the iBlot Gel Transfer bottom stack. Keep the stack in the red plastic tray and place the anode stack onto the blotting surface of the transfer device with the tray tab facing toward the right.
5. Carefully place the pre-run gel on the transfer membrane. Ensure that the gel does not protrude over the edges of the membrane.
6. Remove the bubbles using the blotting roller.
7. Remove the sealing from the iBlot Gel Transfer top stack and discard the red plastic tray.
8. Place the top stack, with the gel facing down, onto the pre-run gel.
9. Remove the bubbles by the blotting roller.
10. Close the lid of the device and secure the latch.
11. Select the appropriate program and run time by press the "Select" button. Use the "Up/Down (±)" buttons to change the program.

    There are a variety of programs stored in the iBlot transfer system. Check the manufacturer's instruction for detail.

12. Press the "Start/Stop" button in the right lower corner.

    The red light turns to green, indicating the start of the run. At the end of the transfer run, the device will automatically shut off and begin beeping for 1 min.

13. Press the Start/Stop button to silence beeping.
14. Open the lid of the iBlot device.

    The stacks are warm/hot and the total thickness of the stacks reduces

15. Remove the top stack and gel using forceps.
16. Remove the transfer membrane from the stack and proceed with the blocking procedure, or stain the membrane.

    Sometimes there is green staining on the membrane. The stain will reduce by washing the membrane several times.

17. Process membrane according to immunoprobing protocols for chromogenic (Basic Protocol 3), chemiluminescent (Alternate Protocol 6), or fluorescent visualization (Alternate Protocol 8)

    See, e.g., Fig. 10.10.5, using Alternate Protocol 7.

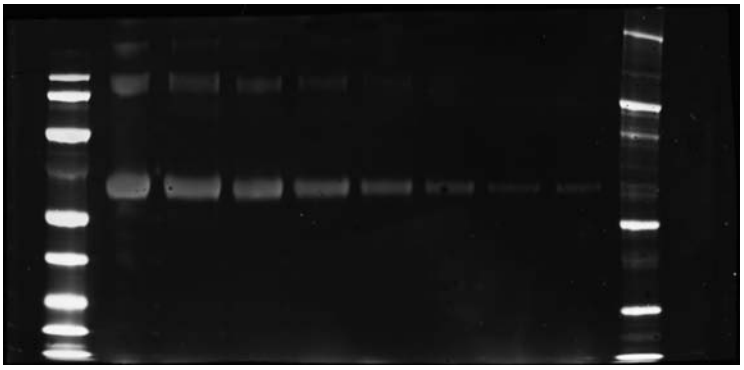

Figure 10.10.5 Sample western blotting results by using iBlot Gel Transfer Device. BSA solutions (start from 1 μg, two-fold series dilution) were loaded. BSA protein was detected by anti-BSA antibody (primary antibody) and IRDye 680RD (secondary antibody). UVP BioSpectrum system was used for image collection.

18. Continue with another run (no cool-down time needed) or turn off the iBlot power switch.
19. Clean the blotting surface and lid with a clean, damp cloth or paper tissue, and store the device according the manufacturer's recommendations.

SLOT AND DOT BLOTTING OF PROTEINS

ALTERNATE PROTOCOL 3

Through use of a vacuum manifold or by simple hand spotting, up to 96 samples can be applied to a single nitrocellulose or PVDF membrane for immunoblotting analysis. In slot and dot blotting, proteins are not separated by electrophoresis. Instead, the entire sample is applied directly to the membrane. Although this approach cannot discriminate between the protein of interest and a cross-reactive antigen, it is a quick way to perform preliminary characterization and high-volume routine quantitation of samples.

Additional Materials *(also see Basic Protocol 1)*

<10 μg protein sample in <100 μl water or TBS (containing no detergent)
Tris-buffered saline (TBS; *APPENDIX 2E*)

Slot or dot blotting apparatus (e.g., Hoefer, Bio-Rad; Fig. 10.10.6)
Vacuum source

1. Prewet the membrane in distilled water or 100% methanol as described in Basic Protocol 1, steps 6 and 7.
2. Prepare the slot or dot blot manifold according to manufacturer's instructions.
3. Apply samples under a low house vacuum according to manufacturer's instructions.

   Typically, samples should contain <10 μg protein in <100 μl. Overloading the wells will prevent flow of liquid through the well.

   Typical systems use a low house vacuum to pull liquid through each slot, binding the proteins to the membrane.
4. Follow sample application with two equivalent volumes of water or TBS, depending on which was used to dissolve the sample, to rinse the wells of unbound protein.

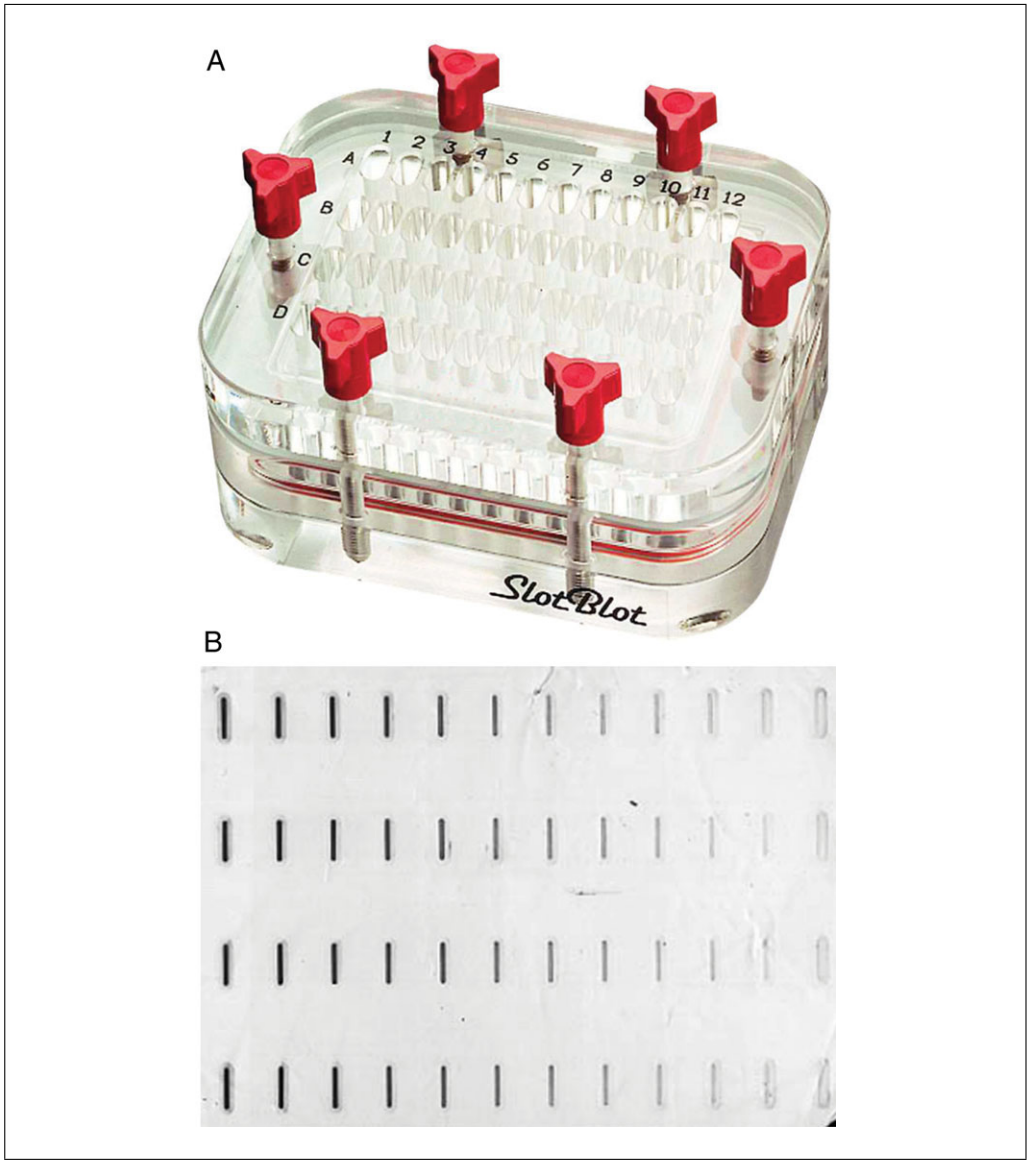

Figure 10.10.6 (A) Slot-blot unit. Through use of a vacuum manifold or by simple hand spotting, up to 96 samples can be applied to a single nitrocellulose or PVDF membrane for immunoblotting. Although this approach cannot discriminate between the protein of interest and a cross-reactive antigen, it is a quick way to perform preliminary characterization and high-volume, routine quantitation of samples. (B) Dilution series of immunoprobed BSA developed with peroxidase-tagged secondary antibody and DAB/$NiCl_2$.

ALTERNATE PROTOCOL 4

BLOTTING OF STAINED GELS

Gels stained with Coomassie blue R250 (*UNIT 10.5*; Echan and Speicher, 2002) can be effectively immunoblotted by the following procedure, based on Perides et al. (1986) and Dionisi et al. (1995). Briefly, the stained gel is soaked in a series of solutions, as described, in order to increase the solubility of the proteins after staining and fixation. After transfer, the membranes are treated with 45% or 100% methanol to decrease the Coomassie blue bound to the membrane prior to processing for chromogenic development. For chemiluminescent development, removal of the Coomassie blue is generally not needed.

Materials

Destained gel containing proteins of interest (*UNIT 10.5*; Echan and Speicher, 2002)
25 mM Tris base/192 mM glycine/1% SDS
25 mM Tris base/192 mM glycine/0.1% SDS

25 mM Tris base/192 mM glycine/0.1% SDS with 6 M urea (optional)

1. Soak destained gel containing proteins of interest in distilled water for 15 min.
2. Equilibrate gel with 25 mM Tris base/192 mM glycine/1% SDS for 1 hr with gentle agitation.
3. Transfer gel to 25 mM Tris base/192 mM glycine/0.1% SDS and equilibrate 30 min with gentle agitation.
4. *Optional:* To increase transfer efficiency of larger proteins >50 kDa, transfer gel to the above solution with 6 M urea for an additional 30 min.
5. Proceed with transfer (see Basic Protocol 1, steps 2 to 12).

   For the most efficient transfer and binding to the membrane, the transfer buffer should contain 0.1% (w/v) SDS.

6. After transfer, soak membranes for 10 to 30 min in 45% methanol (nitrocellulose) or 100% methanol (nylon or PVDF) to remove the bound Coomassie blue.

   This step is not needed if using chemiluminescent reactions or radiolabeled protein A for immunodevelopment. Destaining of the nitrocellulose membrane is enhanced by adding a small wad of laboratory tissue to the methanol to absorb the Coomassie blue.

7. Proceed with immunoprobing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 4 and 6).

REVERSIBLE STAINING OF TRANSFERRED PROTEINS WITH PONCEAU S

SUPPORT PROTOCOL 1

To verify transfer efficiency, nitrocellulose and PVDF membranes can be reversibly stained. This method will not work on nylon membranes, due to the high staining background on charged nylon membranes.

Additional Materials *(also see Basic Protocol 1)*

Ponceau S solution (see recipe)

Additional reagents and equipment for photographing membranes (*UNIT 10.5*; Echan and Speicher, 2002)

1. Following protein transfer to nitrocellulose or PVDF (see Basic Protocol 1 or Alternate Protocol 1), place membrane in Ponceau S solution for 5 min at room temperature.
2. Destain 2 min in water. Photograph membrane if required (*UNIT 10.5*; Echan and Speicher, 2002) and mark the molecular-weight-standard band locations with indelible ink.
3. Completely destain membrane by soaking an additional 10 min in water.

QUANTITATION OF TRANSFERRED PROTEINS WITH PONCEAU S

SUPPORT PROTOCOL 2

In addition to qualitatively visualizing proteins on membranes after blotting, Ponceau S provides a convenient method for quantitating the amount of protein in a given lane. By eluting the dye off a strip of membrane and reading in a spectrophotometer (OD_{525}), an internal control value of protein on a lane is obtained. This value is used to correct for any differences in protein loading from lane to lane. Comparison of the Ponceau S value to the chemiluminescent or chromogenic immunodetection value determined by densitometry provides a straightforward correction for lane-to-lane variation. This method works best

for complex mixtures where the immunodetected protein represents a small proportion of the total protein (Klein et al., 1995).

Additional Materials *(also see Support Protocol 1)*

UV/vis spectrophotometer
2-ml cuvette

1. Following protein transfer to nitrocellulose, PVDF, or nylon (see Basic Protocol 1 or Alternate Protocol 1), stain membrane, photograph, and destain (see Support Protocol 1).

   Membranes should be destained until the background becomes white.

2. Mark lanes with a soft pencil and cut lanes into strips.

3. Place each strip into 7 ml of distilled water for 7 min and remove the resulting solution. If any particulates are visible, centrifuge to remove them.

4. Read OD_{525} in a 2-ml cuvette.

   Any variation in gel-to-gel sample loading and blotting efficiency will be reflected in a change in OD of the sample lanes when compared to the control.

   The change in OD can be calibrated to a known amount of protein loaded on the control lane. This will be a relative value, however, since the efficiency of transfer out of the gel and binding to the membrane is rarely 100%.

BASIC PROTOCOL 2

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

Immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is immersed in blocking buffer to fill all remaining protein-binding sites with a nonreactive protein or detergent. Next, it is placed in a solution containing the antibody directed against the antigen (primary antibody). The blot is washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG for a rabbit primary antibody). Antigens are identified by chromogenic or luminescent visualization (see Basic Protocol 3 and Alternate Protocol 5 or 6) of the enzyme bound to the membrane.

Materials

Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocols 1 to 3)
Blocking buffer (see recipe) appropriate for membrane and detection protocol
Primary antibody specific for protein of interest
TTBS (nitrocellulose or PVDF) or TBS (nylon; see *APPENDIX 2E* for recipes)
Secondary antibody conjugate: species-specific anti-Ig conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP; e.g., Cappel, Vector Labs, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25-µl aliquots until use)

Heat-sealable plastic bags
Plastic bag heat-sealer
Orbital shaker or rocking platform
Incubation trays for membrane strips
Powder-free gloves
Plastic box

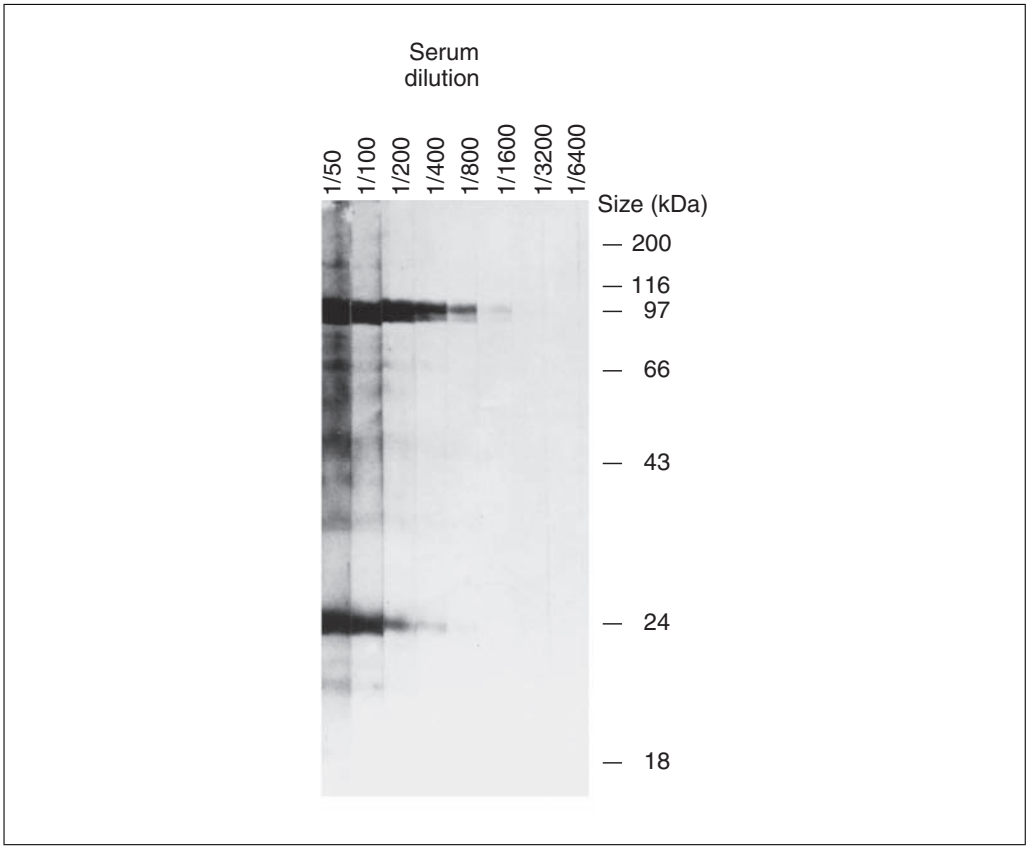

Figure 10.10.7 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. Blot was developed with HRPO-coupled avidin-biotin reagents according to Alternate Protocol 4 and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

1. Place membrane in a heat-sealable plastic bag with 5 ml blocking buffer and seal bag. Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker or rocking platform.

   Usually, 5 ml of buffer is sufficient for two to three membranes (14 × 14–cm size). If the membrane is to be stripped and reprobed (see Support Protocol 3), the blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute primary antibody in blocking buffer.

   Primary antibody dilution is determined empirically, but is typically 1/100 to 1/1000 for a polyclonal antibody, 1/10 to 1/100 for hybridoma supernatants, and ≥1/1000 for murine ascites fluid. Ten- to one-hundred-fold higher dilutions can be used with AP- or luminescence-based detection systems. To determine the appropriate antibody concentration, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 10.10.7).

   Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature with constant agitation.

Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash four times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.

5. Dilute secondary antibody conjugate in blocking buffer.

   Commercially available enzyme–conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1999).

6. Place membrane in new heat-sealable plastic bag, add diluted secondary antibody conjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

   When using plastic incubation trays, see step 3 annotation for proper antibody volumes.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 6).

ALTERNATE PROTOCOL 5

IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO SECONDARY ANTIBODY

The following procedure is based on the Vectastain ABC kit from Vector Labs. It uses an avidin-biotin complex to attach the enzyme to a biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity due to the multiple reporter enzymes bound to each secondary antibody.

Additional Materials *(also see Basic Protocol 2)*

Vectastain ABC (HRP) or ABC-AP (AP) kit (Vector Labs) containing the following: reagent A (avidin), reagent B (biotinylated HRP or AP), and biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate membrane in appropriate blocking buffer in a heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature. For nylon, incubate ≥2 hr at 37°C.

   TTBS is well suited for avidin-biotin systems. For nylon, protein-binding agents are recommended. Because nonfat dry milk contains residual biotin, which will interfere with the immunoassay, it must be used in the blocking step only. If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Prepare primary antibody solution in TTBS (nitrocellulose or PVDF) or TBS (nylon).

   Dilutions of sera containing primary antibody generally range from 1/100 to 1/100,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems. The correct dilution should give low background and high specificity (Fig. 10.10.7).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.

   Usually 5 ml of diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from bag and place in plastic box. Wash membrane three times over a 15-min span in TTBS (nitrocellulose or PVDF) or TBS (nylon). Add enough TTBS or TBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting two drops of biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF) or TBS (nylon).

   This dilution gives both high sensitivity and enough volume to easily cover a large 14 × 14–cm membrane.

6. Transfer membrane to a fresh plastic bag containing secondary antibody solution, and heat seal the bag. Incubate 30 min at room temperature with slow rocking, then wash as in step 4.

   When using plastic incubation trays, see step 3 annotations for proper antibody volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRP or -AP complex. Mix two drops of Vectastain reagent A and two drops of reagent B into 10 ml TTBS (nitrocellulose or PVDF) or TBS (nylon). Incubate 30 min at room temperature, then further dilute to 50 ml with TTBS or TBS.

   Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity.

   Sodium azide is a peroxidase inhibitor and should not be used as a preservative. Casein, nonfat dry milk, serum, and some grades of BSA may interfere with the formation of the avidin-biotin complex and should not be used in the presence of avidin or biotin reagents (Gillespie and Hudspeth, 1991; Vector Labs).

8. Transfer membrane to avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.

9. Develop membrane according to the appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 5 or 6).

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

BASIC PROTOCOL 3

Bound antigens are typically visualized with chromogenic enzyme substrates. The substrates 4CN, DAB/$NiCl_2$, and TMB are commonly used with HRP, while BCIP/NBT is recommended for AP (see Table 10.10.3). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins, probed with antibody-enzyme complex (see Basic Protocol 2 or Alternate Protocol 4)

TBS (*APPENDIX 2E*)

Chromogenic visualization solution (Table 10.10.3; also see recipe for 4CN and DAB/$NiCl_2$ visualization solutions)

Table 10.10.3 Chromogenic and Luminescent Visualization Systems[a]

System	Reagent[b]	Reaction/detection	Comments[c]
Chromogenic			
HRP-based	4CN	Oxidized products form purple precipitate	Not very sensitive (Tween 20 inhibits reaction); fades rapidly upon exposure to light
	DAB/$NiCl_2$ [d]	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned
	TMB[e]	Forms dark purple stain	More stable, less toxic than DAB/$NiCl_2$; may be somewhat more sensitive[e]; can be used with all membrane types; kits available from Kirkegaard & Perry, Invitrogen, Sigma-Aldrich, and Vector Labs
AP-based	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRP-based	Luminol/H_2O_2/*p*-iodophenol	Oxidized luminol substrate gives off blue light; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction detected within a few seconds to 1 hr. Numerous commercial kits are available through GE, ThermoFisher (Pierce), BioRad, and others.
AP-based	Substituted 1,2-dioxetane-phosphates (e.g., AMPPD, CSPD, Lumigen, Lumi-Phos 530[f])	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting); kits available from Thermo Fisher

[a]Abbreviations: AMPPD or Lumigen, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-napthol; CSPD, AMPPD with substituted chlorine moiety on adamantine ring; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine.
[b]Recipes and suppliers are listed in Reagents and Solutions except for TMB, for which use of a kit is recommended.
[c]See Commentary for further details.
[d]DAB/$NiCl_2$ can be used without the nickel enhancement, but it is much less sensitive.
[e]McKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.
[f]Lumi-Phos 530 contains dioxetane phosphate, $MgCl_2$, CTAB, and fluorescent enhancer in a pH 9.6 buffer.

Additional reagents and equipment for gel photography (*UNIT 10.5*; Echan and Speicher, 2002)

1. If final membrane wash was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

   The Tween 20 in TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place membrane into chromogenic visualization solution.

   Bands should appear in 10 to 30 min.

3. Terminate reaction by washing membrane in distilled water. Air dry and photograph (*UNIT 10.5*; Echan and Speicher, 2002) for a permanent record.

VISUALIZATION WITH LUMINESCENT SUBSTRATES

Bound antigens can also be visualized with luminescent substrates. Detection with light offers both speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for HRP or dioxetane phosphate for AP (Table 10.10.3). Film contact exposure or CCD-based digital imaging is used to capture the chemiluminescent signal. Exposures range from a few seconds to several hours, although typically signals appear within a few seconds or minutes.

Additional Materials *(also see Basic Protocol 3)*

Luminescent substrate buffer: 50 mM Tris·Cl, pH 7.5 (for HRP; *APPENDIX 2E*) *or* dioxetane phosphate substrate buffer (for alkaline phosphatase; see recipe)
Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Applied Biosystems) in dioxetane phosphate substrate buffer, prepared just before use
Luminescent visualization solution (Table 10.10.3; also see recipe for luminol visualization solution and dioxetane phosphate visualization solution)
Clear plastic wrap

Additional reagents and equipment for autoradiography (*UNIT 10.11*; Bundy, 1997)

NOTE: See Troubleshooting for suggestions concerning optimization of this protocol, particularly when employing AP-based systems.

1. Equilibrate membrane in two 15-min washes with 50 ml substrate buffer.

   For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.

2. *For AP reactions using nitrocellulose or PVDF membranes:* Incubate 5 min in Nitro-Block solution, followed by 5 min in substrate buffer (volumes as in step 1).

   Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen concentrate. It is required for nitrocellulose, and it is recommended for PVDF membranes. It is not needed for Lumi-Phos 530, AP reactions on nylon membranes, or HRP reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.

3. Transfer membrane to luminescent visualization solution (volumes as in step 1). Soak 30 sec (HRP) to 5 min (AP).

   Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step.

4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane to form a liquid-tight enclosure and quickly place against film (step 5a) or in CCD imaging system (step 5b).

   To insure an optimal image, only one layer of plastic should be between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.

5a. *For film:* In a darkroom, place membrane face down onto film and expose film for a few seconds to several hours.

   Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that insures a tight fit.

   Typically, immunoblots produce very strong signals within a few seconds or minutes. Weak signals may require several hours to overnight exposure (see Troubleshooting).

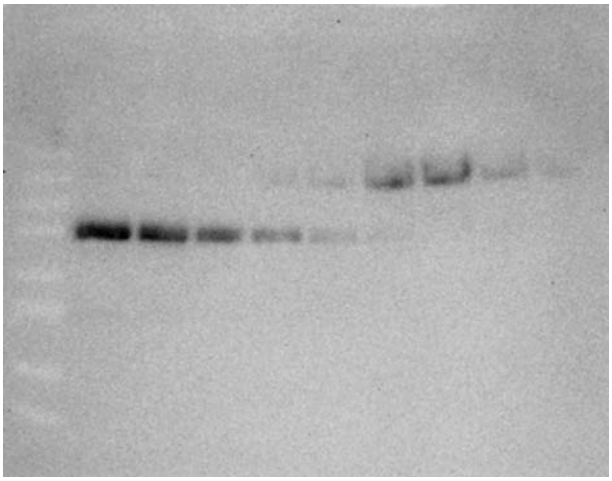

Figure 10.10.8 Chemiluminescent blot captured with a 5-min exposure using a 1-megapixel cooled (–100°C) CCD scientific camera. In this case, the reaction had decayed to a point where film was not able to record an image, while the CCD camera shows the dilution series. The blot, a two-fold IgG dilution series from 500 to 2 ng, was probed using peroxidase-conjugated affinity-purified sheep anti-mouse IgG (H&L; Rockland Immunochemicals) and visualized with luminol.

5b. *For CCD imaging:* Place the membrane in the imager, focus, and close the door. Preview the exposure with a high pixel binning (4 × 4 to 8 × 8). Once satisfied with the preview image, capture at 1 × 1 (highest resolution) to 4 × 4 (lower resolution, but faster more sensitive capture).

Exposures with cooled CCD cameras fitted with low-light optics are rarely longer than 10 min. Once captured, archive the image for later analysis [Fig. 10.10.8].

6. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Basic Protocol 3).

Chemiluminescent and chromogenic immunoblotting can be easily combined on a single blot to provide a permanent visual marker of a known protein. First probe membrane with the chemiluminescent reactions to record on film. If stripping and reprobing are needed, process by wetting and NaOH treatment (see Support Protocol 3). For the last reaction, use chromogenic development to produce a permanent visual record of the blot. Alternatively, once the film record of the chemiluminescent blot is recorded, the blot can be rinsed briefly with distilled water and placed in the appropriate chromogenic solution for chromogenic development of the blot. This results in a permanent reference stain on the blot for comparison to the more easily scanned and quantitated film record.

ALTERNATE PROTOCOL 7

SIGNAL AMPLIFICATION WITH AVIDIN-BIOTIN VISUALIZATION REAGENTS

Specialized kits are available that provide significant signal amplification and can accommodate the chromogenic, chemiluminescent, or fluorescent development of a substrate. The Vectastain ABC-AmP Western Blotting Immunodetection Kit (Vector Labs) features a preformed complex between streptavidin and biotinylated alkaline phosphatase. The substrate is either the chromogenic substrate BCIP/NBT, which produces a blue-purple precipitate, or a chemiluminescent/fluorescent substrate, DuoLux, which provides very high sensitivity and has sustained light-emission characteristics. Blots can be exposed to film several times over an 8-hr period for the optimization of band intensities or resolution. In addition to providing an amplified signal, this approach

produces a very low background on both nitrocellulose and PVDF membranes. These kits have been developed to produce maximum sensitivity with minimal background staining.

Materials

Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocols 1 to 3)
Primary antibody (from mouse or rabbit) specific for protein of interest
Vectastain ABC-AmP Chromogenic or Chemiluminescent Western Blotting Immunodetection Kit (Vector Labs) containing:
10× casein solution (250 ml)
Biotinylated secondary antibody (0.25 ml): anti-mouse IgG (for mouse primary antibodies) *or* anti-rabbit IgG (for rabbit primary antibodies)
Reagents A and B (0.5 ml each)
Substrate: chemiluminescent/fluorescent substrate (DuoLux; 100 ml) or BCIP/NBT chromogenic substrate kit (stock reagents for 200 ml)
PBS: 10 mM sodium phosphate buffer, pH 7.5 (*APPENDIX 2E*) containing 150 mM NaCl
M Tris·Cl, pH 9.5 (*APPENDIX 2E*)

Staining trays
X-ray film (e.g., Kodak BioMax)
UV transilluminator or UV imaging transillumination, cooled CCD acquisition system (e.g., Biospectrum Imaging System, UVP, Inc.)

NOTE: The components supplied in each Vectastain ABC-AmP Western Blotting Immunodetection Kit provide sufficient reagents to develop approximately twenty 100-cm^2 blots. The volumes of the reagents in the protocol below are optimized for the development of a 100-cm^2 membrane. Volumes may be proportionally adjusted for blots of a different size. All kit reagents may be used immediately following dilution. For optimal results, it is recommended that all diluted reagents from the kit be used the same day that they are prepared. Vectastain ABC-AmP Kit stock reagents should be stored under refrigeration and kept in the box in which they are supplied.

Block membrane

1. For a 100-cm^2 (10 × 10-cm) blot, prepare 120 ml of 1× casein solution by adding 12 ml of 10× casein solution (provided with Vectastain kit) to 108 ml distilled water. Block membrane by incubating in 10 ml of 1× casein solution for 5 min at room temperature with gentle agitation. Save remaining 1× casein to be used in subsequent steps.

   In rare cases, some nonspecific bands may develop. This can usually be eliminated by increasing the concentration of NaCl to 0.3 to 0.5 M in the Vectastain ABC-AmP reagent (see step 5, below). Some enzymes isolated from tissues may have covalently attached biotin as a cofactor. If high salt does not prevent the Vectastain ABC-AmP reagent from binding to particular bands, use an avidin/biotin blocking step (Vector Laboratories, cat. No. SP-2001) between steps 1 and 2 in this protocol.

2. Incubate the membrane for 30 min (or for a time established to be optimal for the concentration of primary antibody used) with gentle agitation at room temperature in an appropriate concentration of primary antibody diluted in PBS.

3. Wash the membrane in 10 ml of 1× casein solution three times, each time for 4 min at room temperature with gentle agitation.

4. Prepare the biotinylated secondary antibody solution by adding 10 µl of biotinylated anti–mouse IgG or anti–rabbit IgG to 10 ml of 1× casein solution (final

concentration 1.5 μg/ml). Incubate membrane 30 min at room temperature with gentle agitation in 10 ml of the biotinylated secondary antibody solution. At end of incubation, wash in 1× casein solution as in step 3.

5. Prepare the Vectastain ABC-AmP reagent by adding 20 μl reagent A and 20 μl reagent B to 10 ml of 1× casein solution. Incubate the membrane in 10 ml reagent for 10 min at room temperature with gentle agitation. At end of incubation, wash with 1× casein solution as in step 3.

Incubate membrane in substrate solution

It is recommended that the membrane be transferred to a different staining tray for the substrate development step.

To detect chemiluminescent and/or fluorescent signal

6a. Equilibrate membrane for 5 min in 0.1 M Tris·Cl, pH 9.5. Remove excess buffer by holding membrane vertically and touching edge of membrane to absorbent paper.

7a. Place membrane target-side-up on plastic wrap on a level surface. Pipet 5 ml of substrate on to membrane surface. Incubate 5 min under subdued light or in dark.

8a. Rinse membrane in 0.1 M Tris·Cl, pH 9.5, for a few seconds, then remove excess buffer by holding membrane vertically and touching edge of membrane to absorbent paper.

If necessary, background may be further reduced by washing membrane in 0.1 M Tris·Cl, pH 9.5, for 5 min at room temperature and removing excess buffer before exposure to film.

9a. Place membrane between two pieces of acetate, plastic wrap, or clear sheet protector. Acquire image of membrane with cooled CCD imaging system according to manufacturer's instructions. Alternatively, use X-ray film (e.g., Carestream KODAK BioMax film) exposure for the appropriate time.

10a. *Optional:* To obtain fluorescent signal, following step 9a, wash membrane in 0.1 M Tris·Cl, pH 9.5, for 5 min at room temperature. Remove excess buffer. Place membrane target-side-up on a UV transilluminator (254 to 365 nm) or UV imaging trans- and CCD acquisition system, and observe results.

To detect chromogenic signal

6b. Equilibrate membrane for 5 min in 0.1 M Tris·Cl, pH 9.5.

7b. Prepare chromogenic reagent by adding 4 drops of reagent from each of the three dropper bottles in the BCIP/NBT substrate kit into 10 ml of 0.1 M Tris·Cl, pH 9.5. Incubate membrane in the substrate solution at room temperature with gentle agitation until the appropriate density of colored bands develops.

Incubation times may vary from 30 min to several hours.

8b. Briefly rinse the membrane in PBS and air dry. Store blot protected from light.

ALTERNATE PROTOCOL 8

FLUORESCENT BLOT PREPARATION AND ANALYSIS

With the extensive range of excitation (Table 10.10.4) and emission filters (Table 10.10.5) available, researchers can detect and quantify virtually any fluorescent dye, from visible to near infrared (NIR). The process for using fluorescently tagged antibodies is straightforward, employing SDS-PAGE to first separate the proteins by size, followed

Table 10.10.4 Recommended Excitation Filters for Fluorescent Protein Blot Imaging

	Wavelength of light passed by filter (nm)							
	450sp[a]	455-495	502-547	533-587	600-645	687-748	700-740	750-780
Peak nm	NA	475	525	560	630	715	720	765
Alexa Fluor 488		X						
Alexa Fluor 546, 555			X					
Alexa Fluor 568, 594				X				
Alexa Fluor 633, 647					X			
Alexa Fluor 750						X		
CFP (mice)	X							
Cy2		X						
Cy3			X					
Cy5					X			
Cy7						X		
Fluo, FITC, FAM		X						
GFP (mice, gels, blots, plants)		X						
Oregon Green 488		X						
RFP, propidium iodide			X					
Qdot 525, 655	X							
Rhodamine Green, 110		X						
Rhodamine Red, 6 G, B				X				
SYBR Green, Gold, Safe		X						
SYPRO Orange		X						
SYPRO RedSYPRO				X				
SYPRO Ruby	X							
SYPRO Tangerine		X						
Texas Red				X				
YFP		X						
IRDye 680, CF 680					X		X	
IRDye 800, CF 770							X	X

[a]450 short-pass filter. Can accommodate wavelengths 380 to 450 nm based on typical fiber-optic light engine.

by electrotransfer of the proteins on to nitrocellulose or PVDF membranes. Fluorescent-dye-tagged secondary antibodies are used to identify the primary antibody binding site. If only one protein species is being identified, then only one primary antibody and label is used. If multiple proteins are being identified on the same blot, then the analysis relies on probing each protein with a different primary antibody (e.g., one from mouse, one from goat) and a fluorescently tagged secondary antibody specific to the primary antibody (e.g., FL680-goat anti rabbit secondary), yielding a multiplexed result. For NIR blotting applications, a range of stable, low-background dyes are available (Table 10.10.6).

For fluorescent imaging, the membrane is illuminated with overhead monochromatic excitation light from a light source, and the induced longer-wavelength fluorescence from the fluorescent tag is recorded with a cooled CCD camera using an emission filter

Table 10.10.5 Recommended Emission Filters for Fluorescent Protein Blot Imaging

	Wavelength of light passed by filter (nm)										
	465-495	503-523	513-557	565-625	580-630	607-682	668-722	700-740	767-807	7801p[a]	8001p[b]
Peak nm	480	513	535	595	605	645	695	720	787	NA	NA
Alexa Fluor 488			X								
Alexa Fluor 546, 555				X							
Alexa Fluor 568, 594						X					
Alexa Fluor 633, 647							X				
Alexa Fluor 750									X	X	
CFP (mice)	X										
Cy2			X								
Cy3				X							
Cy5							X				
Cy7											
Fluo, FITC, FAM			X								
GFP (gels, blots, plants)			X								
GFP (mice)		X									
Oregon Green 488			X								
Propidium Iodide, Qdot 655						X					
Qdot 525			X								
RFP					X						
Rhodamine Green, 110			X								
Rhodamine Red, 6 G, B						X					
SYBR Green, Gold, Safe			X								
SYPRO Orange, Ruby				X							
SYPRO Red, Tangerine						X					
Texas Red						X					
YFP			X								
IRDye 680, CF^{TM} 680								X			
IRDye 800, CF 770											X

[a]780 long-pass filter. Can accommodate wavelengths above 780 nm.
[b]800 long-pass filter. Can accommodate wavelengths above 800 nm.

that selects for the specific wavelength of the fluorescence while blocking the shorter-wavelength excitation light. For the results shown in Figure 10.10.9, an NIR blot was processed to identify two proteins in the sample.

Materials

Membrane processed according to Basic Protocols 2 and/or 3 using fluorescently tagged ABC primary or secondary antibodies: e.g., Biotium (*http://www.biotium.com/*), GE Amersham, Life Technologies, LI-COR (*http://www.licor.com*), Pierce (fluorescent standards are also quite useful; e.g., LI-COR Chameleon Protein Ladders)

Imaging system with multiple emission filters and overhead ("epi") variable-excitation-light illumination (e.g., UVP BioSpectrum with fiber optic–based

Table 10.10.6 Filters Used for NIR Blotting with 680 and 770 to 800 nm Fluorescent Tags[a]

Filter		Protein tag emission		
Ex (nm)	Em (nm)	680 nm	770- 800 nm	Comments
				NIR tags are available from a variety of sources (Biotium, Li-Core, GE/Amersham, Life Technologies)
720/40	800LP	X	X	Both bands visible
630/50	720/40	X		Only 680 nm band visible
765/30	800LP		X	Only 800 band visible

[a]Excitation filters select the wavelength of light needed to illuminate the fluorescent tags inducing fluorescence. The resulting fluorescence from the antibody protein complex, at a longer wavelength than the excitation light, is specifically selected by the emission filters that block the excitation light and let only the fluorescence wavelengths of light through to the CCD sensor in the camera.

quartz halogen or xenon arc light source; Protein Simple; BioRad); alternatively, dedicated laser scanning systems are available for NIR blotting imaging (Odyssey series, LI-COR)

Appropriate excitation and emission light filters (Tables 10.10.4, 10.10.5 and 10.10.6)

NOTE: Nitrocellulose or low-fluorescence PVDF blotting membrane (e.g., Millipore Immobilon-FL PVDF) is required for fluorescence imaging to avoid a high background

1. Process membrane according to Basic Protocols 2 and/or 3 using fluorescently tagged ABC primary or secondary antibodies.

   Note that once the final wash step is complete, no further development is required because no enzyme reactions are involved. The blot can be wet or dry, with higher fluorescence typically coming from dry membranes.

2. Select appropriate excitation and emission filters (Tables 10.10.4 to 10.10.6)

3. Position and focus the blot for fluorescence imaging:

   a. Briefly, the processed blot is positioned on the sample platen with the door open to provide light for positioning and focus.
   b. Use the imaging system camera preview function to center the membrane on the imaging platform. Fluorescence produced during blot imaging is typically much less bright than with typical applications such as ethidium bromide gels, and can take up to several minutes of exposure for a high-quality image.
   Positioning and focusing the membrane is facilitated by slightly opening the door to the imaging system to produce dim white light, or using a low-intensity setting on the excitation light with no filter present.
   c. Due to the very low intensity of the fluorescence, the lens must be focused at the most sensitive setting, typically *f*/1.2 to *f*/1.4 or less, to achieve a sharp image. Under this setting, bright white light will saturate the image.

4. Close the door on the imaging cabinet and set the lens at *f*/1.2.

5. Adjust the exposure time while viewing the preview at a camera binning set to 4 × 4, and capture at a binning of 1 × 1 to 4 × 4.

   Exposure adjusted for maximal signal without saturation will typically range from 30 sec to 2 min, depending on the sample and filter set. Frequently, the fluorescence is strongest when using a dry membrane. Some fluorescent tags will photobleach, so check for light

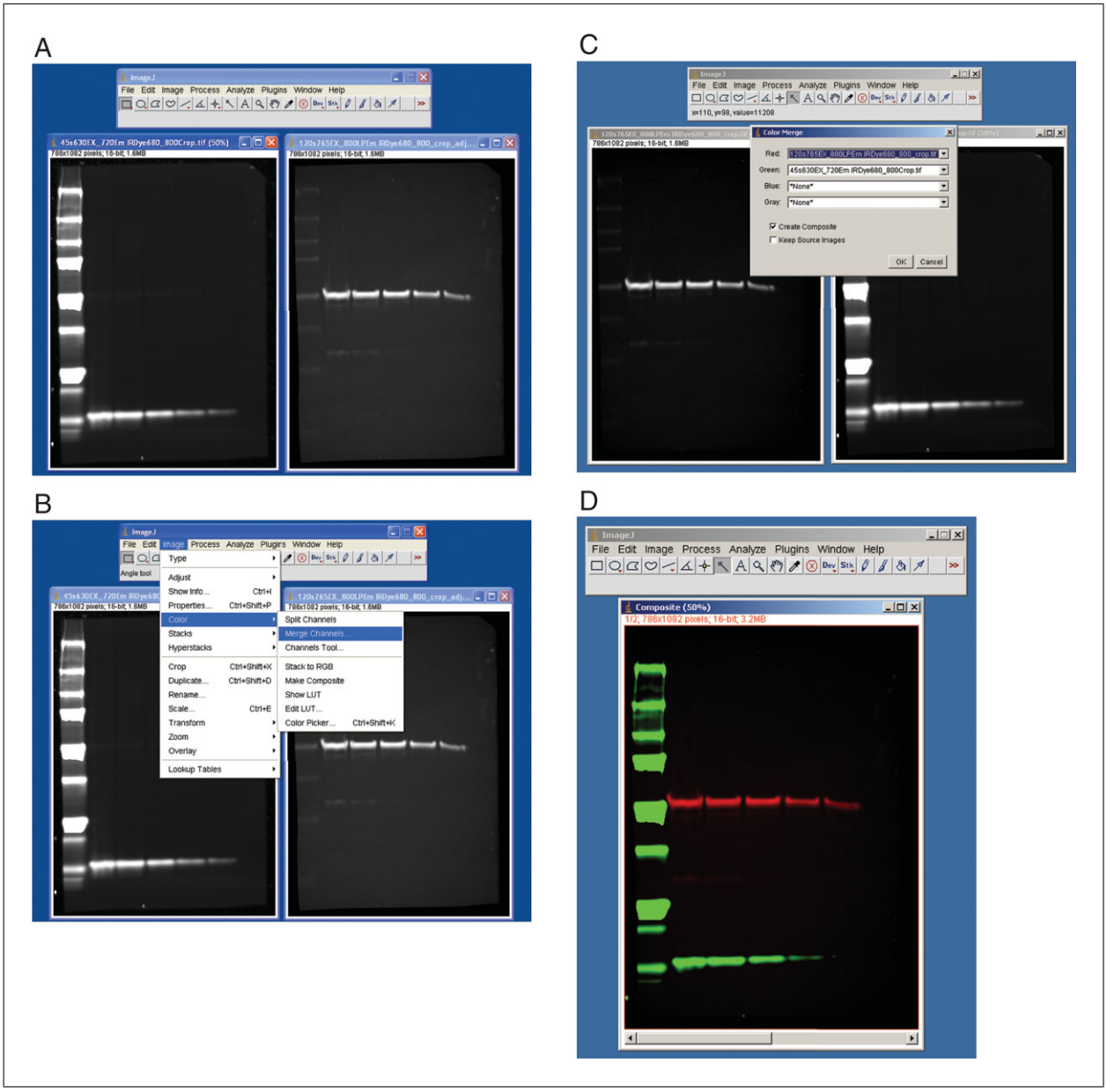

Figure 10.10.9 Fluorescence-based immunoblotting enables multiplex imaging of two different protein species from a single blot. Pseudo-coloring each class of proteins and combination of the two separate fluorescent images (green proteins show excitation/emission at 630/720 nm, red proteins show excitation/emission at 765/800 nm) into a combined image gives a final combined image showing separate proteins in different pseudo-colors, along with standards.

stability under visualization conditions by taking sequential exposures and looking for fluorescence intensity falloff.

6. Process digital image according to image analysis software recommendations to pseudocolor and composite the images as needed.

In brief, images are typically adjusted globally to remove background, contrast is enhanced, and images pseudocolored for ease of viewing and analysis. In the example shown in Fig. 10.10.9, the freely available image analysis software package Image J (see, e.g., Gallagher, 2014) is used to pseudo-color to separate images and composite them together to illustrate multiplexed image in red and green.

STRIPPING AND REUSING MEMBRANES

This stripping procedure works with blotted membranes from one- and two-dimensional gels, including previously stained gels (Suck and Krupinska, 1996). Reprobing PVDF membranes that have been developed with chemiluminescent reagents is simple and straightforward. All residual antibodies are removed from the membrane by first rewetting it in water and then briefly treating with NaOH. Although repeated reprobing can lead to loss of signal, up to five reprobings are generally feasible. The blot should have been blocked with 5% nonfat dry milk prior to treatment.

Chromogenic development leaves a permanent stain on the membrane that is difficult to remove, and should not be used when reprobing. The stain can interfere with subsequent analysis if reactive bands from sequential immunostainings are close together.

Materials

0.2 M NaOH

1. Wash blot 5 min in distilled water.

   In order to effectively reprobe the membranes, casein (for AP systems) or nonfat dry milk must be used as the blocking agent.

2. Transfer to 0.2 M NaOH and wash 5 min.
3. Wash blot 5 min in distilled water.
4. Proceed with immunoprobing procedure (see Basic Protocol 2 and Alternate Protocol 4).

   Casein or nonfat dry milk is recommended as blocking agent when reprobing membranes.

REAGENTS AND SOLUTIONS

Deionized, distilled water should be used to prepare all solutions. For common stock solutions, see APPENDIX 2E. For selection of appropriate chromogenic or luminescent solutions, and for definition of abbreviations, see Table 10.10.3.

4CN visualization solution

Mix 20 ml ice-cold methanol with 60 mg 4-chloro-1-naphthol (4CN). Separately, mix 60 µl of 30% H_2O_2 with 100 ml TBS (*APPENDIX 2E*) at room temperature. Rapidly mix the two solutions and use immediately.

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5 (*APPENDIX 2E*)
100 mM NaCl
5 mM $MgCl_2$

BCIP/NBT visualization solution

NBT stock: 50 mg/ml nitroblue tetrazolium in 70% *N,N*-dimethylformamide (DMF). Store <1 year at 4°C.
BCIP stock: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100% DMF. Store <1 year at 4°C.
Mix 33 µl NBT stock and 5 ml alkaline phosphate substrate buffer (see recipe).
Add 17 µl BCIP stock and mix. Stable 1 hr at room temperature.
Recipe is from Harlow and Lane (1999). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, and Vector Labs.

Blocking buffer

For colorimetric detection:

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in Tris-buffered saline (TTBS; *APPENDIX 2E*). Store up to 1 week at 4°C.

For neutral and positively charged nylon: Tris-buffered saline (TBS; *APPENDIX 2E*) containing 10% (w/v) nonfat dry milk. Prepare just before use.

For luminescent detection:

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): With constant mixing, add 0.2% casein (e.g., Hammarsten grade or I-Block; Applied Biosystems) to warm (65°C) TTBS (*APPENDIX 2E*). Stir for 5 min. Cool before use. Prepare just before use.

For positively charged nylon: With constant mixing, add 6% (w/v) casein and 1% (v/v) polyvinyl pyrrolidone (PVP) to warm (65°C) TTBS (*APPENDIX 2E*). Stir for 5 min. Cool before use. Prepare just before use.

DAB/$NiCl_2$ visualization solution

5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)
100 µl 3,3′-diaminobenzidine (DAB) stock (40 mg/ml in H_2O, stored in 100-µl aliquots at –20°C)
25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100-µl aliquots at –20°C)
15 µl 3% H_2O_2
Mix just before use
CAUTION: *Handle DAB carefully, wearing gloves and mask, as it is a carcinogen.*

Dioxetane phosphate substrate buffer

1 mM $MgCl_2$
M diethanolamine
0.02% sodium azide (optional)
Adjust to pH 10 with HCl and use fresh

Dioxetane phosphate visualization solution

Prepare 0.1 mg/ml AMPPD or CSPD (Applied Biosystems) *or* Lumigen concentrate (Lumigen) (see Table 10.10.3) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use.

Luminol visualization solution

0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml DMSO]
0.5 ml 10× *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)
25 µl 3% H_2O_2
H_2O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). *Alternative recipes are also available (Haan and Behrmann*, 2007). *Premixed luminol substrate mix (Amersham ECL; Perkin Elmer; Kirkegaard & Perry LumiGLO) may also be used. p-Iodophenol is an optional enhancing agent that increases light output.*

Luminol and p-iodophenol stocks can be stored for ≤6 months at −20°C.

Ponceau S solution

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare just before use.

Table 10.10.7 Applications of Immunoblotting

Application	Comment
Antibody development and characterization	Characterize antibody specificity
Subcellular localization of an expressed protein	Analyze isolated organelles (e.g., Golgi apparatus, plasma membrane, nucleus) for presence of specific proteins; probe intact cells and tissue for subcellular localization using visible light, fluorescence, and electron microscopy
Protein purification	Demonstrate enrichment of well-characterized antibody by sampling, electrophoresing, and blotting at various stages of purification
Diagnostics	Separate and blot a viral lysate to test serum for antibodies that bind key viral proteins, indicating presence of antibodies against an infection (e.g., HIV testing)
Gene expression	Detect presence or absence and amount of a specific protein during gene expression; track markers and reporter genes (e.g., luciferase) in transgenic organisms to get a complete picture of transcription and translation
Post-translational modifications	Phosphoproteomics: determine phosphorylation status of the protein complement in the cell or tissue using antibodies specific for phospho amino acids
Protein sequencing by mass spectrometry	Characterize the readily available proteins blotted onto membranes using sensitive methods, e.g., peptide sequencing by MALDI (Carr and Annan, 1996)

Transfer buffer

Add 18.2 g Tris base and 86.5 g glycine to 4 liters of water. Add 1200 ml methanol and bring to 6 liters with water. The pH of the solution is ~8.3 to 8.4. For use with PVDF filters, decrease methanol concentration to 15%; for nylon filters, omit methanol altogether.

CAPS transfer buffer can also be used. Add 2.21 g cyclohexylaminopropane sulfonic acid (CAPS; free acid), 0.5 g DTT, 150 ml methanol, and water to 1 liter. Adjust to pH 10.5 with NaOH and chill to 4°C. For proteins >60 kDa, reduce methanol content to 1%.

COMMENTARY

Background Information

Immunoblotting

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal. However, there are several problems inherent in this method, including the requirement for radiolabeling of antigen, co-precipitation of tightly associated macromolecules, occasional difficulty in obtaining precipitating antibodies, and insolubility of various antigens (Talbot et al., 1984).

To circumvent these problems, electroblotting (Towbin et al., 1979; Kurien and Scofield, 2006)—subsequently popularized as western blotting or immunoblotting (Burnette, 1981)—was conceived. Immunoblotting is a rapid and sensitive assay for the detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccharides by SDS-PAGE (*UNIT 10.1*; Gallagher, 2012) or urea-PAGE, followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon. This technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies, and is highly sensitive (1 ng of antigen can be detected).

Protein blotting has many important research and clinical applications. For example, it is the confirmatory test for human immunodeficiency virus Type 1 (HIV-1).

SDS-PAGE-separated virus proteins are blotted onto nitrocellulose and processed with patient serum. After washing and probing with an anti–human IgG coupled to HRP or alkaline phosphatase, the antigen-antibody complexes are identified by chromogenic development, indicating the presence of anti-HIV antibodies in the serum. For other selected applications, see Table 10.10.7.

Electroblotting of previously stained gels is a convenient way to visualize and document the gel prior to immunoblotting. Transfer efficiencies at all molecular weights will be lower with fixed and stained gels. This is particularly true of proteins >50 kDa (Perides et al., 1986). The additional incubation in 6 M urea will significantly increase transfer efficiency of all proteins, and is required for proteins >50 kDa.

Ponceau S staining provides an easy method for calibrating and quantitating the amount of material on a nitrocellulose or PVDF blot. An alternative to this method is to use an internal protein control with a separate antibody probe, but these tend to be expensive and time-consuming to use. Other applications for Ponceau S calibration include monitoring transfer efficiency under varied conditions for optimization of tank and semidry blotting.

Desired antigens are probed using indirect immunochemistry with secondary antibodies conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP). Signal amplification can be achieved by adding a biotin-avidin step using Vectastain ABC kits (Vector Labs). In Alternate Protocol 4, avidin acts as a bridge between biotinylated secondary antibody and biotinylated enzyme (HRP or AP). Sensitivity is improved because the multiple avidin-biotin binding sites increase the amount of enzyme per molecule of secondary antibody. Higher sensitivity is achieved using the Vectastain ABC-AmP kit (Alternate Protocol 6), which uses a preformed complex of streptavidin and biotinylated alkaline phosphatase.

While the basics of immunoblotting are largely unchanged, great improvements have been made to many of the key steps in the process. Electrophoresis using precast minigels and rapid separation speeds can be completed in under a half hour, while the blotting step using the new generation of rapid blotters can be completed a few minutes. Visualization reagents offer, in addition to the routine low-cost chromogenic stains, high-sensitivity chemiluminescent and fluorescent tag visualization technologies. With chemiluminescence and fluorescence, the sensitivity is dramatically increased, and the blots can be imaged quickly without the use of film in times ranging from a few seconds up to several minutes. Additionally, the use of fluorescence allows multiplexing of signal from different tags of a single blot. While the actual post-transfer blot processing time still varies considerably depending on the protocol and reagents used, improvements in this process include the current generation of low-noise blocking reagents to improve the signal-to-noise ratio. The current standard size for immunoblotting is approximately 10 × 10 cm to fit mini gels, although this varies considerably, as well. On the much smaller end of the scale, Hughes et al. (2014) developed a procedure using a thin (30-μm) coat of polyacrylamide on a microscope slide to monitor up to 1000 single-cell westerns as a way to monitor single-cell differentiation of rat neural stem cells. Though not routine, these efforts in miniaturization and higher throughput will continue to give researchers a wider range of choices for protein analysis.

Detection methods

Immunoblotted proteins can be detected using chromogenic or luminescent enzyme substrates (see Basic Protocol 3 and Alternate Protocol 5; see Table 10.10.2 for comparison). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRP and phosphatase systems without the need for radioisotopes (for alkaline phosphatase, see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Bronstein et al., 1992). Luminescent detection can be completed in as little as a few seconds; exposures rarely go more than 10 min. Depending on the system, the luminescence can last for several hours, permitting multiple exposures of the same blot.

AP-based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted. The procedure described in Alternate Protocol 5 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

Alternate Protocol 6 describes an amplification protocol that can be used in chromogenic, chemiluminescent, or fluorescent detection schemes. Chemiluminescent and fluorescent detection are achieved using Vectastain's DuoLuX Chemiluminescent/Fluorescent Substrate, a novel acridan-based substrate that can be used not only in immunoblots, as described

here, but also in nucleic acid detection methods (e.g., Southern and northern blotting, colony lifts, ELISAs). The substrate is available for either AP or HRP development. Generally, signal development is faster when using HRP. Thus, HRP may be preferred when digital imaging systems are used or when abundance of target reduces the need for a high signal-to-noise ratio. However, for applications where optimal sensitivity is required, AP is recommended, as it will provide a higher signal-to-noise ratio than HRP.

The DuoLuX Chemiluminescent/Fluorescent Substrate has very high sensitivity and prolonged light-emission characteristics. This enables image documentation with either film or digital imaging systems (see below). Unlike some chemiluminescent substrates, blots can be re-exposed to film as often as necessary over many hours. Because many digital imaging systems require a longer exposure time than film, the faster signal development of HRP relative to AP may be preferred when using these systems. PVDF, nitrocellulose, or nylon membranes can be used, although the chemiluminescent signal develops faster on nylon and PVDF.

In addition to its chemiluminescent properties, the reaction product is also fluorescent. Fluorescence can be recorded with a digital imaging system or a conventional camera months after chemiluminescence has faded. For fluorescence detection, nitrocellulose is recommended due to the intrinsic fluorescence of nylon. Acquisition of fluorescent signal requires a much shorter exposure time than chemiluminescence, often a fraction of a second.

Imaging methods

CCD-based digital imaging (typical) or film contact exposure (historical) can be used to capture the signal from chemiluminescent blots. Nowadays, photographic film is rarely used, and the typical application of western blotting is now fully digital for documentation and quantitation, greatly simplifying the acquisition and analysis of white-light (containing all visible light frequencies), fluorescent, and chemiluminescent images. There are number of advantages noted below that are associated with a CCD-based digital workflow for image acquisition and analysis. Historically, film-based capture has been inexpensive to get started, and requires very little in the way of equipment: film, processing chemicals, and cassettes that hold the blot close to the film. Film produces a physical record that is robust, and the film can be scanned for quantitation. However, in spite of the high initial cost, CCD-based imaging hardware has a number of advantages over film, including speed, ease of use, and data quality. First, CCD cameras with appropriate optics, cooled to minimize thermal noise, are much more sensitive than film (Fig. 10.10.6). Second, CCD image capture requires many fewer steps. The camera's preview function gives an accurate estimate of exposure time for capture of the image. Trial exposures and developing are not needed as with film. Finally, CCD capture yields higher-quality data with a wider linear range compared to film, and digital files can be automatically date stamped and archived on backup systems.

Scientific CCD cameras, cooled from ambient temperature to –20°C to –100°C, are needed for the range of exposures required for chemiluminescent imaging. Cooling lowers the dark noise on the CCD sensor, so that longer exposures (up to 60 min or more) are possible without a significant increase in background noise (*UNIT 10.12*; Medberry et al., 2005).

In addition to cooling, CCD binning, where groups of pixels are imaged at one time, is important for high-sensitivity low-light imaging. By grouping pixels into a single large pixel, signal is collected much more quickly, giving a higher signal-to-noise ratio (*UNIT 10.12*; Medberry et al., 2005). The disadvantage of this technique is that the output resolution is also reduced. By using a typical configuration of 4 × 4 binning, 16 pixels on the sensor become effectively one, lowering output resolution.

Successful digital imaging of chemiluminescent and fluorescent immunoblots depends not only on the camera sensor and cooling, but also on the optics, which determine how much light is collected and imaged onto the CCD sensor. The standard designation of light-gathering capacity in the lens is the *f* number or *f*-stop. Typical *f*-stops range from *f*/22, letting in the least light, to *f*/1.2 or lower, letting in the most light (aperture blades wide open). Lenses designed for low-light imaging typically have designations at or below *f*/1.2.

Image quantitation is accomplished with image analysis software. Internal standards can be added to the separation (a probe or protein of known amount) to serve as a relative reference. Luminescent blots can be easily erased and reprobed because the reaction products are soluble and do not deposit on the membrane. Compared to chromogenic development, the luminescent image is easier to photograph and

to quantitate by densitometry. In addition to a wider range of quantitation, improved sensitivity, straightforward preview prior to final capture of the image, the ability to readily overlay the detection of different proteins labeled with different fluorescent antibodies enables multiplexing. Also, with chemiluminescence and fluorescence detection, a white light image showing the protein standards can be composited onto the detection image to create an image for display and molecular weight determination. While instrumentation designed for high sensitivity, cooled CCD-based chemiluminescent and fluorescent detection does add to the initial experimental cost, the cost of the cooled chemiluminescent digital imaging systems continues to decrease.

Critical Parameters

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. Time of transfer and primary antibody and conjugate dilutions should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1999). These include Tween 20, PVP, nonfat dry milk, casein, BSA, and serum. A 0.1% (v/v) solution of Tween 20 in TBS (TTBS), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution (BLOTTO), TTBS is stable and has a long shelf life at 4°C. Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink (*UNIT 10.8*; Goldman et al., 2016). However, even with the application of such standard blocking procedures as 5% to 10% nonfat dry milk protein or 0.05% to 0.1% Tween 20, background can still be a significant problem. If this happens, using a blocking protein (e.g., goat, horse, or rabbit normal serum) from the same species as the primary antibody can reduce the background, presumably by reducing cross-reactivity between the primary antibodies and the blocking agent. If secondary antibody detection is used, the blocking protein (i.e., normal serum) should be from the same species as the secondary antibody. Combinations of blocking agents can also be effective. Thus, 0.1% human serum albumin (HSA) and 0.05% Tween 20 in TBS is recommended when probing PVDF (e.g., Immobilon-P) membranes with human serum (Craig et al., 1993). However, this can also lead to overall loss of antigen signal, requiring a ten-fold increase in the primary antibody (serum) concentration to achieve an adequate background-free antigen signal.

When using chemiluminescent detection for immunoblotting, high background frequently occurs, particularly for strong signals (Pampori et al., 1995). Several methods are available for reducing the background from chemiluminescent reactions. These include changing the type and concentration of blocking agents, optimizing antibody concentrations, letting the reaction proceed for several minutes before imaging, or simply limiting the exposure time of the blot. These procedures are not always successful, however, and can lead to inconsistent results. An alternative approach is to reduce the concentration of reagents ten-fold. This effectively removes the background and has a number of advantages which include lower cost, increased signal-to-noise ratio, and reduced detection of cross-reacting species.

Parameters for processing fluorescently tagged blots are similar to chemiluminescence in that background must be kept at a minimum. Critical to obtaining a strong signal-to-noise ratio is the use of specialized blocking agents designed for low fluorescence background, nitrocellulose or specialized PVDF membranes that are designed to be low fluorescence, and high-quality laboratory water, again to avoid any possible contamination. In addition, to obtain strong signal to noise, adjusting the primary and secondary antibodies to a higher or lower concentration might be needed. Laser scanning systems tend to be more sensitive, while overhead illumination ('epi') imaging systems tend to require more signal, particularly in the long-wavelength near IR (>800 nm). Fluorescent kits and chemistries are available from a number of manufacturers, as noted in the materials lists of the individual protocols. Lastly, fluorescently tagged western blots include using specific excitation/emission filters to illuminate the blot and detect the fluorescence. The lighting systems are typically either already built into the system or come with manufacturer's recommendations for excitation/emission filter pairs that should be followed. Currently, due to a combination of low background and ease of processing, PVDF and nitrocellulose membranes are the most commonly used membranes for protein blotting. Nylon membranes, typically used for nucleic acid blotting applications, are robust and can be used for protein blotting

as well. However, nylon requires more stringent blocking to minimize background (see below), limiting its popularity for protein blotting applications. Two types of nylon membrane are used for protein transfer—neutral (e.g., Pall Biodyne A) and positively charged (e.g., Pall Biodyne B). Although the positively charged membranes have very good protein-binding characteristics, they tend to give a higher background. These membranes remain positively charged from pH 3 to pH 10. Neutral nylon membranes are also charged, having a mix of amino and carboxyl groups that give an isoelectric point of 6.5. Because of their high binding capacity, positively charged membranes have been popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here, 10% nonfat dry milk in TBS is recommended for chromogenic development. During luminescence development, however, background is a more significant problem. Compared to dry milk, purified casein has minimal endogenous alkaline phosphatase activity (AP activity leads to high background) and is therefore recommended as a blocking agent for nitrocellulose, PVDF, and nylon membranes. Positively charged nylon requires much more stringent blocking with 6% (w/v) casein and 1% (v/v) polyvinylpyrrolidone (PVP). Because nonfat dry milk and casein may contain biotin that will interfere with avidin-biotin reactions, subsequent steps are done without protein-blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

If reprobing is desired, blots can be air dried and stored at 4°C for 3 months after chemiluminescence detection. After drying, store in a sealed freezer bag until use. Repeated probing will lead to a gradual loss of signal and increased background. However, this will depend in part on the properties of the sample.

If the primary procedure is problematic due to loss of sensitivity or an increase in background, two possible alternative procedures for stripping membranes are recommended. The first uses 2-mercaptoethanol and SDS (Kaufmann et al., 1987; Tesfaigzi et al., 1994). Briefly, the membranes are incubated in 2% SDS/100 mM Tris·Cl, pH 7.4/100 mM 2-mercaptoethanol for 30 min at 70°C, effectively removing primary and secondary antibodies. As with the primary procedure recommended above, repeated probing should be done with caution due to the potential loss of detection signal, and 5% nonfat dry milk is required as a blocking agent. The milk blocking agent facilitates antibody removal from the blot (Kaufmann et al., 1987). The second uses guanidine·HCl. For nylon and PVDF membranes (do *not* use with nitrocellulose), incubate the immunoblot in 7 M guanidine·HCl for 10 min at room temperature. (The short wash time is critical, as guanidine·HCl is a very strong denaturant, so do not leave the filter in this solution >15 min.). Pour off excess guanidine·HCl and then rinse the membrane several times in 1× TTBS. Reblock the membrane and proceed with the standard immunoblotting procedure. Membranes stripped using this procedure can generally be reused three or four times.

In Alternate Protocol 6, extensive washing will reduce signal strength, so the wash time should not be extended unless high background is observed. If background is excessive, use a wash time of 5 to 10 min in step 8a (may require optimization). The long emission lifetime of the substrate allows the blot to be re-exposed until an optimal signal-to-noise ratio is achieved. Blotting can be done onto either nylon or nitrocellulose. Nylon requires shorter exposure times, and is therefore preferred for chemiluminescent applications. However, because of nylon's intrinsic fluorescence, nitrocellulose is preferred for fluorescence detection.

Troubleshooting

There are several problems associated with immunoblotting. The antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. For example, zwitterionic detergents (e.g., Zwittergent 3-14 from EMD Chemicals, http://www.emdbiosciences.com) have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can also be performed (*UNIT 10.3*; Gallagher, 1995).

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and discussion of immunoblotting problems and artifacts, see Bjerrum et al. (1988).

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that the proper electrical connections were made and that power was delivered during transfer. In addition, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the transfer efficiency using the tank system appears to be low, increase the transfer time or power. Cooling (using the unit's built-in cooling cores) is generally required for transfers >1 hr. At no time should the buffer temperature go above 45°C. Prolonged transfers (>1 hr) are not possible in semidry transfer units due to rapid buffer depletion.

Alternatively, the transfer buffer can be modified to increase efficiency. Adding SDS to the transfer buffer at a concentration of 0.1% improves the transfer of all proteins out of the gel, particularly those above 60 to 90 kDa in size. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. These procedures are tradeoffs. Methanol improves the binding of proteins to PVDF and nitrocellulose, but at the same time hinders transfer. With SDS present, transfer efficiency is improved, but the SDS can interfere with protein binding to the membrane. Nylon and PVDF membranes are particularly sensitive to SDS interference. If needed, 0.01% to 0.02% SDS may be used in PVDF membrane transfer buffers (Pluskal et al., 1986). SDS and methanol should not be used in the transfer buffer for nylon (Peluso and Rosenberg, 1987), but many be used for nitrocellulose or PVDF.

Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5- to 0.75-mm-thick gels will transfer much more efficiently than thicker gels (e.g., 1.5 mm thick). Gels with a higher acrylamide percentage will also transfer less efficiently. Proteins can be particularly difficult to transfer from gradient gels, and a combination of longer transfer times, thin gels, and the addition of SDS to the transfer buffer may be needed.

If the protein bands are diffuse, check the transfer cassette. The gel must be held firmly against the membrane during transfer. If the transfer sandwich is loose in the cassette, add another thin sponge or more blotter paper to both sides.

Occasionally, a grid pattern will be apparent on the membrane after tank transfer. This is caused by having either the gel or the membrane too close to the sides of the cassette. Correct this by adding more layers of filter paper to diffuse the current flowing through the gel and membrane. Use a thinner sponge and more filter paper if necessary.

If air bubbles are trapped between the filter and the gel, they will appear as clear white areas on the filter after blotting and staining. Take extra care to make sure that all bubbles are removed.

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using preimmune (i.e., not previously immunized) serum or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. 10.10.5).

Due to the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow nearby weaker signals on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad diffuse image on the film. The signal can also saturate the film, exposing the film to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the alkaline phosphatase substrate AMPPD, nitrocellulose, PVDF, and nylon membranes require 2, 4, and 8 to 12 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Applied Biosystems Western Light instructions). Positively charged nylon requires special blocking procedures to minimize background (Gillespie and Hudspeth, 1991). These procedures include using a blocking and primary antibody solution containing 6% casein, 1% polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN_3, 10 mM EDTA, and PBS, pH 6.8. Prior to use, the casein must be heated to 65°C to denature alkaline phosphatase and markedly reduce its enzymatic activity in the casein. In addition, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma).

Anticipated Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should

produce a single band, degradation of the sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If simultaneously testing multiple antibodies directed against a complex protein mixture (e.g., using patient sera against viral proteins in AIDS western blot test), multiple bands will be visualized.

For immunoblot or protein dot blot chemiluminescent applications, the sensitivity using HRP is ~1 pg of target protein. For chemiluminescent blots, DuoLuX Chemiluminescent/Fluorescent Substrate (see Alternate Protocol 6) can be used on either nitrocellulose or PVDF membranes.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and under 1 hr on a minigel. Transfer time can be a manner of minutes with the new generation of fast blotters to 1 hr (high-power transfer) to overnight. Blocking, antibody probing, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min (chromogen) and a few seconds to several hours (luminescence). With fluorescence, once the final wash is complete, the membrane is ready for imaging since the fluorescent requires no further processing.

With the newer fast blotters, very rapid protein blotting workflows can be created using the manufacturer's recommended reagents. From sample prep to electrophoresis to blotted protein can be easily accomplished within an hour. The subsequent immunodetection of the proteins of interest on the blot can vary from a couple of hours to overnight or longer depending on the level sensitivity, convenience, and application. The digital analysis can also be accomplished quite quickly and be completed for membrane on the order of 5 to 10 min.

For the DuoLuX Chemiluminescent/Fluorescent Substrate (see Alternate Protocol 6), optimal exposure times can vary from 5 sec to 15 min. It is recommended that initial exposures be taken between 1 and 5 min. Band intensities or resolution can then be optimized by lengthening or shortening exposure times based on the initial results.

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Details alternative detection methods.

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Describes the use of Ponceau S staining for immunoblotting.

Schneppenheim et al., 1991. See above.

Details peroxidase-based luminescent detection methods.

Internet Resources

http://www.bio-rad.com/en-us/category/western-blotting?pcp_loc=catprod

Introduction to Trans-Blot Turbo Transfer System by BioRad.

http://www.piercenet.com/product/pierce-power-blotter.

Introduction to Pierce Power Blotter by Thermo Scientific.

https://www.thermofisher.com/order/catalog/product/IB21001?ICID=cvc-Western-Blot-Transfer-c4t1

Introduction of iBlot Dry Blotting System by Life Technologies.

http://tools.lifetechnologies.com/content/sfs/manuals/iblotsystem_qrc.pdf

iBlotDry Blotting System quick reference.

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Received May 8, 2017
Revised July 6, 2017
Accepted July 13, 2017

Short Communication

Checking transfer efficiency and equal loading via qualitative optical way in western blotting

The ability to determine that successful transfer and equal loading occur prior to using primary antibodies is important. And total protein staining is commonly used to check transfer efficiency and normalization, which play a crucial role in western blotting. Ponceau S and coomassie blue are commonly used, but there are disadvantages reported in recent years. Therefore, we are interested in finding another method, which is cheap, easy and fast. As we know, protein binding region of PVDF membrane is still hydrophilic when carbinol volatilizes, however, the non-protein binding region of PVDF membrane became hydrophobic again. And this different wettability between non-protein binding region and protein binding region of Polyvinylidene difluoride membrane may be used to check transfer efficiency and equal loading in western blotting. Based on the principle above, we describe an optical approach where an experimenter can observe that the proteins have been transferred to the membrane without any staining within minutes.

Keywords:
Equal loading / Hydrophobicity / Polyvinylidene difluoride / Transfer efficiency / Western blotting
DOI 10.1002/elps.201700266

Western blotting is the most widely used techniques in the life sciences [1], also called immunoblotting, which was introduced by Towbin in 1979 and is now a routine analytical method for protein analysis [2]. Western blotting involves the separation of proteins by electrophoresis, transfer to a membrane, and selective immunodetection of an immobilized antigen [3]. Each step in western blotting assay can independently and significantly alter the result [4]. Transfer efficiency and normalization play important roles in western blotting, which depend on protein quantification, composition of the SDS-polyacrylamide gel, transfer time, size of proteins, composition of the transfer buffer and so forth.

Before the procedure continues with the western blotting, it is often needed to stain total proteins on the membrane by Ponceau S or to stain the SDS-polyacrylamide gel by coomassie blue to check transfer efficiency. Generally total protein stains are commonly used; however, there are complications such as toxicity and difficulty [5]. In addition, stain-free technique, which is proved as a superior loading control to β-actin for western blotting assay [6], can also check transfer efficiency and normalization by UV induced fluorescence in the gel. Although this technology is reliable, it requires a cooled-CCD imager and the purchase of commercial gels [7]. These disadvantages above are stumbling blocks for researchers. So an alternative method, only use our naked eye to check transfer efficiency and normalization without any staining within minutes, which can be done during the procedure of western blotting, is needed. And it is good for researchers, who will perform lots of western blotting in research of life science.

Polyvinylidene difluoride (PVDF) membrane, a fluoropolymer, has a high mechanical strength and hydrophobicity, and has been widely adopted in biomedical applications, such as western blotting [8–10]. Because of hydrophobicity, PVDF membrane usually need soak in carbinol for 30 s to become hydrophilic before transfer. PVDF membrane efficiently adsorbs proteins, leading to fast liquid transfer through the membrane [11]. Because the wettability of the PVDF membrane is dominated by two factors: surface composition and surface roughness [12], protein binding region of PVDF membrane is still hydrophilic when carbinol volatilizes, while, the non-protein binding region of PVDF membrane became hydrophobic again. Maybe, we could check transfer efficiency and normalization of western blotting via this principle (Fig. 1A). In order to address this issue, western blotting with protein samples has been performed.

There are three types of protein samples. First, human hepatocellular carcinoma tissues were collected from the Department of Hepatobiliary Surgery in the Second Affiliated Hospital at Chongqing Medical University. Peritumor and tumor were stored individually at –80°C, and the protocol was

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Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; **HepG2**, Human hepatoma-derived cell line; **Nrp1**, Neuropilin-1; **PVDF**, Polyvinylidene difluoride; **SDS**, Sodium dodecyl sulfate; **SMMC-7721**, Human hepatoma cell line; **TBST**, Tris-buffered saline with Tween-20

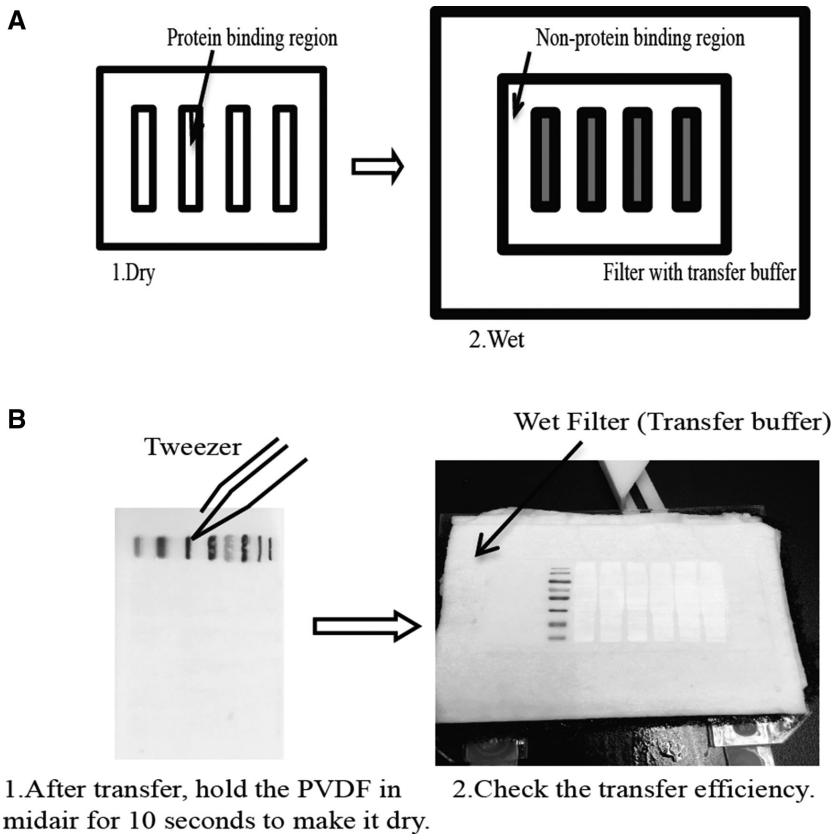

Figure 1. A diagram showing how this method probably work (A) and practical steps to check transfer efficiency and equal loading in western blotting with naked eye (B). We soaked the "sandwich" into transfer buffer for 30 s, and tweezered the PVDF membrane out of the "sandwich", and then held the PVDF membrane in midair for 10 s, in order to dry it. After that, we put it on the wet filter paper, and checked transfer efficiency and equal loading in western blotting by sunlamp light (B).

approved by the Ethic Review Committee of Second Hospital Affiliated to Chongqing Medical University. Second, whole livers of mouses were excised, and stored at –80°C. Animal treatment followed the Guide for the care and Use of Laboratory Animals of the National Institute of Health. The protein of the human hepatocellular carcinoma cell lines (HepG2 and SMMC-7721, from Institute of Life Science, Chongqing Medical University) and tissues were obtained via Total Protein Extraction Kit (BestBio, China) according to the instruction, and were stored individually at –80°C. And the concentration of protein samples was measured by BCA protein assay kit respectively (P0012, Beyotime Biotechnology, China).

Protein samples were diluted with 5 × loading buffer (Beyotime Biotechnology, China), then heated at 98.5°C for 5 min, and then were loaded into 10% SDS-polyacrylamide gels (90 V, 90 min, Room temperature). After electrophoresis, protein samples were transferred (100 V, 60 min, 4°C) to the PVDF membranes (Millipore, USA). As for post transfer, we soaked the "sandwich" into transfer buffer (fresh, 4°C) for 30 s, and tweezered the PVDF membrane out of the "sandwich" (Gel/Membrane/Filter), and then held the PVDF membrane in midair for 10 s, in order to dry it. After that, we put it on the wet filter paper (was soaked in transfer buffer, contained 20% carbinol), and checked transfer efficiency and equal loading in western blotting with naked eye (Fig. 1B). After blocking the membranes with non-fat milk (2 g milk powder in 40 mL tris-buffered saline with Tween-20, TBST) at room temperature, membranes were washed in a few minutes in TBST. Then, membranes were incubated with mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ARG10112, Arigo, China, 1: 1000) or β-actin (B1033, Biodragon, China, 1: 1000) overnight at 4°C. The membranes were washed 3 × 10 min in TBST at room temperature, and then membranes were incubated with secondary goat anti-mouse antibody (BA1050, Boster, China, 1: 5000) or goat anti-rabbit antibody (BA1054, Boster, China, 1: 5000) for 1 h at room temperature. After that, the membranes were washed 3 × 10 min in TBST at room temperature again. Finally, membranes were incubated with ECL plus reagent (Beyotime Biotechnology, China) and visualized on ChemiDoc MP Imaging System (Bio-Rad, USA). And the experiments were totally repeated for three times with three repetitions for each group.

Repeatability and reliability of this novel approach was compared with equal levels of housekeeping protein (GAPDH or β-actin were used as loading control). In mouse liver (Fig. 2A and B), human hepatocellular carcinoma tissue samples (Fig. 2C and D) and human hepatocellular carcinoma cell lines (HepG2, SMMC-7721, Fig. 2E and F), we could see the lanes clearly, and we also found a nearly uniform width and the similar reflected light intensity of the lanes (Fig. 2A, C, and E). In a similar way, the density of the bands was well consistent with the results above (Fig. 2B, D, and F). Interestingly, we could even observe the bands on the PVDF

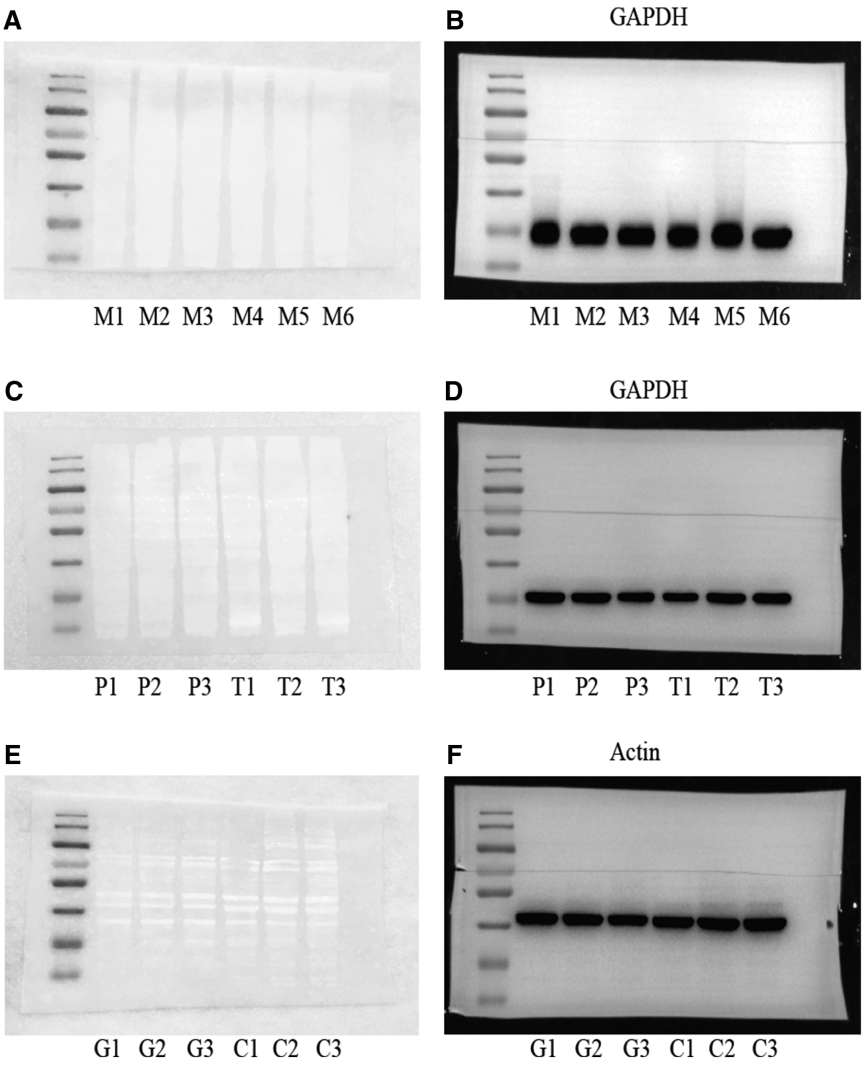

Figure 2. Reliability and repeatability of this approach were compared with housekeeping protein by equal loaded protein western blotting. In mouse liver (A and B), human hepatocellular carcinoma tissue samples (C and D) and human hepatocellular carcinoma cell lines (HepG2, SMMC-7721, E and F), M for mouse, P for peritumor, T for tumor, G for HepG2, C for SMMC-7721. Photos (A, C, and E) were captured by normal camera.

membrane without staining in the human hepatocellular carcinoma cell lines (HepG2, SMMC-7721, Fig. 2E and F), while we just observe the lanes on the PVDF membrane in group of tissues. This in an interesting phenomenon, but we do not know the reason.

With employing the protein samples, the linear dynamic range of this method and GAPDH were compared by running serial dilutions of protein samples (5–50 μg) in follow-up experiments. As shown in Fig. 3, with the increase in loading quantities, the lane became a little wider and had higher reflected light intensity (Fig. 3A and B). And we also found that the density of GAPDH (Fig. 3C and D) was nearly linear with the amounts (5–50 μg) of protein loaded onto the gel (Fig. 3E and F). Interestingly, we found that there was relative low reflected light intensity of protein binding region of PVDF (Fig. 3B, black arrow). According to the results above, we speculated that the big molecular protein with PVDF membrane bind not well. Therefore, another antibody, an anti-Nrp1 antibody (Neuropilin-1, which plays crucial role in hepatocellular carcinoma, ab81321, abcam, UK, 1:1000, observed band size 120 kDa), was used to address this issue. Compared with Fig. 3G and H, we found low density of the band (40 μg, Fig. 3H).

These data have shown that we can use this different wettability between non-protein binding region and protein binding region of PVDF membrane to check transfer efficiency and equal loading in western blotting without staining. These data have also shown that this novel approach does not impair reliability or repeatability of the following experiment of western blotting. And we also found that the low reflected light intensity of lane reveals that protein with PVDF bind not well.

Currently, there are many methods to check transfer efficiency and normalization in western blotting, including prestained protein markers, total protein staining and stain-free technology [13, 14]. Prestained protein markers are used as molecular weight standards in western blotting [15], and the protein samples are transferred to membrane. However, this cannot ensure efficient binding of protein on the PVDF membrane. If the protein samples of interest are

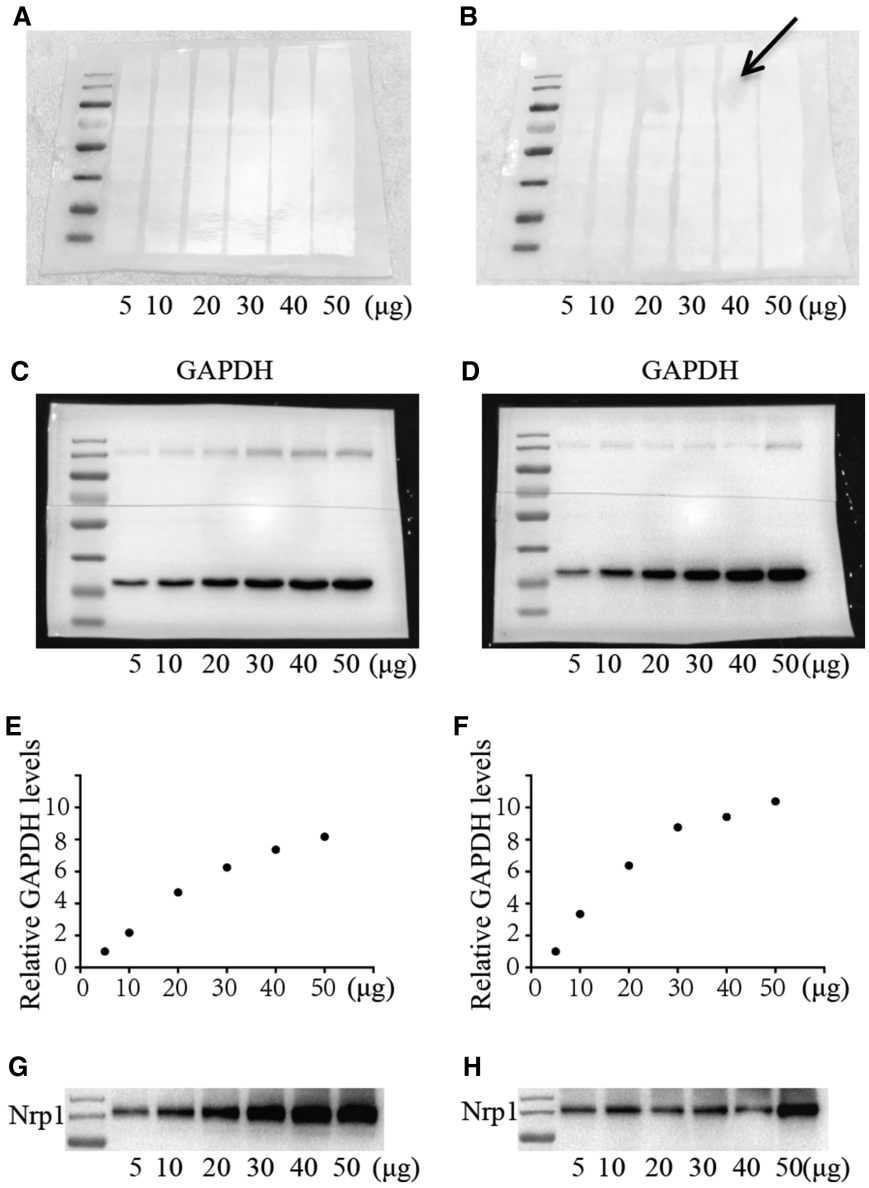

Figure 3. A dilution series (5–50 μg) of tissue lysate from human hepatocellular carcinoma was loaded onto the gel, after transfer, relative low reflected light intensity of protein binding region of PVDF (black arrow) was observed (A, tumor; B, peritumor, Photos were captured by normal camera), membranes were detected by anti-GAPDH antibody (C and D). The graph shows the relative intensity of GAPDH, and the relative intensity of GAPDH is nearly linear with the amounts (5–50 μg) of loaded protein (E and F). Comparing A and G, B and H, we found that low reflected light intensity of lane (black arrow, B) reveals that protein with PVDF bind not well (H).

electrophoresed in duplicate and transferred to a PVDF membrane, half the membrane can be stained with India ink, Gold or SYPRO Ruby, which can check transfer efficiency and the other half can be used for western blotting [16], though loading a separate gel is not advised because of loading error. Total protein stains are commonly used, such as Ponceau S and coomassie blue. Ponceau S stain is inexpensive and reversible, but it has limited sensitivity and fades with time. Although the gel can be stained by coomassie blue, which determine that protein left the gel, this cannot ensure transfer efficiency and normalization of protein level. And staining transfer membranes with coomassie blue is sensitive and inexpensive, while there are complications, for example, difficulty, toxicity and irreversibility have been reported [5]. Another method to check transfer efficiency utilizes stain-free technology [17, 18]. Recent studies suggested that stain-free technology outperforms Ponceau S staining and it is better than housekeeping proteins as the loading control [14, 19]. Although stain-free technology shows the characteristics of repeatability and reliability, it requires commercial gels and imager.

The advantage of this approach is that we can check transfer efficiency of western blotting without staining, which is more convenient than staining with Ponceau S and coomassie blue and is more reliable than prestained protein marker. This method, which does not need any other auxiliary equipment, also can roughly check equal loading of gels before membranes are incubated with primary antibody. And we can stop incubating if we find protein samples are not loaded equally, which is important for us to make full use of the primary antibody. Besides, as for western blotting in life science, we sometimes need examine lots of proteins on one PVDF

membrane. And it is easy for us to cut the PVDF membrane through this method when we can incubate primary antibodies respectively (protein of interest, protein of control). Generally, this method that can check transfer efficiency and normalization of protein levels in western blotting is cheap, easy and fast.

Nevertheless, there are several limitations to this method. First, there is no actual quantitative way to measure the width or the reflected light intensity of the lanes. However, the aim of this study is to describe an easy and quick way to visually monitor the relative transfer efficiency and normalization of protein during western blotting, in my opinion, this useful approach is worth a try. Indeed, it could not be better if we could get quantitative parameters that could be used to document the efficiency or normalization, therefore, future studies are needed. Second, the influential factors of this method are unclear at present, so further studies are needed to provide more results and details. In conclusion, the different wettability between non-protein binding region and protein binding region of PVDF membrane can be used to check transfer efficiency in western blotting. And this novel qualitative optical method, which is cheap, easy and fast, can also roughly check equal loading of gels.

The authors would like to thank Yun Liang for critical revision for manuscript. We would also thank all the anonymous reviewers for their helpful suggestions on the quality improvement of our paper.

The authors have declared no conflict of interest.

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Protein Blotting: Immunoblotting

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Immunoblotting (also referred to as western blotting) uses antibodies to probe for a specific protein in a sample bound to a membrane. Typically, a protein sample is first size separated via electrophoresis (e.g., SDS PAGE). However, antibodies used for specific protein detection are restricted by the polyacrylamide gel and, to make the separated proteins accessible, the proteins need to be moved out of the gel and bound to a rectangular sheet of PVDF or nitrocellulose membrane. Specialized blotting equipment electrophoretically transfers the negatively charged proteins from the gel onto the membrane. The nitrocellulose or PVDF membrane binds the proteins as they move out of the gel, producing an exact replica, on the membrane surface, of the original protein gel separation. The membrane is then blocked to prevent any nonspecific protein binding and visualized by specific antibodies to detect the presence or absence of a particular protein. Applications of immunoblotting are many, and include antibody characterization, diagnostics, gene expression, and post-translational modification analysis.

Keywords: alkaline phosphatase • chemiluminescence • chromogenic • DAB • dot blot • fluorescence • nitrocellulose • peroxidase • protein blotting • PVDF • slot blot • TMB • western blotting

OVERVIEW AND PRINCIPLES

Immunoblotting (often referred to as "western blotting") is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. With numerous applications from protein quantitation to diagnostics, western blotting remains a core technique for protein analysis (see Table 8.3.1). The typical steps for performing western blotting are described in Basic Protocols 1 and 2 and follow the sequence starting with SDS-PAGE (detailed in *UNIT 7.3*; Gallagher, 2012), then moving on to electroblotting, blocking, antibody probing, and visualization of the reaction.

While antibodies are exquisitely specific for the target antigen (sites on proteins in the case of western blotting), the small pores of the polyacrylamide gel used in SDS-PAGE (*UNIT 7.3*; Gallagher, 2012) prevent the antibody from binding to the electrophoretically separated proteins. Western blotting creates an exact replica of the protein gel, typically on a nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membrane, by electrophoretically removing the protein from the gel and, in the process, irreversibly binding the protein to the readily accessible membrane surface where reagents such as antibodies are free to interact with the bound protein (Towbin et al., 1979; Burnette, 1981; Kurien and Scofield, 2006). Membranes have an enormous capacity for protein binding, and

Current Protocols Essential Laboratory Techniques 8.3.1-8.3.40, May 2016
Published online May 2016 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/9780470089941.et0803s12

Table 8.3.1 Applications of Western Blotting

Application	Comment
Antibody development and characterization	Characterize antibody specificity
Subcellular localization of an expressed protein	Analyze isolated organelles (e.g., Golgi apparatus, plasma membrane, nucleus, etc.) via SDS-PAGE and immunoblotting for the presence of specific proteins; probe intact cells and tissue for subcellular localization using visible light, fluorescence, and electron microscopy for confirmation
Protein purification	Demonstrate enrichment of well characterized antibody by sampling, electrophoresing, and blotting at various stages of purification
Diagnostics	Separate and blot a viral lysate to test serum for antibodies that bind key viral proteins, indicating presence of antibodies against an infection (e.g., HIV testing)
Gene expression	Detect presence or absence and amount of a specific protein during gene expression; track markers and reporter genes (e.g., luciferase) in transgenic organisms to get a complete picture of transcription and translation
Post-translational modifications	Phosphoproteomics: determine phosphorylation status of the protein complement in the cell or tissue using antibodies specific for phospho amino acids. Determining phosphorylation signal transduction cascades has become one of main uses of protein blotting.
Protein sequencing by mass spectrometry	Characterize the readily available proteins blotted onto membranes using sensitive methods, e.g., peptide sequencing by MALDI (see *CP Protein Science* UNIT 16.1; Carr and Annan, 1996)

before probing, any remaining binding capacity is blocked after transfer by soaking the membrane in a solution containing a nonspecific protein (e.g., purified casein) or, more typically, a detergent. Nylon membranes can also be used for protein blotting, but are not as popular due to additional steps needed to minimize background signal.

Recent improvements in protein blotting have been driven by the need for faster and more convenient results. Precast small-format gels and blotting equipment are ideal (see Figs 8.3.1 and Figs 8.3.2). In the simplest and most common approach, the proteins are electrophoretically transferred in a semidry transfer apparatus to a nitrocellulose or PVDF membrane in a process that can be monitored for efficiency of transfer by staining the membrane for transferred proteins and the gel for any residual sample. Alternate Protocol 1 describes a procedure for rapid transfer using the iBlot dry blotting system from Life Technologies.

Dot and slot blots (Alternate Protocol 2) are alternatives useful for both preliminary and routine characterizations, providing a quick and simple way to determine the amount of an antigen in a sample without performing electrophoresis first. Briefly, the proteins are deposited onto the membrane either manually or through use of a vacuum manifold that pulls a solution containing the antigen through a nitrocellulose membrane (see Fig. 8.3.3), depositing the protein of interest onto the membrane. The membrane is probed with the same chromogenic or luminescence protocol as the western blot.

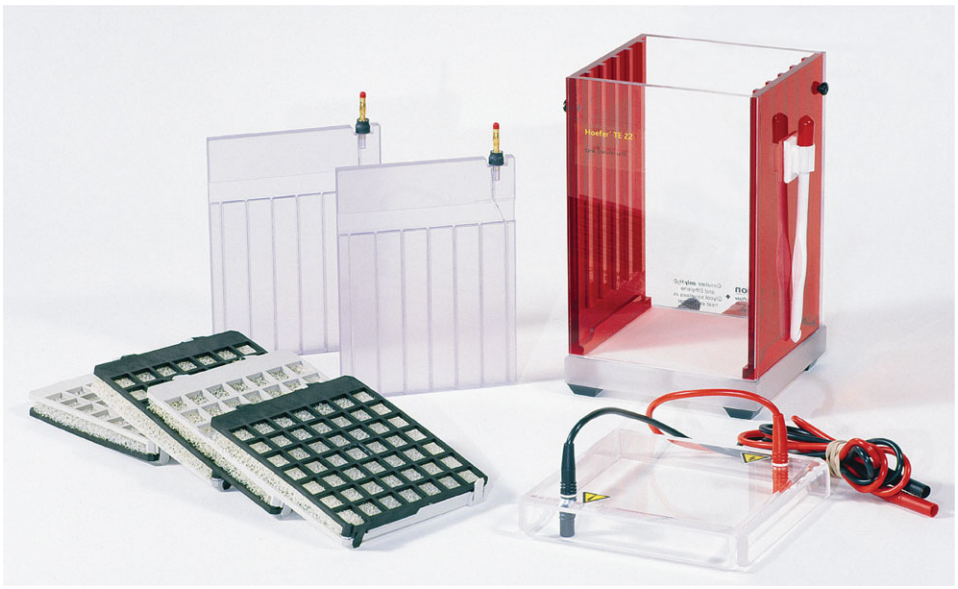

Figure 8.3.1 Minigel tank electrotransfer unit. Designed for smaller 8 × 10–cm gels, these units will process four gels at a time. Note that the two outside panels hold the electrode grid. Figure courtesy of Hoefer, Inc.

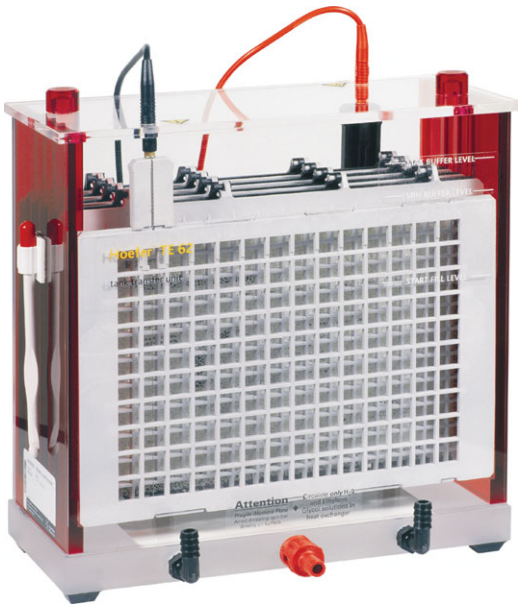

Figure 8.3.2 Large gel tank electrotransfer unit. Designed for larger 15 × 21–cm gels, these units will process four gels at a time. Note that the two outside panels hold the electrode grid. Figure courtesy of Hoefer, Inc.

Visualizing Immunoblots

After probing with the primary antibody, the membrane is washed and the antibody-antigen complexes are identified with horseradish peroxidase (HRPO) or alkaline phosphatase (AP) enzymes coupled to the secondary anti-IgG antibody (e.g., goat anti–rabbit IgG). The enzymes are attached to the secondary antibody either directly (Basic Protocol 3) or via an avidin-biotin bridge (Alternate Protocol 3). Chromogenic (Basic Protocol 4) or luminescent (Alternate Protocol 4) substrates are then used to visualize the activity.

Figure 8.3.3 Slot-blot unit. Through use of a vacuum manifold or by simple hand spotting, up to 96 samples can be applied to a single NC or PVDF membrane for immunoblotting analysis. Although this approach cannot discriminate between the protein of interest and a cross-reactive antigen, it is a quick way to perform preliminary characterization and high-volume, routine quantitation of a sample. Figure courtesy of Hoefer, Inc.

Table 8.3.2 Properties and Compatibilities of Total Protein Stains for Detecting Membrane Transfers

		Membrane types		Gel types		
Stain	Detection limit	Nitrocellulose	PVDF	SDS-PAGE	Native PAGE	Comments
Ponceau S	2 μg	+	+	+	+	Reversible, visible
India ink	50 ng	+	+	+	+	Permanent, visible
Gold	3 ng	+	+	+	+	Permanent, visible
SYPRO Ruby	2 ng	+	+	+	+	Reversible, fluorescent

Abbreviations: PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Alternatively, instead of using enzymatic reactions, the secondary anti-IgG antibody is coupled to a fluorescent tag, and the fluorescence from the antibody-antigen complexes are visualized through illumination by overhead excitation light (Alternate Protocol 5).

Total Protein Stains

Protocols for staining membranes to detect electroblotted proteins are straightforward and use either white light or fluorescence imaging (see *UNIT 7.4*; Gallagher and Sasse, 2012). Detection limits for each staining method and the compatible blot transfer membranes and gels are presented in Table 8.3.2. Depending on the transfer membrane used and other considerations (e.g., the use of SDS-polyacrylamide gels or polyacrylamide gels without SDS and the level of sensitivity required), different total protein staining procedures must be selected.

Ponceau S staining

Ponceau S staining (Salinovich and Montelaro, 1986; see Support Protocol 1) is a simple method for visualizing proteins on nitrocellulose and PVDF membranes. Although relatively insensitive (see Table 8.3.2), Ponceau staining permits a quick visual inspection of the blot to verify transfer and to mark the positions of the molecular weight standards. The stain readily washes away and does not interfere with subsequent immunostaining.

India ink staining

Probably the simplest approach is staining with India ink (Support Protocol 2), which is also compatible with chemiluminescent imaging (Eynard and Lauriere, 1998). The protocol for India ink staining of polypeptides is based on the procedure described by Hancock and Tsang (1983).

Gold staining

Gold staining (which stains the proteins pink) is very sensitive and easy to perform. Staining of membrane-bound polypeptides by gold sol is mediated by hydrophobic interactions and ionic interactions of negatively charged gold particles with positive groups on the proteins. Support Protocol 3 provides methods described by Moeremans et al. (1985).

Increasing staining by alkali treatment

Brief exposure to alkali as described in Support Protocol 4 significantly enhances staining of proteins by either India ink or colloidal gold. Sutherland and Skerritt (1986) suggest that the alkali treatment enhances retention of proteins on the nitrocellulose surface during extensive washing of the membrane; hence, more protein is available for staining.

SYPRO Ruby staining

Fluorescent methods for total protein staining have gained popularity because of their high sensitivity and direct compatibility with colorimetric, fluorogenic, and chemiluminescent techniques. A method is described for staining blots with SYPRO Ruby (see Support Protocol 5), a sensitive fluorescent stain that works equally well on both nitrocellulose and PVDF blots. Support Protocol 6 provides detail for photographing fluorescently stained blots and recording the results.

Compatibility considerations

SYPRO Ruby staining, in contrast to India ink and colloidal gold, is compatible with colorimetric, fluorogenic, and chemiluminescent immunodetection techniques. Stains such as colloidal gold often block epitopes required for subsequent immunodetection. In addition, the dark color of the colloidal gold or India ink stains makes it difficult to visualize colorimetric or fluorogenic immunodetection reagents in subsequent immunodetection assays on the membrane.

Chromogenic versus Luminescent and Fluorescent Assays

Immunoblotted proteins can be detected by chromogenic or luminescent assays; see Table 8.3.3 for a description of the reagents available for each system, their reactions, and a comparison of their advantages and disadvantages. Luminescence detection methods offer several advantages over traditional chromogenic procedures: (1) In general, luminescent substrates increase the sensitivity of both HRPO and AP systems without the need for radioisotopes. Substrates for AP systems are generally available as proprietary kits, although there is significant literature describing these reagents (see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Kricka et al., 2000). (2) Luminescence detection can be completed in as little as a few seconds; exposures rarely go more than

Table 8.3.3 Chromogenic, Luminescent, and Fluorescent Visualization Systems

System	Reagent[a]	Detection/reaction	Comments[b]
Chromogenic			
HRPO-based	4CN	Purple precipitate formed by oxidized products	Not very sensitive (Tween 20 inhibits the reaction); fades rapidly upon exposure to light
	DAB/$NiCl_2$[c]	Dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned
	TMB[d]	Dark purple stain	More stable, less toxic than DAB/$NiCl_2$; may be somewhat more sensitive[d]; can be used with all membrane types; kits available from KPL, Invitrogen, Sigma-Aldrich, and Vector Labs
AP-based	BCIP/NBT	Dark blue-gray stain: BCIP oxidized by NBP hydrolyzes to produces indigo precipitate; reduced NBT precipitates	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRPO-based	For example, luminol/H_2O_2/ *p*-iodophenol	Blue light produced by oxidized luminol substrate; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction detected within a few seconds to 1 hr; also see Haan and Behrmann (2007) for a lab-made reagent mix. Numerous commercial kits are available through GE, Thermo Fisher (Pierce), BioRad, and others.
AP-based Fluorescent antibody labels	For example, substituted 1,2-dioxetane-phosphates	Light produced by dephosphorylated substrate fluorescence from excitation light.	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting); kits available from Thermo Fisher adapt to existing protocols with little modification.
	See Tables 8.3.8 to 8.3.10	Ex/Em Wavelength pairs range from 400 to 900 nm.	Once the secondary fluorescent tagged Ab is incubated with blot and blot washed, imaging can begin without further development. Blots can be dried and saved for later imaging without further development.

[a]Recipes and suppliers are listed in Reagents and Solutions except for TMB for which use of a kit is recommended.
[b]See Commentary for further details.
[c]DAB/$NiCl_2$ can be used without the nickel enhancement, but it is much less sensitive.
[d]McKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.
Abbreviations: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-napthol; DAB, 3,3′-diaminobenzidine; HRPO, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine.

1 hr. (3) Depending on the system, the luminescence can last for 3 days, permitting multiple exposures of the same blot. (4) Furthermore, the signal is easily detected by digital charge-coupled device (CCD) systems, allowing simple digital overlay and comparison to blots stained for total protein. Compared to chromogenic development, the luminescent image recorded by CCD imaging is easier to photograph and quantitate because of the ability to record a wide dynamic range of signal over prolonged exposure. (5) Luminescent blots can be easily erased and re-probed because the reaction products are soluble and do not deposit on the membrane (see below). On the other hand, AP-based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted. Fluorescent blots, while more complex because of the requirement for an excitation light source, offer many advantages: (1) most importantly, a wide range of near-infrared (NIR) and visible fluorescent labels permit multiplexing so that several proteins in a sample can be detected and analyzed at the same time on a single protein blot; (2) NIR labels, in particular, offer very low background and high signal-to-noise ratio for quantitative imaging; and (3) fluorescent labels afford high stability of the label on the processed and dried blot. With a shelf life of many months, the blot can be repeatedly imaged dry.

Examples of Where Immunoblotting is Used

Protein blotting has important clinical applications; for one, it is the confirmatory test for human immunodeficiency virus type 1 (HIV-1). SDS-PAGE-separated virus proteins are blotted onto nitrocellulose membranes and processed with patient sera. After washing (using a Tween 20/nonfat dry milk diluent and washing solution) and incubating with an anti-human IgG coupled to HRPO or alkaline phosphatase, the antigens are identified by chromogenic development. Typically, the prepared blots and all reagents needed for the test are purchased commercially (e.g., Immunetics; *http://www.immunetics.com/*).

Other areas of growing importance for immunoblotting are detection of post-translational modifications such as reversible protein phosphorylation, a widely used regulatory strategy in signal transduction. A challenge with phosphoprotein analysis is the sheer complexity of the number and type of phosphoproteins in a cell. Reducing the complexity requires some form of prefractionation and separation, including SDS-PAGE followed by immunoblotting and visualization with phosphospecific antibodies (Morandell et al., 2006). For a typical immunoblotting experiment to identify the phosphoprotein, a combination of cell fractionation, one-dimensional or two-dimensional electrophoresis, fluorescent phosphoprotein staining (ProQ Diamond), and protein immunoblotting with phosphospecific antibodies is used.

STRATEGIC PLANNING

It is mandatory that all glassware and plasticware used with the gels and blot membranes be thoroughly cleaned in order to avoid staining artifacts. All blot membranes should be handled with forceps only. The total protein stains cannot be used for detecting polypeptides blotted onto nylon membranes, because these membranes are positively charged. Furthermore, due to its very high sensitivity, the gold stain is susceptible to impurities present in buffers and on the surface of staining boxes.

Choice of Materials

The following choices should be considered and made prior to beginning the immunoblotting experiment.

Primary antibody

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always

be run in parallel. Dilution and incubation time for primary antibodies and conjugates should always be optimized.

Membrane blocking agent

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1999). These include Tween 20, polyvinylpyrrolidone (PVP), nonfat dry milk, casein, bovine serum albumin (BSA), and serum. A 0.1% solution of Tween 20 in TBS (TTBS; see recipe in Reagents and Solutions), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution (BLOTTO), TTBS is stable and has a long shelf life at 4°C. Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink.

Even with the application of such standard blocking reagents as 5% to 10% milk protein or 0.05% to 0.1% Tween 20, background can still be a significant problem. If this happens, using a blocking protein (e.g., goat, horse, or rabbit normal serum) from the same species as the primary antibody can reduce the background, presumably by reducing cross-reactivity between the primary antibodies and the blocking agent. If a secondary antibody detection is used, the blocking protein (i.e., normal serum) should be from the same species as the secondary antibody.

Combinations of blocking agents can also be effective. Thus, 0.1% human serum albumin (HSA) and 0.05% Tween 20 in TBS is recommended when probing PVDF membranes with human serum (Craig et al., 1993). However, this can also lead to overall loss of antigen signal, requiring a 10-fold increase in the primary antibody (serum) concentration to achieve an adequate background-free antigen signal.

Compared to dry milk, purified casein has minimal endogenous alkaline phosphatase activity (AP activity leads to high background), and is therefore recommended as a blocking agent for nitrocellulose and PVDF. Because nonfat dry milk and casein may contain biotin that will interfere with avidin-biotin reactions, subsequent steps are performed without protein-blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

Type of membrane

Two membrane types, nitrocellulose (Burnette, 1981) and PVDF (Pluskal et al., 1986), are in wide use for protein blotting applications. Nitrocellulose is the historical choice, and it is still in wide use due to its lower cost and straightforward handling. Nitrocellulose is prepared for blotting by simply wetting it with water or aqueous buffer. In addition, it is tolerant of SDS in the transfer buffer, which is added for more efficient transfer of proteins, particularly high-molecular-weight proteins, out of the gel and onto the membrane. In contrast, PVDF membranes will not wet in aqueous buffers and must be equilibrated in 100% methanol prior to wetting in transfer buffer. However, PVDF has a much higher protein binding capacity (100 to 200 $\mu g/cm^2$ for PVDF) compared to nitrocellulose (80 to 100 $\mu g/cm^2$). PVDF is tough and will not crack or tear under normal use, unlike nitrocellulose. In addition, PVDF is popular for protein sequencing off of the membranes (Fernandez and Mische, 1995). For fluorescent protein blotting, both nitrocellulose and PVDF work well. PVDF, however, requires a low-fluorescent-background version specifically made for fluorescent visualization.

General Considerations

When using chemiluminescent detection for immunoblotting, high background frequently occurs, particularly for strong signals (Pampori et al., 1995). Several methods are

available for reducing the background from chemiluminescent reactions. These include changing the type and concentration of blocking agents (see above), optimizing antibody concentrations, letting the reaction proceed for several minutes before exposing to film, or simply limiting the exposure time of the film on the blot. These procedures are not always successful, however, and can lead to inconsistent results. An alternative approach is to reduce the concentration of reagents. A 10-fold reduction is a good place to start. This effectively removes the background and has a number of advantages which include lower cost, increased signal-to-noise ratio, and reduced detection of cross-reacting species. Fluorescent detection also benefits from optimization to minimize background. Using low-fluorescence PVDF is recommended, as well as varying the blocking agents, with the proprietary blocking solutions frequently providing the lowest background signal. Highly specific excitation and emission filters that minimize out of band-pass light leakage are also required for low background, high signal-to-noise imaging. Fluorescent blots typically can be stored dry in the dark for several months.

If reprobing is desired, blots can be air dried and stored at 4°C for 3 months after chemiluminescence detection. After drying, store in a sealed freezer bag until use (the probe is not typically removed before drying and storage). Repeated probing will lead to a gradual loss of signal and increased background. However, this will depend in part on the properties of the sample.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and 1 hr on a minigel. Transfer time can be 1 hr (high-power transfer) to overnight (Ponceau S staining takes < 1 hr). The India ink staining procedure requires ~6 hr, while gold staining can be performed in ~3 hr, and an additional 30 min is required at the beginning of either protocol to perform the alkali pretreatment. SYPRO fluorescent staining is completed in < 1 hr. Blocking, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min for chromogen, and a few seconds to several hours for luminescence.

PROTOCOLS

The typical steps for performing western blotting are described in Basic Protocols 1 (semidry systems) and 2 (tank transfer systems). Alternate Protocol 1 describes a rapid dry blotting systems (iBlot from Life Technologies). Dot and slot blots (Alternate Protocol 2) are alternatives useful for preliminary or routine repeated characterizations. Antibody-antigen complexes on the blotting membranes are identified with horseradish peroxidase (HRPO) or alkaline phosphatase (AP) enzymes coupled to the secondary anti-IgG antibody (e.g., goat anti–rabbit IgG) either directly (Basic Protocol 3) or via an avidin-biotin bridge (Alternate Protocol 3). Chromogenic or luminescent substrates are then used to visualize the activity (see Basic Protocol 4 and Alternate Protocol 4). The use of fluorescent tags for detection in place of coupled enzymes is illustrated in Alternate Protocol 5. Support Protocols 1 to 6 are related to total protein staining used to visualize standards and to verify the transfer of proteins from the gels to the blotting membranes prior to immunostaining.

NOTE: All steps requiring membrane washing, staining, and equilibration should use continuous, gentle reciprocating or rotating shaking (e.g., on an orbital shaker at 50 rpm) for efficient and even coverage of the reagents across the membrane. For PVDF membranes, be sure to float the membrane face down on the solution. Staining in separate containers for each membrane is recommended to avoid contact transfer of the blotted proteins. Handle gels and membranes with forceps or powder-free gloves.

Basic Protocol 1: Protein Blotting with Semidry Systems

Even and efficient transfer of most proteins can be accomplished with semidry blotting. The gel is held horizontally between buffer-saturated blotting paper that is in contact with the electrodes (Fig. 8.3.4) and requires only a small amount of buffer. The electrodes are close together, giving high field strengths and rapid transfer with a standard electrophoresis power supply. Prolonged transfers (>1 hr) are not recommended; tank blotting (see Basic Protocol 2) should be used for proteins that require long blotting times for efficient transfer. Because transfer efficiency depends on many factors (e.g., gel concentration and thickness and protein size, shape, and net charge), results may vary. This protocol provides a guideline for 0.75-mm-thick SDS-PAGE gels transferred by semidry blotting.

Materials

Samples for analysis

Protein molecular weight standards (*UNIT 8.4*; Haushalter, 2008): prestained (Sigma or BioRad), biotinylated (Vector Labs or Sigma), fluorescent (e.g., BenchMark fluorescent protein standards; Thermo Fisher Scientific/Invitrogen), or compatible with other colorimetric and fluorescent detection method (e.g., MagicMark and MagicMark XP western protein standards; Thermo Fisher/Invitrogen); see Table 8.3.4.

Transfer buffer (see recipe)

100% methanol

UV transilluminator or overhead illuminator (e.g., UVP)

Transfer membrane: 0.45-μm nitrocellulose (e.g., Millipore or Schleicher & Schuell) or polyvinylidene difluoride (PVDF; Millipore Immobilon P)

Plastic trays (polypropylene for PVDF membranes), larger than the gels

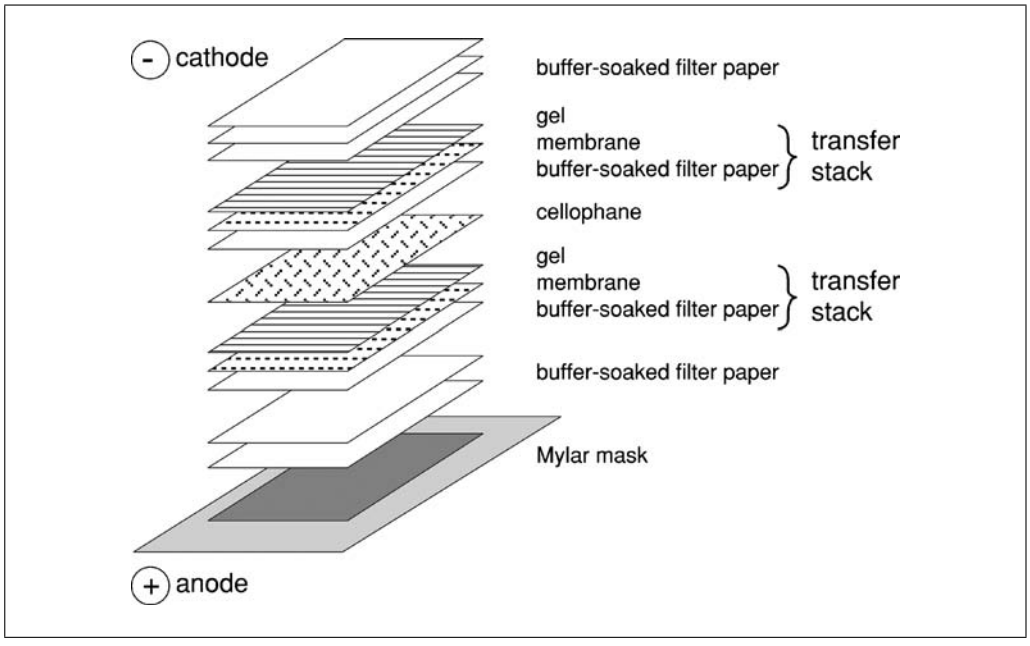

Figure 8.3.4 Immunoblotting with a semidry transfer unit. Generally, the lower electrode is the anode, and one gel is transferred at a time. A Mylar mask (optional in some units) is put in place on the anode. This is followed by three sheets of transfer buffer–soaked filter paper, the membrane, the gel, and finally, three more sheets of buffer-soaked filter paper. To transfer multiple gels, construct transfer stacks as illustrated, and separate each with a sheet of porous cellophane. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Transfer is achieved by applying a maximum current of 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-size gel (14 × 14 cm), this means 60 and 200 mA, respectively.

Table 8.3.4 Protein Standards for Western Blotting

Protein standard	Application
Unstained	Molecular weight calibration and transfer efficiency; can be visualized with total protein stains
Tagged	Molecular weight calibration and transfer efficiency; visualized during immunodetection steps; a variety of potential tags, including biotinylated and antibody-specific amino acid sequence engineered into standard proteins
Prestained	Excellent for checking transfer efficiency and visual inspection of the blot; typically do not produce as sharp a band as other standards, making precise molecular weight calculations difficult

Razor blade or spatula
Six sheets of Whatman 3 MM filter paper or equivalent, cut to size of gel
Semidry transfer unit (Hoefer, BioRad)
Glass test tube (for removal of air bubbles by rolling over membrane)
Porous cellophane (Hoefer, Gel Company) or dialysis membrane), equilibrated with transfer buffer (see recipe), optional
Indelible pen (e.g., PaperMate) or soft lead pencil

Additional reagents and equipment for performing one-dimensional gel electrophoresis (*UNIT 7.3*; Gallagher, 2012) and staining proteins in gels (*UNIT 7.4*; Gallagher and Sasse, 2012)

Electrophorese samples

1. Prepare samples and separate proteins using small or standard-size one-dimensional gels (*UNIT 7.3*; Gallagher, 2012) or gradient gels. Include protein markers when running the gels.

   The protein markers will be transferred to the membrane along with the proteins of interest and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

   MagicMark western protein standards allow direct visualization of protein size standards on membrane blots without the need for protein modification or special detection reagents. The standard proteins are derived from E. coli cells containing a construct with repetitive units of a fusion protein forming the size variation and an IgG binding site. These protein standards do not have to be heated or reduced; they are in a ready-to-use format.

   The proteins can be visualized with the colorimetric, chemiluminescent, or fluorescent detection system of choice simply by processing the membrane for the specific protein. The IgG binding site will allow all the standard proteins to react with the specific primary and secondary antibodies.

   Alternatively, BenchMark Fluorescent Protein Standards are visualized directly via UV transillumination on a UV transilluminator (available from UVP) when wet, or via overhead UV illumination (apparatus also available from UVP) when dry.

   A variety of gel sizes and percentages of acrylamide can be used (Table 8.3.5). Most routinely used are either 14 cm × 14 cm × 0.75–mm gels or 8 cm × 10 cm × 0.75–mm minigels. Acrylamide concentrations vary from 5% to 20%, but are usually in the 10% to 15% range.

Prepare transfer membrane

2. Cut the membrane to the same size as the gel plus 1 to 2 mm on each edge.

Table 8.3.5 Recommended Acrylamide Percentages for Resolving Proteins

Percent acrylamide (resolving gel)	Size range transferred (~100% efficiency) in kDa
5-7	29-150
8-10	14-66
13-15	< 36
18-20	< 20

For nitrocellulose membranes

3a. Slowly place the membrane into distilled water in a plastic tray, holding one edge at a 45° angle.

IMPORTANT NOTE: *The water will wick up into the membrane, wetting the entire surface. If it is inserted too quickly into the water, air gets trapped and will appear as white blotches in the membrane, and protein will not transfer onto these areas.*

Precut membranes, matched to precast minigels, are convenient and minimize handling that can damage the membrane and lead to artifacts.

4a. Decant the water and equilibrate 10 to 15 min in transfer buffer.

For PVDF membranes

3b. Immerse membrane 1 to 2 sec in 100% methanol in a polypropylene plastic tray.

PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer.

4b. Decant the methanol and equilibrate 5 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet the membrane once again with methanol and transfer buffer as described above.

Assemble transfer stack

5. Disassemble the gel sandwich after completion of SDS-PAGE separation. Excise the stacking gel (if present) with a razor blade or spatula and discard.

6. *Optional:* Equilibrate the separating gel 10 to 15 min in transfer buffer.

Equilibration of the separating gel with transfer buffer is not normally required for semidry blotting, but it may improve transfer in some cases.

7. Place three sheets of filter paper saturated with transfer buffer on the anode (Fig. 8.3.4) of the semidry transfer unit.

Most transfer units are designed so that negatively charged proteins move downward toward either a platinum or graphite positive electrode (anode).

The filter paper should be cut to the exact size of the gel. This forces the current to flow only through the gel and not through overlapping filter paper. Some manufacturers (e.g., Hoefer) recommend placing a Mylar mask on the lower platinum anode. With an opening that is slightly less than the size of the gel, the mask forces the current to flow through the gel and not the surrounding electrode area during transfer.

8. Place the equilibrated transfer membrane on top of the filter paper stack. Remove all bubbles between the membrane and filter paper by rolling a test tube over the surface of membrane.

Any bubbles in the filter paper stack or between the filter paper, membrane, and gel will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

9. Place the gel on top of the membrane. Gently roll a test tube over the surface of gel to ensure intimate contact between gel and membrane and to remove any interfering bubbles.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

10. Complete the transfer stack by putting the three remaining sheets of filter paper on top of the gel. Roll out bubbles as described above.

    Multiple gels can be transferred using semidry blotting. Simply put a sheet of porous cellophane (Hoefer) or dialysis membrane (BioRad or Sartorius) equilibrated with transfer buffer between transfer stacks (Fig. 8.3.4). Transfer efficiency is dependent on the position of the transfer stack in the blotting unit, and for critical applications, transferring one gel at a time is recommended. The gel next to the anode tends to be more efficiently transferred when blotting more than one gel at a time.

Transfer proteins from gel to membrane

11. Place top electrode onto transfer stack.

    Most units have safety-interlock features and can only be assembled one way. Consult manufacturer's instructions for details.

    Once the transfer stack has been assembled with both electrodes, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane. Some transfer will occur as soon as the gel contacts the membrane, and any shifting of the transfer stack after assembly will distort the transfer pattern.

12. Carefully connect high-voltage leads to the power supply [see *UNIT 7.1* (Gallagher, 2014a) for safety precautions]. Apply constant current to initiate protein transfer.

    Transfers of 1 hr are generally sufficient.

    In general, do not exceed 0.8 mA/cm^2 of gel area, or overheating and drying of the gel will result in poor transfer efficiency and resolution. For a typical minigel (8 × 10 cm) and standard-size gel (14 × 14 cm), this means ~60 and 200 mA, respectively.

    Monitor the temperature of the transfer unit directly above the gel by touch. The unit should not exceed 45°C. If the outside of the unit is warm, too much current is being applied. Note that units with graphite electrodes are more prone to heating, because graphite has much more resistance to current flow than platinum or steel electrodes.

13. After transfer, turn off the power supply and disassemble the unit. Remove the membrane from the transfer stack and mark the side facing the gel and the top orientation with a soft lead pencil.

14. Proceed with staining for total protein (Support Protocols 1 to 6) and immunoprobing (see Basic Protocol 3 or Alternate Protocol 3).

Basic Protocol 2: Tank Transfer

In this method, the gel and membrane are placed vertically into a tank filled with transfer buffer (Fig. 8.3.5). Tank blotting is the historical method of choice for immunoblotting. In contrast to the semidry technique (Basic Protocol 1), tank systems are capable of the high power and prolonged (overnight) transfers needed for difficult-to-transfer proteins (e.g., high-molecular-weight proteins from gradient gels). However, tank blotting is more complex to set up and uses large volumes of liquid. For simple routine work, the semidry technique is more convenient.

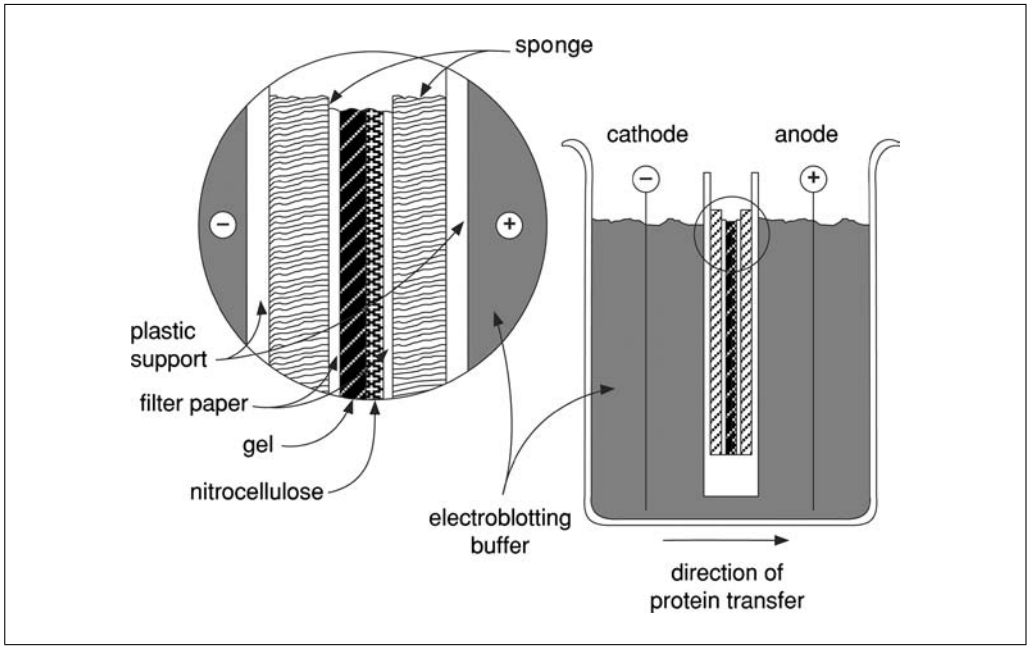

Figure 8.3.5 Immunoblotting with a tank transfer unit. The gel/membrane sandwich is held in a transfer cassette between two pads and assembled in the following order: three sheets of transfer buffer–soaked filter paper, transfer buffer–equilibrated gel, wet membrane, and three sheets of transfer buffer soaked filter paper. The sandwich is placed in the cassette and immersed in transfer buffer between the electrodes. For transfer of negatively charged protein, the membrane is positioned on the anode (+) side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel.

Materials

Samples for analysis

Protein molecular weight standards (*UNIT 8.4*; Haushalter, 2008): prestained (Sigma or BioRad), biotinylated (Vector Labs or Sigma), fluorescent (e.g., Benchmark fluorescent protein standards; Thermo Fisher/Invitrogen), or compatible with other colorimetric and fluorescent detection method (e.g., MagicMark and MagicMark XP western protein standards; Thermo Fisher/Invitrogen); see Table 8.3.4

Transfer buffer (see recipe)

100% methanol

Razor blade or spatula

Plastic tray (polypropylene for PVDF membranes), larger than the gel

0.45-μm nitrocellulose (Millipore or Schleicher & Schuell) or polyvinylidene difluoride (PVDF; Millipore Immobilon P)

Transfer tank blotting apparatus and cassette with sponge (Hoefer, BioRad, or Thermo Fisher/Invitrogen; see Fig. 8.3.5)

Six sheets of Whatman 3 MM filter paper or equivalent, cut to size of gel

Glass test tube (optional)

Heat exchanger and cooling recirculating water bath (optional)

Additional reagents and equipment for one-dimensional or gradient gel electrophoresis (*UNIT 7.3*; Gallagher, 2012) and staining proteins in gels (*UNIT 7.4*; Gallagher and Sasse, 2012)

Prepare gel

1. Prepare samples and separate proteins using small or standard-size one-dimensional gels (*UNIT 7.3*; Gallagher, 2012) or gradient gels. Include protein markers when running the gels.

2. Remove the gel from the cassette or glass sandwich after completion of SDS-PAGE separation and excise the low-percentage stacking gel (if present) with a razor blade or spatula.

   Precast gradient gels typically do not have stacking gels included.

3. Place the gel in a plastic tray with enough transfer buffer for the gel to freely float, and equilibrate 5 to 15 min, with gentle shaking.

   Due to the differences between the electrophoresis gel buffer and the transfer buffer (pH, buffer strength, alcohol content), it is important to equilibrate the gel in the transfer buffer. This ensures equivalent pH and ionic strength between the gel and the transfer buffer, and also ensures that any shrinkage of the gel occurs before the actual transfer. Note that the gel will also shrink in typical transfer buffer because of the 20% methanol, and shrinkage during the actual blotting will decrease transfer resolution. Low-percentage or thin gels (< 10%, 1 mm) should keep equilibration to a minimum (5 to 10 min), so no proteins are lost by diffusion out of the gel.

Prepare membrane

4. Cut the membrane to same size as the gel plus 1 to 2 mm on each edge.

For nitrocellulose membranes

5a. Slowly place the membrane into distilled water in a plastic tray, holding one edge at a 45° angle.

   IMPORTANT NOTE: *The water will wick up into the membrane, wetting the entire surface. If it is inserted too quickly into the water, air gets trapped and will appear as white blotches in the membrane, and protein will not transfer onto these areas.*

   Precut membranes, matched to precast minigels, are convenient and minimize handling that can damage the membrane and lead to artifacts.

6a. Decant the water and equilibrate 5 min in transfer buffer.

For PVDF membranes

5b. Immerse membrane 1 to 2 sec in 100% methanol in a polypropylene plastic tray.

   PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer.

6b. Decant the methanol and equilibrate 5 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet the membrane once again with methanol and transfer buffer as described above.

Assemble transfer sandwich

7. Immerse the sponge and filter paper in transfer buffer prior to assembly.

8. Start with one layer of sponge material and add two layers of buffer-saturated filter paper, equilibrated gel, membrane, and two sheets of buffer-saturated filter paper.

9. With a gloved hand, push out any air trapped between the layers. Alternatively, roll a glass test tube over each layer during assembly to ensure good contact between the membrane and gel and the removal of trapped bubbles between the layers.

   The membrane side should face the positive electrode.

10. Fill the transfer tank with enough buffer to cover the top of the panel by at least 2 cm. Place the assembled cassette into the tank.

    This ensures complete heat removal during transfer.

Table 8.3.6 Rapid High-Power Transfer Conditions for Tank Transfer

Gel/unit size	Number of gels	Voltage	Current	Transfer time at 15°C[a]
9 × 10 cm	1-4 gels	100 V	400 mA	1 hr
15 × 21 cm	1-4 gels	100 V	1 A	1-3 hr

[a]High-power transfer requires a transfer unit with a heat exchanger and a cooling recirculating water bath. For uncooled transfer, set the voltage to 10 V overnight constant voltage.

Transfer proteins from gel to membrane

11. Connect the power supply and set the cooling temperature (if available) to 15°C to 20°C.

    For high-power transfers, cooling is required (see Table 8.3.6). For optimal reproducibility, use the same temperature for the transfer.

12. Carefully connect high-voltage leads to the power supply [see *UNIT 7.1* (Gallagher, 2014a) for safety precautions]. Electrophoretically transfer proteins from gel to membrane for 30 min to 1 hr at 100 V with cooling (see Table 8.3.6) or overnight at 14 V (constant voltage), in a cold room.

    Overnight transfers can be performed in a cold room at 10°C to 14°C without an external cooling unit. Lower-percentage (protein of interest migrates to < 0.5 R_f; see UNIT 7.3) and thinner gels (0.75 mm thick) are recommended for efficient transfer. Improved transfer efficiency may require SDS (up to 0.1%) in the transfer buffer and reduction or elimination of methanol. However, this may lead to reduced binding of the protein to the membrane, and conditions need to be optimized.

13. After transfer, turn the voltage on the power supply to 0 and turn off the power. Remove the safety lid and pull the transfer cassette out of the tank. Remove the membrane from the transfer stack and mark the side facing the gel and the top orientation with a soft lead pencil.

14. Stain and destain the gel (e.g., Coomassie blue; see *UNIT 7.4*; Gallagher and Sasse, 2012).

    Any protein remaining in the gel indicates an incomplete transfer. Transfers are seldom 100%.

15. Stain the membrane with Ponceau S (Support Protocol 1) or SYPRO Ruby (Support Protocol 5) to confirm protein transfer from the gel to the membrane.

Alternate Protocol 1: Rapid Western Transfer with iBlot Dry Blotting System

An extension of the semidry concept (see Basic Protocol 1) is the new generation of fast semidry blotters. Using specialized buffers and integrated programmable power supplies, several dedicated dry/semi-dry transfer systems can transfer 10- to 220-kDa proteins from polyacrylamide gels to nitrocellulose or PVDF membranes in 3 to 12 min (Table 8.3.7). Among these rapid transfer systems, the iBlot Dry Blotting system from Life Technologies (Silva and McMahon, 2014) is a dry transfer system that only supports the rapid transfer process—all others are semidry systems effective for both rapid (3- to 12-min) or standard (30 to 60 min) transfer protocols. Most of these systems require special kits (blotting paper and nitrocellulose or PVDF membranes are included) from the same company to perform the rapid process; however, the Pierce Power Blotter system only requires the Pierce 1-Step Transfer Buffer from the company.

Here we use iBlot Dry Blotting system and one minigel as an example to introduce the rapid transfer process for western blotting. Refer to the user manual for optimization condition before proceeding. Pretreatment of the gel after electrophoresis is generally

Table 8.3.7 List of Representative Rapid Transfer Systems

Name	Manufacturer	Proteins range (kDa)	Transfer time(min)	Web site
Lightning Blot system	Perkin Elmer	10-250	6-12	*http://www.perkinelmer.com/catalog/category/id/lightning%20blot%20system*
Trans-blot Turbo system	BioRad	5-300	3-10	*http://www.bio-rad.com/en-us/product/semi-dry-rapid-blotting-systems/trans-blot-turbo-transfer-system*
Pierce Power Blotter	Thermo Fisher Scientific	10-300	<10	*http://www.piercenet.com/product/pierce-power-blotter*
iBlot Dry Blotting system	Life Technologies	11-220	7-10	*http://www.lifetechnologies.com/us/en/home/life-science/protein-expression-and-analysis/western-blotting/western-blot-transfer/iblot-dry-blotting-system.html*.

not required, but transfer is improved for proteins >150 kDa by equilibration of the gel in 20% ethanol for 5 to 10 min prior to the transfer.

Materials

Pre-run gel containing protein samples and protein standard (*UNIT 7.3*; Gallagher, 2012)
iBlot Dry Blotting System (Life Technologies)
Gel Transfer Stacks Mini (including bottom stack, top stack and sponge) for blotting one minigel (Life Technologies
Blotting roller (included in the iBlot transfer device package)
Forceps

Set up iBlot device

1. Place the device on a stable flat surface, plug in the power cord, and turn on the power switch.
2. Open the lid of the iBlot Gel Transfer device.
3. Remove the sealing from the disposable sponge and place it on the inside of the lid. Make sure the metal contact is in the upper right corner of the lid.
4. Remove the sealing from the iBlot Gel Transfer bottom stack. Keep the stack in the red plastic tray and place the anode stack onto the blotting surface of the transfer device with the tray tab facing toward the right.

Prepare the gel for blotting

5. Carefully place the pre-run gel on the transfer membrane, ensuring that the gel does not protrude over the edges of the membrane.
6. Remove the bubbles using the blotting roller.
7. Remove the sealing from the iBlot Gel Transfer top stack and discard the red plastic tray.
8. Place the top stack, with the gel facing down, onto the pre-run gel.
9. Remove the bubbles by the blotting roller.
10. Close the lid of the device and secure the latch.

Perform blotting

11. Select the appropriate program and run time by pressing the Select button. Use the "Up/Down (±)" buttons to change the program.

    There are a variety of programs stored in the iBlot transfer system. Check the manufacturer's instructions for details.

12. Press the Start/Stop button in the right lower corner.

    The red light turns to green, indicating the start of the run. At the end of the transfer run, the device will automatically shut off and begin beeping for 1 min.

13. Press the Start/Stop button to silence beeping.

14. Open the lid of the iBlot device.

    The stacks are warm to hot, and the total thickness of the stacks reduces

15. Remove the top stack and gel using forceps.

16. Remove the transfer membrane from the stack and proceed with the blocking procedure or stain the membrane.

    Sometimes there is a green stain on the membrane; the stain will reduce by washing the membrane several times.

Process the blot and continue the procedure

17. Process membrane according to immunoprobing protocols for chromogenic, chemiluminescent or fluorescent visualization (Alternate Protocols 3 to 5).

18. Continue with another run (no cool down time needed) or turn off the iBlot power switch.

19. Clean the blotting surface and lid with a clean, damp cloth or paper tissue, and store the device according the manufacturer's recommendations.

Alternate Protocol 2: Slot and Dot Blotting

Through use of a vacuum manifold or by simple hand spotting, up to 96 samples can be applied to a single nitrocellulose (NC) or polyvinylidene difluoride (PVDF) membrane for immunoblotting analysis. In slot and dot blotting, proteins are not separated by electrophoresis before immunoblot analysis. Instead, the entire sample is directly applied to (or spotted on) the membrane. Although this approach cannot discriminate between the protein of interest and a cross-reactive antigen, it is a quick way to perform preliminary characterization and high-volume routine quantitation of a sample.

Additional Materials *(also see Basic Protocol 1)*

<10 μg protein sample in < 100 μl water or TBS (no detergent)
Tris-buffered saline (TBS; *UNIT 3.3*)

Slot and dot blotting apparatus (e.g., Hoefer, BioRad)
Vacuum source

1. Prewet the membrane in distilled water (for nitrocellulose) or 100% methanol (PVDF) as described in Basic Protocol 1, steps 4 and 5.

2. Prepare the slot or dot blot manifold according to manufacturer's instructions.

3. Apply samples under a low house vacuum, followed by two equivalent volumes water or TBS, depending upon which was used to dissolved the sample, to rinse the wells of unbound protein.

   Typically, samples should contain < 10 μg of protein in under 100 μl of solution. Overloading the wells will prevent the flow of the liquid through the well.

   Typical systems use a low house vacuum to pull liquid through each slot, binding the proteins to the membrane.

Support Protocol 1: Ponceau S Staining of Transferred Proteins

Nitrocellulose and PVDF membranes can be reversibly stained with Ponceau S to verify transfer efficiency and indicate the positions of the molecular weight markers. The stain is compatible with subsequent immunostaining procedures.

Materials

Membrane with transferred proteins (Basic Protocol 1 or 2 or Alternate Protocol 2)
Ponceau S solution (see recipe)
Plastic boxes
Pen with indelible ink

Additional reagents and equipment for photographing membranes (*UNIT 7.5*; Moomaw et al., 2014)

1. Stain the membrane in Ponceau S solution for 5 min at room temperature, with gentle agitation.
2. Destain 2 min in water.
3. Photograph membrane if required (*UNIT 7.5*; Moomaw et al., 2014) and mark the molecular-weight-standard band locations with indelible ink.
4. Completely destain membrane by soaking an additional 10 min in water and proceed to immunoprobing.

Support Protocol 2: India Ink Staining of Transferred Proteins

India ink is used to stain total protein on blot transfer membranes. The transferred proteins (~50 ng) appear as black bands on a gray background.

Materials

Membrane with transferred proteins (Basic Protocol 1 or 2 or Alternate Protocol 2)
Tween 20 solution (see recipe)
India ink solution (see recipe)
Plastic boxes

1. *Optional:* Pretreat proteins transferred onto nitrocellulose membrane with alkali (see Support Protocol 4).
2. Place blot transfer membrane(s) in a plastic box containing enough Tween 20 solution to cover the membrane(s) and wash in Tween 20 solution three times for 30 min each time at 37°C, with gentle shaking on an orbital shaker.
3. Stain the membrane in India ink solution 3 hr or overnight, room temperature.
4. Rinse the membrane twice in Tween 20 solution, leave in Tween 20 solution to destain until an acceptable background is obtained, and then air dry the membrane for storage.

   Black bands appear against a gray background. While the membranes can be photographed dry or wet, the contrast of the bands will be greater in a wet membrane.

Support Protocol 3: Gold Staining of Transferred Proteins

A colloidal gold sol is used to stain proteins on blot transfer membranes. The transferred proteins (~3 ng) will appear as red bands on an almost white background.

NOTE: Do not attempt to stain nylon membranes using colloidal gold.

Materials

Nitrocellulose membrane with transferred proteins (Basic Protocol 1 or 2 or Alternate Protocol 2)
Tween 20 solution (see recipe)
Colloidal gold staining solution (BioRad, Sigma)

Plastic boxes
Glass dish or heat-sealable plastic bags
Filter paper

1. *Optional:* Pretreat proteins on the nitrocellulose membrane with alkali (see Support Protocol 4).
2. Place blot transfer membrane(s) in a plastic box containing enough Tween 20 solution to cover the membranes. Wash in Tween 20 solution three times for 30 min each time at 37°C, with gentle shaking on an orbital shaker.
3. Continue to wash the membrane in Tween 20 solution three times for 5 min each time at room temperature.
4. Rinse well with water.
5. Place nitrocellulose blot or dot membrane with transferred proteins in a glass dish containing enough colloidal gold staining solution to cover the membrane. Stain the membrane 30 min to 1 hr at room temperature, with continuous shaking.

   A heat-sealed plastic bag is a convenient container for the staining. Extended staining periods do not impair visualization.

6. Rinse the membrane briefly in water and air dry on filter paper.

Support Protocol 4: Alkali Enhancement of Protein Staining

A brief pretreatment of nitrocellulose-bound protein with alkali enhances subsequent staining with either India ink or colloidal gold. This protocol is specifically for use with nitrocellulose membranes.

Materials

Nitrocellulose membrane with transferred proteins (Basic Protocol 1 or 2 or Alternate Protocol 2)
1% (w/v) KOH
Phosphate-buffered saline (PBS; *UNIT 3.3*)
Glass or Pyrex dish

1. Place nitrocellulose blot or dot membrane with transferred proteins in a glass dish containing enough 1% KOH to cover the membranes. Soak 5 min at room temperature, with gentle agitation.
2. Rinse twice in PBS for 10 min each time at room temperature.

Support Protocol 5: Fluorescent Protein Blot Staining of Transferred Proteins

The fluorescent SYPRO Ruby protein blot stain provides a rapid, simple, and highly sensitive method for detecting proteins on nitrocellulose or PVDF membranes (blots).

Staining total protein before applying specific protein detection techniques provides an assessment of protein transfer efficiency, and makes it possible to detect contaminating proteins in the sample and to compare the sample with molecular weight standards. For blots of two-dimensional gels, total protein staining makes it easier to localize a protein to a particular spot in the complex protein pattern. The bright, orange-red fluorescent stain can be easily visualized using UV illumination or a laser scanner. The staining procedure is simple to perform and can be completed within 1 hr. SYPRO Ruby has a sensitivity limit of 2 to 8 ng/band, making it about 60 times more sensitive than reversible stains like Ponceau S and 20 to 30 times more sensitive than Amido Black or Coomassie Brilliant Blue stains. The SYPRO Ruby protein blot stain will not stain nucleic acids and is compatible with immunodetection and colorimetric, fluorogenic, and chemiluminescent detection techniques, as well as with Edman sequencing and mass spectrometry.

Materials

Nitrocellulose or PVDF membrane with transferred proteins (Basic Protocol 1 or 2 or Alternate Protocol 2)
7% (v/v) acetic acid/10% (v/v) methanol
SYPRO Ruby protein blot stain (Sigma, Thermo Fisher Scientific; also see recipe)
150 mM Tris·Cl, pH 8.8 (*UNIT 3.3*)/20% (v/v) methanol

Small polypropylene staining dish
Orbital shaker
Forceps

NOTE: Perform all washing, staining, and other incubation steps with continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm). For PVDF membranes, be sure to float the membrane face down on the solution.

After electroblotting to nitrocellulose membranes

1a. Completely immerse the membrane in 7% acetic acid/10% methanol and incubate 15 min at room temperature in a small polypropylene staining dish.

2a. Wash the membrane in four changes of deionized water for 5 min each time.

3a. Completely immerse the membrane in SYPRO Ruby protein blot stain for 15 min.

4a. Wash the membrane in deionized water four to six times for 1 min each time to remove excess dye.

Membranes stained with SYPRO Ruby blot stain should be periodically monitored using UV overhead illumination to determine if background fluorescence has been washed away.

After electroblotting to PVDF membranes

1b. Allow the membrane to dry completely.

2b. Float the membrane face down in 7% acetic acid/10% methanol and incubate 15 min.

3b. Float the membrane for 5 min each time in four changes of deionized water, then float the membrane in SYPRO Ruby protein blot stain for 15 min.

4b. Wash the membrane two or three times in deionized water for 1 min each time to remove excess dye.

For subsequent Edman-based microsequencing

1c. Allow a PVDF electroblotted membrane to dry completely.

2c. Stain with SYPRO Ruby protein blot stain as described in steps 2b and 3b.

3c. Partially destain the blot by placing it face down in a solution of 150 mM Tris·Cl, pH 8.8 (*UNIT 3.3*)/20% methanol for 10 min, with gentle agitation.

4c. Rinse the blot four times in deionized water for 1 min each time.

5. Allow the membranes to air dry.

After staining, wet membranes should not be touched because residue found on latex gloves may destroy the staining pattern. Use forceps to handle wet blots. Once dry, the membranes can be handled freely.

6. Proceed to Support Protocol 6 to view and photograph the protein blots.

Support Protocol 6: Viewing and Photographing SYPRO Ruby-Stained Protein Blots

SYPRO Ruby protein blot stain (Support Protocol 5) has two excitation maxima, one at ~280 nm and one at ~450 nm, and it has an emission maximum near 618 nm. Proteins stained with the dye can be visualized using a 300-nm UV overhead or transilluminator, a blue-light transilluminator, or a laser scanner. The stain is photostable, allowing long exposure times for maximum sensitivity. It is important to photograph or otherwise document the SYPRO Ruby stain before immunostaining because over 90% of the stain is washed off the blot during the blocking step. Detection limits and compatibilities of transfer membranes and gel types are summarized in Table 8.3.2.

UV overhead illuminator or transilluminator

Proteins stained with SYPRO Ruby protein blot stain are readily visualized using UV illumination. The front face of the membrane can be illuminated using a hand-held UV-B (~300 nm) light source. Alternatively, a UV light box can be placed on its side to illuminate the blots, or a top-illuminating system such as the UVP Biospectrum System or the BioRad imager can be used to visualize the stain. Satisfactory results can also be obtained from direct UV transillumination through the blotting membrane. In either case, the use of a photographic or CCD camera and the appropriate filters is essential to obtain the greatest sensitivity. The camera's integrating capability can make bands visible that cannot be detected by eye. It is important to clean the surface of the transilluminator after each use with deionized water and a soft cloth (like cheesecloth); otherwise, fluorescent dyes (e.g., SYPRO stains, SYBR stains, and ethidium bromide) will accumulate on the glass surface and cause a high background fluorescence.

Digital documentation is the method of choice, and various types of cameras are available, ranging from simple digital compact consumer cameras to quantitative high–dynamic range cooled scientific imaging cameras and systems (*UNIT 7.5*; Moomaw et al., 2014). When using a CCD camera, the best images are obtained with high resolution (1.4 megapixels or greater) and 12- or 16-bit grayscale levels per pixel. The manufacturer of the particular imaging system should be contacted for recommendations on filter sets to use.

For those still in the analog world and using black-and-white print film, the highest sensitivity is achieved with a 490-nm long-pass filter (e.g., the SYPRO protein gel stain photographic filter; Molecular Probes S-6656). Blots are typically photographed using an *f*-stop of <4.5 for under 1 sec, using ASA 400 film.

Laser-scanning instruments

Blots stained with the SYPRO Ruby protein blot stain can be visualized using imaging systems equipped with lasers that emit at 450, 473, 488, or 532 nm.

Basic Protocol 3: Immunoprobing with Directly Conjugated Secondary Antibody

Immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent (Tween 20 is a common alternative to protein-blocking agents when using nitrocellulose or PVDF filters). Next, it is placed in a solution containing the primary antibody (the antibody directed against the antigen). The blot is washed and exposed to the conjugated secondary antibody (an enzyme-antibody conjugate directed against the primary antibody; e.g., goat anti-rabbit IgG). Antigens are identified by chromogenic or luminescent visualization (see Basic Protocol 4 or Alternate Protocol 4, respectively) of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. The antibody probes are commercially available for a wide range of proteins and can also be prepared in the lab for a new protein so that simplified assays (e.g., for expression, subcellular location, or amount) can be developed. Custom antibodies based on a purified antigen can be prepared commercially (e.g., Covance Immunological Services). A quick way to purify a protein for antibody analysis is by SDS-PAGE. Although denatured, SDS-PAGE-isolated proteins typically generate antibodies that react well against separated and blotted proteins. Several hundred micrograms of highly purified protein can be obtained in this way, and equipment (e.g., Hoefer) is available for preparative electrophoresis. Once the protein is separated, it can be electroeluted from the gel (using, e.g., BioRad PrepCell), concentrated, and used to generate polyclonal antibodies in the animal of choice (e.g., rabbit, goat, or chicken).

Materials

Membrane with transferred proteins (Basic Protocol 1 or 2 or Alternate Protocol 2)
Blocking buffer for colorimetric detection (see recipe) or blocking buffer for luminescence detection (see recipe)
Primary antibody specific for protein of interest (working concentration optimized; see Fig. 8.3.6)
TTBS (nitrocellulose or PVDF membranes) or TBS (nylon membranes; see *UNIT 3.3* for recipes)
Secondary antibody conjugate: horseradish peroxidase (HRPO)- or alkaline phosphatase (AP)-anti-Ig conjugate (MP Biomedical, Vector Labs, KPL, or Sigma-Aldrich; dilute as indicated by manufacturer)

Heat-sealable plastic bag, plastic box, or slotted incubation tray
Orbital shaker or rocking platform

1. Place the membrane in a heat-sealable plastic bag with 5 ml blocking buffer and seal the bag. Incubate 30 min to 1 hr at room temperature, with agitation on an orbital shaker or rocking platform.

   Usually 5 ml buffer is sufficient for two to three membranes (14 × 14–cm size). If the membrane is to be stripped and reprobed (Gallagher et al., 2004), the blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute primary antibody in blocking buffer.

   Primary antibody dilution is determined empirically but is typically 1/100 to 1/1000 for a polyclonal antibody (Fig. 8.3.6) or 1/10 to 1/100 for hybridoma supernatants and ~1/1000 for murine ascites fluid containing monoclonal antibodies. Ten to one hundred-fold higher dilutions can be used with alkaline phosphatase-based or luminescence-based detection systems. Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended. Consult the antibody supplier's instructions for alternative dilution and blocking solution recommendations.

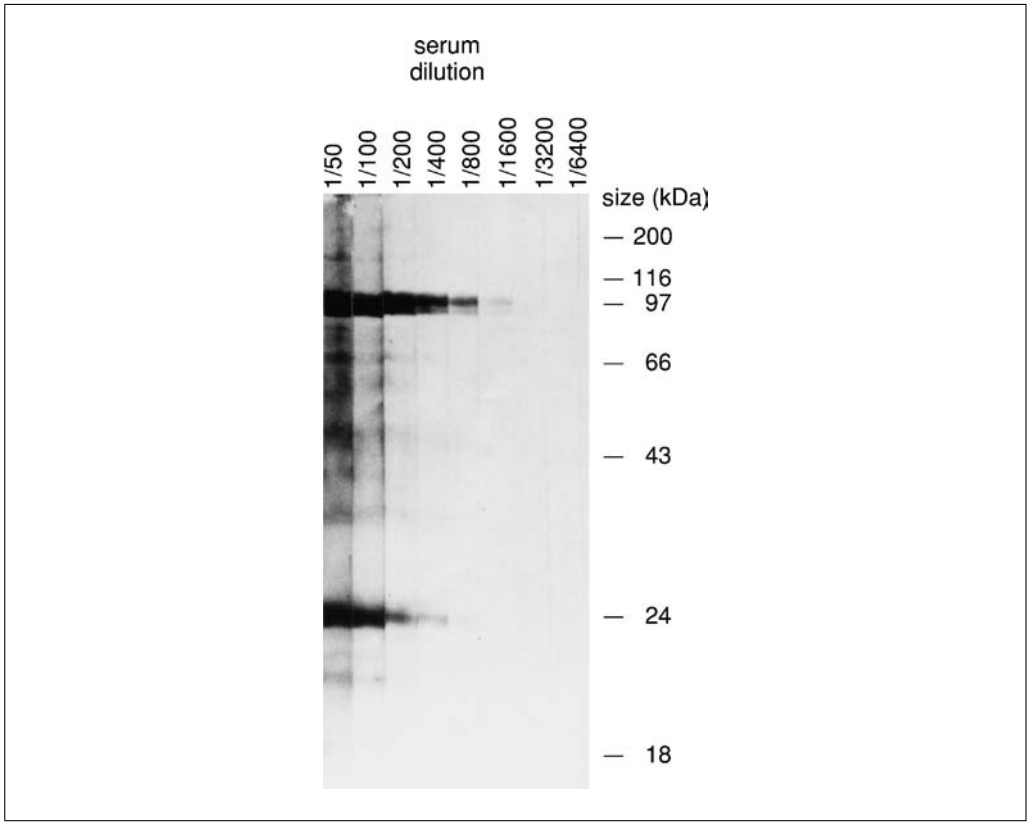

Figure 8.3.6 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. The blot was developed with HRPO-coupled avidin-biotin reagents according to Alternate Protocol 3 and visualized with 4-chloro-1-naphthol (4CN; Basic Protocol 4). Note how background improves with dilution. In this case, a dilution of 1/400 to 1/800 is optimal, keeping the background to a minimum.

To determine the appropriate concentration of the primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 8.3.6).

3. Open the bag and pour out the blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature, with constant agitation.

   Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on the conjugate used.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove the membrane from the plastic bag with a gloved hand. Place in plastic box and wash four times with 200 ml TTBS (nitrocellulose or PVDF; see recipe) or TBS (nylon) for 10 to 15 min each time, with agitation.

5. Dilute the secondary antibody (HRPO- or AP-anti-Ig conjugate) in blocking buffer.

   Commercially available enzyme–conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1999).

6. Place the membrane in a new heat-sealable plastic bag, add diluted conjugated secondary antibody, seal, and incubate 30 min to 1 hr at room temperature, with constant agitation.

   When using plastic incubation trays, see step 3 annotation for proper antibody solution volumes.

7. Remove membrane from bag and wash as in step 4. Develop the color or luminescence according to appropriate visualization protocol (see Basic Protocol 4 or Alternate Protocol 4).

Alternate Protocol 3: Immunoprobing with Avidin-Biotin Coupling to Secondary Antibody

The following procedure is based on the Vectastain ABC kit from Vector Labs. It uses an avidin-biotin complex to attach horseradish peroxidase (HRPO) or alkaline phosphatase (AP) to the biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity due to the multiple reporter enzymes bound to each secondary antibody. In addition, the detergent Tween 20 is a popular alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Additional Materials *(also see Basic Protocol 3)*

Vectastain ABC (HRPO) or ABC-AP (AP) kit (Vector Labs) containing:
Reagent A (avidin)
Reagent B (biotinylated HRPO or AP)
Biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate the membrane in appropriate blocking buffer in a heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature.

   TTBS (see recipe) is well suited for avidin-biotin systems and is typically the only blocking agent needed. Because nonfat dry milk contains residual biotin, which will interfere with the immunoassay, it is typically not used in avidin-biotin systems.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute the primary antibody solution in TTBS (nitrocellulose or PVDF; see recipe) or TBS (nylon).

   Dilutions of sera containing primary antibody generally range from 1/100 to 1/100,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems.

   To determine the appropriate concentration of the primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Schleicher and Schuell; Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 8.3.6).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.

   Usually 5 ml of diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove the membrane from the bag and place in the plastic box. Wash the membrane three times over a 15-min span in TTBS (nitrocellulose or PVDF; see recipe). Add enough TTBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting two drops biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF).

   This dilution gives both high sensitivity and enough volume to easily cover a large 14 × 14–cm membrane.

6. Transfer the membrane to a fresh plastic bag containing the secondary antibody solution. Incubate 30 min at room temperature, with slow rocking, and then wash as in step 4.

   When using plastic incubation trays, see the step 3 annotation for proper antibody solution volumes.

7. While the membrane is being incubated with secondary antibody, prepare avidin-biotin-HRPO or -AP complex.

   a. Add two drops Vectastain reagent A and two drops reagent B to 10 ml TTBS (nitrocellulose or PVDF).
   b. Incubate 30 min at room temperature.
   c. Add 40 ml TTBS.

   Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity. Sodium azide is a peroxidase inhibitor and should not be used as a preservative. Casein, nonfat dry milk, serum, and some grades of BSA may interfere with the formation of the avidin-biotin complex and should not be used when employing avidin or biotin reagents (Vector Labs; Gillespie and Hudspeth, 1991).

8. Transfer the membrane to avidin-biotin-enzyme solution. Incubate 30 min at room temperature, with slow rocking, and then wash over a 30-min span as in step 4.

9. Develop the color or luminescence according to the appropriate visualization protocol (see Basic Protocol 4 or Alternate Protocol 4).

Basic Protocol 4: Visualization with Chromogenic Substrates

Bound antigens are typically visualized with chromogenic substrates. The substrates 4CN, DAB/$NiCl_2$, and TMB are commonly used with horseradish peroxidase (HRPO)–based immunodetection procedures, while BCIP/NBT is recommended for AP-based procedures (see Table 8.3.3). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins, probed with antibody-enzyme complex (see Basic Protocol 3 or Alternate Protocol 3)
Tris-buffered saline (TBS; *UNIT 3.3*)
Chromogenic visualization solution (see Table 8.3.3)

Additional reagents and equipment for photographing gels (see *UNIT 7.5*; Moomaw et al., 2014)

1. If the final membrane wash (see Basic Protocol 2, step 7 or Alternate Protocol 3, step 8) was performed in TTBS, wash membrane in 50 ml TBS for 15 min at room temperature.

   The Tween 20 in TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place the membrane into the chosen chromogenic visualization solution.

   Bands should appear in 10 to 30 min.

3. Terminate the reaction by washing the membrane in distilled water. Air dry and photograph (*UNIT 7.5*; Moomaw et al., 2014) for a permanent record.

Alternate Protocol 4: Visualization with Luminescent Substrates

Antigens can also be visualized with luminescent substrates. Detection with light offers both speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for horseradish peroxidase (HRPO) systems or dioxetane phosphate for alkaline phosphatase (AP) systems, sealed in thin plastic wrap, and placed firmly against film. Exposures range from a few seconds to several minutes, although strong signals typically appear within 1 min.

Additional Materials *(also see Basic Protocol 3)*

Luminescent substrate buffer: 50 mM Tris·Cl, pH 7.5 (for HRPO; *UNIT 3.3*)
Luminescent visualization solution or kit for HRPO or AP (Table 8.3.3)

Clear plastic wrap
Film cassette

Additional reagents and equipment for autoradiography (Voytas and Ke, 1999) or digital image capture (*UNIT 7.5*; Moomaw et al., 2014) and digital image processing (*APPENDIX 3C*; Gallagher, 2014b)

NOTE: See Troubleshooting section for suggestions concerning optimization of this protocol, particularly when employing AP-based systems.

1. Equilibrate the membrane by washing two times in substrate buffer for 15 min each time. For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.

2a. Transfer the membrane to visualization solution. Soak 30 sec (HRPO reactions) to 5 min (AP reactions) in the same volumes as in step 1.

2b. Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step 4.

3. Remove the membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane to form a liquid-tight enclosure.

   To ensure an optimal image, only one layer of plastic should be between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.

4. In a darkroom, place membrane face down onto film.

   Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that insures a tight fit.

5a. *For film imaging:* Expose the film for a few seconds to several hours.

Typically, immunoblots produce very strong signals within a few seconds or minutes. However, weak signals may require several hours to an overnight exposure. If no image is detected, expose film 30 min to 1 hr, and if needed, overnight (see Troubleshooting).

5b. *For CCD imaging:* Place the membrane in the imager, focus, and close the door. Preview the exposure with a high pixel binning (4 × 4 to 8 × 8). Once satisfied with the preview image, capture at 1 × 1 (highest resolution) to 4 × 4 (or higher, to 8 × 8; this gives lower resolution, but faster, more sensitive capture (Fig. 8.3.7).

Autoexposure for chemiluminescent blotting is also available in advanced low-light CCD-based imaging systems, greatly simplifying the process of image capture with a "one button" approach to acquiring image (e.g., UVP, BioRad)

Exposures with cooled CCD cameras fitted with low-light optics are rarely longer than 10 min. Once captured, archive the image for later intensity analysis.

6b. Process digital image according to *APPENDIX 3C* (Gallagher, 2014b).

In brief, images are typically adjusted globally to remove background, contrast is enhanced, and images pseudocolored for ease of viewing and analysis.

7. *Optional*: Wash the membrane twice in 50 ml TBS for 15 min each time and process for chromogenic development (see Basic Protocol 4).

Chemiluminescent and chromogenic immunoblotting can be easily combined on a single blot to provide a permanent visual marker of a known protein. First, probe membrane with the chemiluminescent reactions to record digitally or on film. For the last reaction, use chromogenic development to produce a permanent visual record of the blot. This results in a permanent reference stain on the blot for comparison to the digitally imaged chemiluminescent blot.

Alternatively, staining with colorimetric stains such as India ink or fluorescent stains such as SYPRO Ruby will image all the proteins on the blot, permitting easy comparisons reference to the subsequent chemiluminescent blot (e.g., Eynard and Lauriere, 1998).

Alternate Protocol 5: Fluorescent Blot Preparation and Analysis

With the extensive range of excitation (Table 8.3.9) and emission filters (Table 8.3.10) available, researchers can detect and quantify virtually any fluorescent dye, from visible to NIR. The process for using fluorescently tagged antibodies is straightforward, using SDS-PAGE to first separate the proteins by size, followed by electrotransfer of the proteins on to nitrocellulose or PVDF membranes. Fluorescent-dye-tagged secondary antibodies are used to identify the primary antibody binding site. If only one protein species is being identified, then only one primary antibody and label is used. If multiple proteins are being identified on the same blot, then the analysis relies on probing each protein with a different primary antibody (e.g., one from mouse, one from goat) and a fluorescent tagged secondary antibody specific to the primary antibody (e.g., FL680-goat anti rabbit secondary), yielding a multiplexed result. For NIR blotting applications, a range of stable, low background dyes are available (Tables 8.3.8, 8.3.9, and 8.3.10).

For fluorescent imaging, the membrane is illuminated with overhead monochromatic excitation light from a light source, and the induced longer-wavelength fluorescence from the fluorescent tag is recorded with a cooled CCD camera using an emission filter that selects for the specific wavelength of the fluorescence while blocking the shorter-wavelength excitation light. For the results shown in Figure 8.3.8, an NIR blot was processed to identify two proteins in the sample.

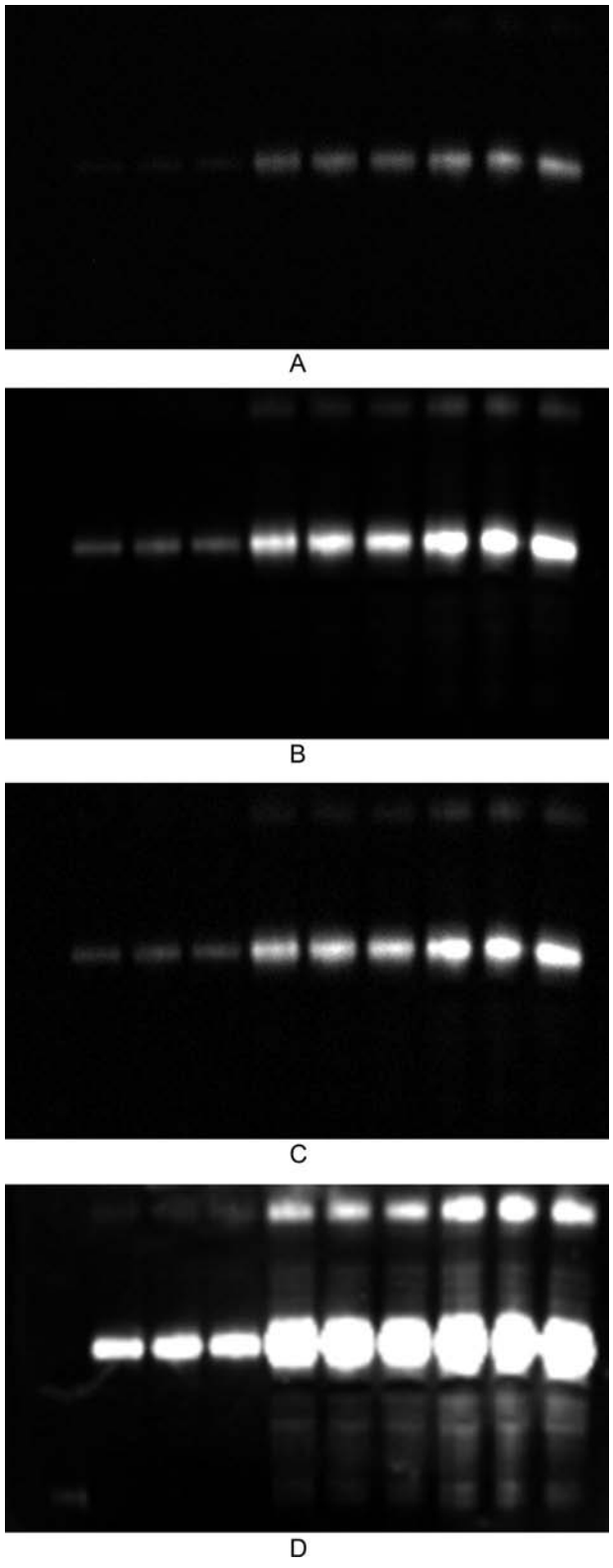

Figure 8.3.7 *(legend appears on next page)*

Table 8.3.8 Filters Used for NIR Blotting with 680 and 770 to 800 nm Fluorescent Tags[a]

Filter		Protein tag emission		
Ex (nm)	Em (nm)	680 nm	770-800 nm	Comments
				NIR tags are available from a variety of sources (Biotium, Li-Core, GE/Amersham, Life Technologies)
720/40	800LP	X	X	Both bands visible
630/50	720/40	X		Only 680 nm band visible
765/30	800LP		X	Only 800 band visible

[a]Excitation filters select the wavelength of light needed to illuminate the fluorescent tags inducing fluorescence. The resulting fluorescence from the antibody protein complex, at a longer wavelength than the excitation light, is specifically selected by the emission filters that block the excitation light and let only the fluorescence wavelengths of light through to the CCD sensor in the camera.

Materials

Fluorescently tagged antibodies: Biotium (*http://www.biotium.com/*), GE Amersham, Life Technologies, Li-Cor (*http://www.licor.com*), Pierce

Membrane with transferred proteins, probed with antibody-enzyme complex (see Basic Protocol 3 or Alternate Protocol 3).

Imaging system with multiple emission filters and overhead LED or Xenon ("epi") variable-excitation-light illumination (e.g., UVP BioSpectrum with fiber optic–based quartz halogen or xenon arc light source; Protein Simple; Bio Rad)

Appropriate excitation and emission light filters (Tables 8.3.8, 8.3.9, and 8.3.10)

Additional reagents and equipment for digital image processing (*APPENDIX 3C*; Gallagher, 2014b)

NOTE: NC or low-fluorescence PVDF blotting membrane (e.g., Millipore Immobilon-FL PVDF) is required for fluorescence imaging to avoid a high background.

1. Process membrane according to Basic Protocols 2 and/or 3 using fluorescently tagged ABC primary or secondary antibodies.

   Note that once the final wash step is complete, no further development is required because no enzyme reactions are involved. The blot can be wet or dry, with higher fluorescence typically coming from dry membranes.

Figure 8.3.7 *(image appears on previous page)* The effect of exposure time and CCD "binning" sensitivity settings on the intensity of sample signal. A serial dilution of rabbit sera was used to illustrate the effect of time and settings on the signal detected during chemiluminescent CCD imaging. Levels were globally adjusted to increase the visibility of bands. (**A**) 1-sec exposure, 1 × 1 binning. Chemiluminescent blot of electrophoretically separated and blotted rabbit antibodies visualized with anti rabbit Ab tagged with horseradish peroxidase and HRP luminescent reagent (Thermo Fisher SuperSignal West Pico Chemiluminescent Substrate). 1-sec exposure at full resolution with 1 × 1 binning and low-light *f*/0.95 lens and −37°C cooled 8 megapixel CCD-based low-light camera (UVP Biospectrum with 815 8-megapixel camera). Note only major bands are seen. (**B**) 1 × 1 binning, 30 sec exposure. Notice with the increased exposure time the less intense bands are now apparent on the blot. (**C**) 1 sec, 4 × 4 binning. Binning, a setting in software and property of CCD sensors, effectively turns groups of CCD pixels (4 × 4 = 16 in this case) into single large pixels, increasing the light collection area per grouped pixel and, as a result, increasing the sensitivity. One consequence is a lower-resolution image, but in most cases, with larger-megapixel sensors, this is not an issue. Notice that the less intense bands are now apparent on the blot at 1 sec exposure instead of 30 sec in B. (**D**) 30-sec exposure, 8 × 8 binning. Compared to 1 × 1 binning, 8 × 8 binning dramatically increases sensitivity so that faint minor bands are now seen. While the resolution is much less due to the high binning level, the ability to identify very dim bands at relatively short exposures is useful for inspection purposes.

Table 8.3.9 Recommended Excitation Filters for Fluorescent Protein Blot Imaging

	Wavelength of light passed by filter (nm)							
	450SP[a]	455-495	502-547	533-587	600-645	687-748	700-740	750-780
Peak nm	NA	475	525	560	630	715	720	765
Alexa Fluor® 488		X						
Alexa Fluor® 546, 555			X					
Alexa Fluor® 568, 594				X				
Alexa Fluor® 633, 647					X			
Alexa Fluor® 750						X		
CFP (mice)	X							
Cy2®		X						
Cy3®			X					
Cy5®					X			
Cy7®						X		
Fluo, FITC, FAM		X						
GFP (mice, gels, blots, plants)		X						
Oregon Green 488®		X						
RFP, Propidium Iodide			X					
Qdot™ 525, 655	X							
Rhodamine Green, 110		X						
Rhodamine Red, 6 G, B				X				
SYBR® Green, Gold, Safe		X						
SYPRO® Orange		X						
SYPRO® RedSYPRO®				X				
SYPRO® Ruby	X							
SYPRO® Tangerine		X						
Texas Red®				X				
YFP		X						
IRDye® 680, CF™ 680					X		X	
IRDye® 800, CF™ 770							X	X

[a]450 short-pass filter. Can accommodate wavelengths 380 to 450 nm based on typical fiber-optic light engine.

Table 8.3.10 Recommended Emission Filters for Fluorescent Protein Blot Imaging

	Wavelength of light passed by filter (nm)										
	465-495	503-523	513-557	565-625	580-630	607-682	668-722	700-740	767-807	7801P[a]	8001P[b]
Peak nm	480	513	535	595	605	645	695	720	787	NA	NA
Alexa Fluor® 488			X								
Alexa Fluor® 546, 555				X							
Alexa Fluor® 568, 594						X					
Alexa Fluor® 633, 647							X				
Alexa Fluor® 750									X	X	
CFP (mice)	X										
Cy2®			X								
Cy3®				X							
Cy5®							X				
Cy7®											
Fluo, FITC, FAM			X								
GFP (gels, blots, plants)			X								
GFP (mice)		X									
Oregon Green 488®			X								
Propidium Iodide, Qdot™ 655						X					
Qdot™ 525			X								
RFP					X						
Rhodamine Green, 110			X								
Rhodamine Red, 6 G, B						X					
SYBR® Green, Gold, Safe			X								
SYPRO® Orange, Ruby				X							
SYPRO® Red, Tangerine						X					
Texas Red®						X					
YFP			X								
IRDye® 680, CF™ 680								X			
IRDye® 800, CF™ 770											X

[a] 780 long pass filter. Can accommodate wavelengths above 780 nm.
[b] 800 long pass filter. Can accommodate wavelengths above 800 nm.

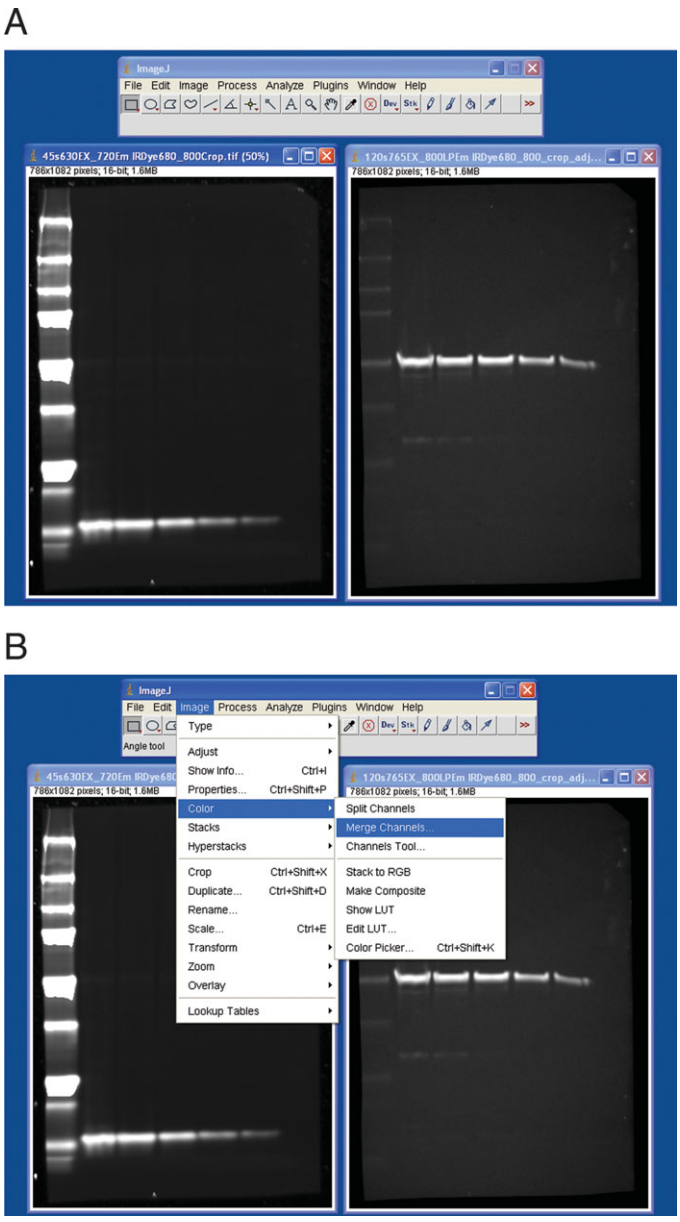

Figure 8.3.8 Pseudo-coloring and combination of two separate images from a single blot into a combined image illustrates the final combined blot.

2. Select appropriate excitation and emission filters (see Tables 8.3.8, 8.3.9, and 8.3.10).
3. Position and focus the blot for fluorescence imaging:
   a. Briefly, the processed blot is positioned on the sample platen with the door open to provide light for positioning and focus.
   b. Use the imaging system camera preview function to center the membrane on the imaging platform. Fluorescence produced during blot imaging is typically much less bright than typical applications such as ethidium bromide gels, and can take up to several minutes of exposure for a high-quality image.

      Positioning and focusing the membrane is facilitated by slightly opening the door to the imaging system to produce dim white light or using a low-intensity setting on the excitation light with no filter present.

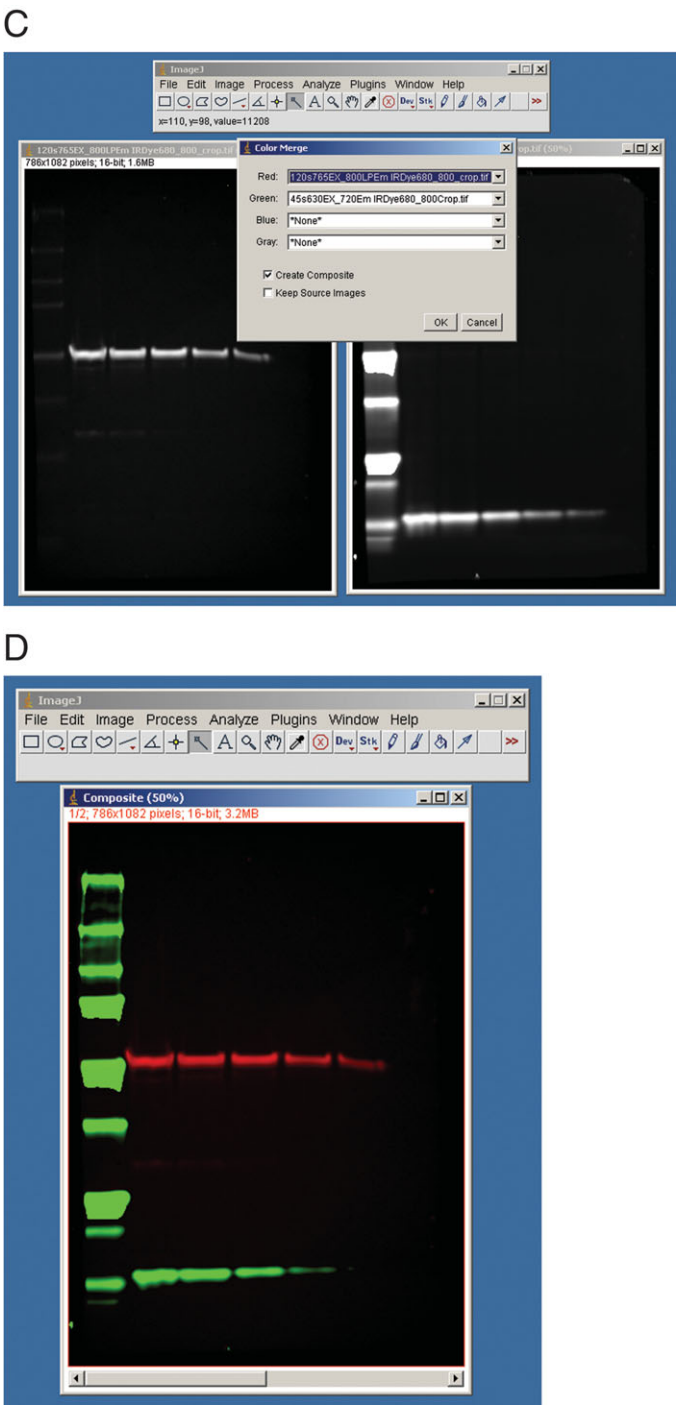

Figure 8.3.8 *continued*

c. Due to the very low intensity of the fluorescence, the lens must be focused at the most sensitive setting, typically *f*/1.2 to *f*/1.4 or less, to achieve a sharp image. Under this setting, bright white light will saturate the image.

4. Close the door on the imaging cabinet and set the lens at *f*/1.2.

5. Adjust the exposure time while viewing the preview at a camera binning set to 4 × 4, and capture at a binning of 1 × 1 to 4 × 4.

Exposure adjusted for maximal signal without saturation will typically range from 30 sec to 2 min, depending on the sample and filter set. Frequently, the fluorescence is strongest when using a dry membrane. Some fluorescent tags will photobleach, so check for light stability under visualization conditions by taking sequential exposures and looking for fluorescence intensity falloff.

6. Process digital image according to *APPENDIX 3C*.

In brief, images are typically adjusted globally to remove background, contrast is enhanced, and images pseudocolored for ease of viewing and analysis.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see UNIT 3.3.

4CN visualization solution

Mix 20 ml ice-cold methanol with 60 mg 4-chloro-l-napthol (4CN). Separately mix 60 µl of 30% (v/v) H_2O_2 with 100 ml TBS (*UNIT 3.3*) at room temperature. Rapidly mix the two solutions and use immediately.

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5 (*UNIT 3.3*)
100 mM NaCl
5 mM $MgCl_2$
Store up to 1 week at 4°C

BCIP/NBT visualization solution

33 µl nitroblue tetrazolium (NBT) stock: 100 mg NBT in 2 ml of 70% dimethylformamide (DMF); store up to 1 year at 4°C
5 ml alkaline phosphate substrate buffer (see recipe)
17 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) stock: 100 mg BCIP in 2 ml of 100% DMF; store up to 1 year at 4°C
Stable 1 hr at room temperature

Recipe is from Harlow and Lane (1999). *Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, and Vector Labs.*

Blocking buffer for colorimetric detection

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in TBS (TTBS; *UNIT 3.3*).
For neutral and positively charged nylon: Tris-buffered saline (TBS; *UNIT 3.3*) containing 10% (w/v) nonfat dry milk. Prepare just before use.

TTBS can be stored ~1 week at 4°C.

Blocking buffer for luminescence detection

0.2% (w/v) casein (e.g., Hammerstein grade or I-Block; Applied Biosystems) in TTBS (*UNIT 3.3*). Prepare just before use.

This blocking buffer can be used for nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A) membranes.

DAB/$NiCl_2$ visualization solution

5 ml 100 mM Tris·Cl, pH 7.5 (*UNIT 3.3*)
100 µl 3, 3′-diaminobenzidine (DAB) stock (40 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
15 µl 3% (v/v) H_2O_2

Mix just before use
CAUTION: *Handle DAB carefully, wearing gloves and mask; it is a carcinogen. Suppliers of peroxidase substrates are Sigma, Kirkegaard & Perry, Moss Substrates, and Vector Labs.*

India ink solution

Prepare 0.1% (v/v) India ink (e.g., Pelikan 17 black) in Tween 20 solution (see recipe). Store up to 1 week at 4°C.

Luminol visualization solution

0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml DMSO]
0.5 ml 10× *p*-iodophenol stock: optional; 10 mg (Aldrich) in 10 ml DMSO
2.5 ml 100 mM Tris·Cl, pH 7.5 (*UNIT 3.3*)
25 µl 3% (v/v) H_2O_2
H_2O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). *Premixed luminol substrate mix (e.g., Pierce; Amersham ECL; Perkin-Elmer Renaissance; Kirkegaard & Perry LumiGLO) may also be used. p-Iodophenol is an optional enhancing agent that increases light output. Luminol and p-iodophenol stocks can be stored up to 6 months at –20°C.*

Ponceau S solution

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare just before use.

SYPRO Ruby protein blot stain

SYPRO Ruby protein blot stain (Molecular Probes) is provided in a unit size of 200 ml. The 200-ml volume is sufficient for staining 10 to 40 minigel electroblots or four large-format electroblots (20 × 20 cm). SYPRO Ruby protein blot stain may be reused up to four times with little loss in sensitivity. The reagent is stable for at least 6 months to 1 year when stored at room temperature, protected from light.

Transfer buffer

Add 18.2 g Tris base and 86.5 g glycine to 4 liters of water. Add 1200 ml methanol and bring to 6 liters with water. For use with PVDF filters, decrease methanol concentration to 15% (v/v); for nylon filters, omit methanol altogether. Store up to 1 week at room temperature.

The pH of the solution is ~8.3 to 8.4.

TTBS

Prepare a 0.1% solution of Tween 20 in TBS (*UNIT 3.3*). Store up to 1 week at 4°C.

Tween 20 solution

0.3% (v/v) Tween 20 in phosphate-buffered saline (PBS; *UNIT 3.3*), pH 7.4. Store up to 1 week at 4°C.

COMMENTARY

Understanding Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should produce a single band, degradation of the sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If simultaneously testing multiple antibodies directed against a complex protein mixture (e.g., us-

ing patient sera against SDS-PAGE-separated viral proteins in an AIDS western blot test), multiple bands will be visualized.

For immunoblot or protein dot blot chemiluminescent applications, the sensitivity using HRPO is ~1 pg of target protein.

Troubleshooting

Immunoblotting problems and artifacts

There are several problems associated with immunoblotting. For instance, the antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. Gel electrophoresis under nondenaturing conditions can also be performed (see Gallagher, 1999).

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and discussion of immunoblotting problems and artifacts, see Bjerrum et al. (1988).

Transfer efficiency

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that the proper electrical connections were made and that power was delivered during transfer. In addition, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the transfer efficiency using the tank system appears to be low, increase the transfer time or power. Cooling (using the unit's built-in cooling cores) is generally required for transfers >1 hr. At no time should the buffer temperature go above 45°C. Prolonged transfers (>1 hr) are not possible in semidry transfer units due to rapid buffer depletion.

If the protein bands are diffuse, check the transfer cassette. The gel must be held firmly against the membrane during transfer. If the transfer sandwich is loose in the cassette, add another thin sponge or more blotter paper to both sides.

Occasionally, a grid pattern will be apparent on the membrane after tank transfer. This is caused by having either the gel or the membrane too close to the sides of the cassette. Correct this by adding more layers of filter paper to diffuse the current flowing through the gel and membrane. Use a thinner sponge and more filter paper if necessary.

If air bubbles are trapped between the filter and the gel, they will appear as clear white areas on the filter after blotting and staining. Take extra care to make sure that all bubbles are removed.

Transfer buffers

Alternatively, the transfer buffer can be modified to increase efficiency. Adding SDS at a concentration of 0.1% to the transfer buffer improves the transfer of all proteins out of the gel, particularly those above 60 to 90 kDa in size. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. These procedures are tradeoffs. Methanol improves the binding of proteins to PVDF and nitrocellulose, but at the same time hinders transfer. With SDS present, transfer efficiency is improved, but the SDS can interfere with protein binding to the membrane. Nylon and PVDF membranes are particularly sensitive to SDS interference. If needed, 0.01% to 0.02% SDS may be used in PVDF membrane transfer buffers.

Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5- to 0.75-mm-thick gels will transfer much more efficiently than thicker gels (e.g., 1.5 mm thick). Gels with a higher acrylamide percentage will also transfer less efficiently. Proteins can be particularly difficult to transfer from gradient gels, and a combination of longer transfer times, thin gels, and the addition of SDS to the transfer buffer may be needed.

Blocking

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high-background stain. A control using preimmune serum or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. 8.3.6).

When casein is used as a blocking agent, it can interfere with subsequent visualization procedures. When using AP staining, the casein must be heated to 65°C prior to use to reduce alkaline phosphatase activity inherent in the casein. In an avidin-biotin system, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma). While routine blocking agents used

in the basic protocols are usually sufficient for fluorescent applications, the proprietary blocking agents that are supplied with the antibody kits can give lower background and higher signal-to-noise results.

Luminescence visualization

Due to the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow nearby weaker signals on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad diffuse image on the film. The signal can also saturate the film, exposing the film to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

Recording the image on the blot can be tailored by exposing bright signals for short exposure times (<5 sec) and weak signals for long exposure (up to 10 min). An advantage of digital imaging with CCD cameras is that the image capture can be programmed to automatically record a series of short to long exposures, ensuring that the full range of luminescence from the blot is represented. Light piping can be minimized through use of thin plastic overlays and minimal liquid.

The membrane can interact with the substrate as well. With the alkaline phosphatase substrate AMPPD, nitrocellulose and PVDF membranes require 2 and 4 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Western Light instructions).

Variations

While the most common procedure for immunoblotting is electrotransfer via a vertical tank or a horizontal "semi-dry" blotter, numerous variations in blotting exist. With the newer fast blotters, very rapid protein blotting workflows can be created using the manufacturer's recommended reagents. Several rapid transfer systems are described in Table 8.3.7. From sample prep to electrophoresis to blotted protein can be easily obtained within an hour. The subsequent immunodetection of the proteins of interest on the blot can vary from a couple of hours to overnight or longer depending on the level sensitivity, convenience, and application. The digital analysis can also go quite quickly and be completed for membrane on the order of 5 to 10 min or shorter.

Other types of protein blotting, although not common, include contact, pressure, and vacuum transfer (Kurien and Scofield, 2006).

Contact transfer is generally a problem to be avoided. During repositioning of the membrane while assembling the blotter paper, membrane, and gel stack for tank or semi-dry transfer, protein immediately transfers and creates a new replicate of the gel every time the membrane is repositioned. However, if quantitation is not required, a quick press-and-lift contact transfer can be very useful for rapid identification.

Vacuum pressure uses a similar approach but places the gel under a vacuum, usually with a stack of buffer saturated blotting paper on top, to pull liquid and the protein from the gel and onto the membrane. The non-electrical procedures, although very inefficient in transferring protein, are useful for charge-neutral transfers. No charge is required on the protein, allowing native, nondenatured proteins to be transferred regardless of the native charge.

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Internet Resources

http://media.cellsignal.com/www/pdfs/resources/white-papers/guide-to-successful-wb.pdf

A guide to successful western blotting from Cell Signaling Technology, 2013.

http://www.perkinelmer.com/catalog/category/id/lightning%20blot%20system

Introduction to the Lightning Blot System by Perkin Elmer

http://www.bio-rad.com/en-us/product/semi-dry-rapid-blotting-systems/trans-blot-turbo-transfer-system
Introduction to Trans-Blot Turbo Transfer System by BioRad.

http://www.piercenet.com/product/pierce-power-blotter
Introduction to Pierce Power Blotter by Thermo Scientific.

http://www.lifetechnologies.com/us/en/home/life-science/protein-expression-and-analysis/western-blotting/western-blot-transfer/iblot-dry-blotting-system.html
Introduction of iBlot Dry Blotting System by Life Technologies.

http://tools.lifetechnologies.com/content/sfs/manuals/iblotsystem_qrc.pdf
iBlot Dry Blotting System quick reference.

Fluorescent Western Blotting: High Sensitivity Detection of Multiple Targets

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Western blotting with fluorescence detection offers the possibility of detecting multiple targets simultaneously on a single blot. Primary antibodies are increasingly available from multiple hosts, and there are now a wide variety of dye labels to exploit multiple imaging channels. If primary and secondary antibodies are selected so that individual targets can be discriminated, multiple antigens can be detected and quantified in a single experiment. Current fluorescence imaging instrumentation offers multiple detection channels and gives sensitivity comparable to other methods. The method described in this article allows multiple targets to be quantified simultaneously and reduces the need for stripping and re-probing. It also allows loading controls to be detected alongside the targets of interest. © 2020 by John Wiley & Sons, Inc.

Basic Protocol: Five-plex western blot detection, including tubulin detection for loading control

Keywords: fluorescence • multiplexed protein detection • western blot

BASIC PROTOCOL

Western blotting is a widely performed and powerful technique for determining the relative abundance of specific proteins in complex mixtures. While there are many methods for detection and quantification (e.g., autoradiography, chemiluminescence, chromogenic reaction), fluorescence detection stands out for its convenience, safety, sensitivity, and straightforward quantification. Fluorescence detection also offers the option of multiplex detection, allowing several targets to be distinguished and quantified simultaneously. This is made possible by the availability of bright, distinguishable fluorescent labels, highly specific secondary detection reagents, and advanced imaging instrumentation.

The experiment described here demonstrates multiplex western blot detection. Five different antibody-detection reagent combinations are employed to illustrate strategies used to detect multiple targets, all of which may be adapted for the design of other multiplex western blot experiments. The protocol is designed for convenience, reproducibility, and speed. A precast gradient gel is used to maximize the resolvable protein size range. The specified TGX gel offers rapid run times and efficient transfer. The membrane transfer is performed on the Trans-Blot Turbo instrument using pre-assembled nitrocellulose transfer packs. The ChemiDoc MP Imaging System provides several fluorescence imaging channels that enable this technique.

STRATEGIC PLANNING

Execution of a western blotting experiment utilizing multiple antibodies to detect multiple targets generally requires advance planning and optimization. If possible, antibodies

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Current Protocols in Pharmacology e72, Volume 88
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpph.72

should be selected that are highly specific and reveal single bands, as nonspecific detection can be particularly confounding in a multiplex experiment. Each antibody should be evaluated individually at a few different dilutions to determine the optimal concentration. In selecting which labeled secondary conjugate to use for detecting which primary antibody, consideration must be given to the relative abundance levels and difficulty of detection. Low-abundance targets, or targets for which a weakly detecting primary is used, should be identified with a bright secondary reagent such as StarBright Goat Anti-Mouse or Goat Anti-Rabbit. Labels with weaker fluorescence can be used for abundant or easily detected targets. In selecting the fluorescent labels for detection, use fluors that have dedicated channels on the imaging system employed. Western blots visualized with the individual fluors should also be imaged on other channels to determine whether signal spillover is tolerably low.

Five-plex western blot detection, including tubulin detection for loading control

Described in this protocol is an example of a multiplex western blot experiment that detects five different targets. Primary and secondary antibodies, and the concentrations at which they are used, are optimized to yield single bands with minimal cross-reactivity or spillover between imaging channels. Blot processing consists of blocking followed by two incubations with antibodies (primary and secondary) with washes in between. The first incubation employs primary antibodies for three targets. These antibodies are from three different species (mouse, rabbit, and chicken), with a biotinylated goat antibody against a fourth target. The second incubation utilizes three fluorescent secondary antibody conjugates with reactivity against mouse, rabbit, and chicken antibodies, respectively. Fluorescently labeled streptavidin is included for detection of the biotinylated primary. A recombinant, fluorescently labeled anti-tubulin is also included in the second incubation. The processed blot is photographed with five different combinations of excitation light and emission filter (imaging channels). The result is a series of five photographs, each displaying a single target as a single band at a unique molecular weight.

Materials

2× Laemmli sample buffer (Bio-Rad, cat. no. 1610737)
2-Mercaptoethanol (Bio-Rad, cat. no. 1610710)
HeLa cell lysate: prepared by lysing HeLa cells in HeLa cell lysis buffer (see recipe); protein concentration determined by DC protein assay (Bio-Rad, cat. no. 5000111)
Precision Plus All Blue Prestained Protein Standards (Bio-Rad, cat. no. 1610373)
4-20% Criterion TGX Precast Protein Gel, 18 well, 30 µl (Bio-Rad, cat. no. 5671094)
1× Tris-glycine SDS running buffer (see recipe)
Trans-Blot Turbo Midi Nitrocellulose Transfer Packs (Bio-Rad, cat. no. 1704159)
Tris-buffered saline (TBS) with 1% casein (Bio-Rad, cat. no. 1610782)
Rabbit anti-USP2 (Bio-Rad, cat. no. VPA00475)
Mouse anti-AKR1C2 (Bio-Rad, cat. no. VMA00346)
Biotinylated Goat anti-TP1 (Everest Biotech, cat. no. EB06643-B)
Chicken anti-SOD1 (Sunshine Antibodies, cat. no. Y00195-002)
TTBS wash buffer (see recipe)
StarBright Blue 700 Goat Anti-Rabbit IgG (Bio-Rad, cat. no. 12004161)
StarBright Blue 520 Goat Anti-Mouse IgG (Bio-Rad, cat. no. 12005866)
hFAB Rhodamine Anti-Tubulin Primary Antibody (Bio-Rad, cat. no. 12004165)
DyLight800 streptavidin (ThermoFisher, cat. no. 21851)
Alexa Fluor Plus 647 Goat Anti-Chicken IgY (ThermoFisher, cat. no. A32933)

Heat block set at 95°C
Criterion cell for SDS-PAGE (Bio-Rad, cat. no. 1656001)

Power supply capable of delivering constant 250 V
Trans-Blot Turbo Transfer System (Bio-Rad, cat. no. 1704150)
Forceps
Plastic tray(s) (e.g., Criterion staining/blotting trays, Bio-Rad, cat. no. 3459920)
Laboratory shaker or rocker
ChemiDoc MP Imaging System (Bio-Rad, cat. no. 12003154)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

1. Prepare a 1 mg/ml protein sample as follows: Use 2× Laemmli buffer, 2-mercaptoethanol, HeLa cell lysate, and water to prepare a sample that is 1 mg/ml protein. Mix in a sealed tube.

   Example: Combine 50 µl of 2× Laemmli buffer, 5 µl of 2-mercaptoethanol, 40 µl 2.5 mg/ml HeLa cell lysate, and 5 µl water for a total of 100 µl of 1 mg/ml protein. Other protein samples may be used.

2. Heat the sample at 95°C for 5 min. Allow to cool.

3. Dilute the All Blue standards 1:10 with 1× Laemmli buffer. Combine 5 µl All Blue standards, 25 µl 2× Laemmli buffer, and 20 µl water for a total of 50 µl. Use freshly prepared standards solution.

   The All Blue standards fluoresce in the same channel as Alexa Fluor 647. Diluting the standards 10-fold and loading a minimal amount ensures that fluorescence from the standards does not overwhelm the fluorescence from the immunochemical detection in that channel. This is essential if the Auto Exposure setting will be used to set the exposure time.

4. Remove a precast Criterion TGX gel from its packaging. Carefully remove the comb. Rinse the wells with water from a squirt bottle. Insert the gel into the Criterion cell. Fill the upper and lower reservoirs with 1× Tris-glycine SDS running buffer.

5. Load wells with standards and sample. Load 20 µl of HeLa cell lysate from step 2 for a total of 20 µg protein. Load one lane per blot with 2 µl of diluted All Blue standard prepared in step 3.

6. Place the lid on the Criterion cell and plug into a power supply. Run the gel at 250 V until the blue dye front reaches the bottom of the gel (approximately 30 min).

Transfer to nitrocellulose membrane

7. Open a Trans-Blot Turbo Midi Nitrocellulose Transfer Pack. Place the membrane and bottom stack on the Trans-Blot Turbo cassette base.

   Nitrocellulose was chosen as the membrane material for this experiment as a matter of convenience and because it yields a high-quality blot image. While low-fluorescence polyvinylidene difluoride (LF-PVDF) may be used instead, nitrocellulose offers certain advantages. The intensity of the fluorescent signal is generally higher when nitrocellulose is employed, and autofluorescence of nitrocellulose is minimal in the fluorescence channels used in this experiment. Pre-packaged transfer packs are available for nitrocellulose, and the membrane material requires no solvent pre-treatment. Secondary incubations can be performed in blocking solution without the need for background-reducing additives.

   Advantages of LF-PVDF include the fact that it is a stronger material and is, therefore, better able to withstand mechanical processing. It may also offer superior performance in situations where membrane autofluorescence obscures the bands of interest, or interferes with signal quantification.

8. Remove the gel from its cassette and carefully place it on top of the membrane. Use the blot roller to remove any air bubbles from between the gel and the membrane, or underneath the membrane.

Use gloves to handle the gel. Hold the gel by the corners. Do not move the gel once it is in contact with the membrane.

9. Place the second transfer stack on top of the gel. Use the roller to remove any air bubbles under the transfer stack.

10. Close and lock the cassette lid and then insert the cassette into the Trans-Blot Turbo instrument.

11. Select the TURBO navigation button and the Mixed MW protocol for midi gels. Begin the transfer.

    The transfer takes 7 min to complete under these conditions.

Blot processing

12. *Blocking:* Disassemble the transfer cassette and remove the upper stack and the gel. Use forceps to lift the membrane and rinse briefly in distilled or deionized water. Transfer to a tray containing TBS plus 1% casein. Make certain that the side of the membrane to which the protein is transferred is facing upwards. Use roughly 10 ml of blocking solution per 30 cm^2 of membrane area. This corresponds to 40 ml for an 8.5 × 13.5 cm midi-gel sized membrane.

    The tray area should accommodate the membrane with as little additional room as possible so as to maximize the depth of the solutions used.

    TBS with 1% casein is very effective at suppressing nonspecific interactions and is selected to ensure visualization of one single band by each of the primary antibodies. Other blocking reagents may be employed if enhanced sensitivity is desired. Enhanced sensitivity, however, often comes at the expense of higher nonspecific detection.

13. Cover the tray and incubate for 1 hr at room temperature with rocking or shaking.

    Rocking or shaking should be sufficiently vigorous to keep the membrane moving within the liquid.

14. *Primary incubation:* Prepare blocking solution with primary antibodies. Add the following antibodies to 20 ml of TBS with 1% casein. The recipe may be scaled depending on the amount of solution required. Prepare immediately before use.

Rabbit anti-USP2	10 µl (1:2000)
Mouse anti-AKR1C2	4 µl (1:5000)
Biotinylated Goat anti-TP1	4 µl (1:5000)
Chicken anti-SOD1	20 µl (1:1000)

    Antibodies are diluted into the solution used for blocking, with no additional additives.

    Other combinations of antibodies may be used as long as they are from different animal species or otherwise provide a means for specific detection (such as biotinylation). Prior optimization of the antibodies, and the concentrations at which they are used, is advised.

15. Pour off the blocking solution from the blot and replace it with the primary antibody solution prepared in the previous step. Use roughly 5 ml blocking solution per 30 cm^2 of membrane area. This corresponds to 20 ml for an 8.5 × 13.5 cm midi-gel sized membrane.

16. Cover the tray and incubate overnight at 4°C with rocking or shaking.

    Similar results may be obtained by incubating for 1 hr at room temperature.

17. *Wash 1:* Pour off the primary solution and replace it with TTBS wash buffer. Use roughly 10 ml of TTBS wash buffer per 30 cm^2 of membrane area. This corresponds

to 40 ml for an 8.5 × 13.5 cm midi-gel sized membrane. Cover the tray and shake or rock at room temperature for 5 min.

The use of a bottle-top dispenser for application of the wash solution makes this step more efficient when multiple blots are being processed simultaneously.

18. Pour off the TTBS wash buffer and replace with the same volume of fresh TTBS wash buffer. Cover the tray and shake or rock the blot at room temperature for 5 min. Perform this step four times, for a total of five 5-min washes.

19. *Secondary incubation:* Prepare blocking solution with fluorescent secondary detection reagents. Add the following to 20 ml of TBS with 1% casein. The recipe may be scaled depending on the amount of solution needed. Prepare immediately before use.

StarBright Blue 700 Goat Anti-Rabbit	8 µl (1:2500)
StarBright Blue 520 Goat Anti-Mouse	8 µl (1:2500)
hFAB Rhodamine Anti-Tubulin	20 µl (1:1000)
DyLight 800 streptavidin	2 µl (1:10,000)
AlexaFluor 647 Goat Anti-Chicken	2 µl (1:10,000)

Reagents are diluted directly in the solution used for blocking with no additional additives.

20. Replace the last wash solution from step 7 with the secondary incubation solution. Use approximately 5 ml of the solution per 30 cm^2 of membrane area. This corresponds to 20 ml for an 8.5 × 13.5 cm midi-gel sized membrane.

21. Cover the tray. Protect the blot from light by covering with aluminum foil or an opaque lid. Allow to incubate 1 hr at room temperature.

Some fluorescent reagents are prone to photobleaching, which may reduce sensitivity, making it advisable to limit exposure to room light.

22. *Wash 2:* Pour off the secondary solution and replace it with TTBS wash buffer. Use roughly 10 ml of TTBS wash buffer per 30 cm^2 of membrane area. This corresponds to 40 ml for an 8.5 × 13.5 cm midi-gel sized membrane. Cover the tray and shake or rock the blot at room temperature for 5 min.

23. Pour off the TTBS wash buffer and replace it with the same volume of fresh TTBS wash buffer. Cover the tray and shake or rock the blot at room temperature for 5 min. Perform this step five times for a total of six 5-min washes.

Blot imaging

24. Pour off the TTBS and place a few drops of water on the platen of the ChemiDoc MP imager. Using forceps, place the blot on the platen in the original protein-side-up orientation.

Placing the blot on a wet platen helps prevent the blot from drying out during imaging. The blot should not be allowed to desiccate during imaging, as drying can change the fluorescence characteristics of some fluors.

25. Use ImageLab software (the software that drives the imager) to prepare a Single Channel or Multichannel protocol. Select the fluorophores to be imaged and set binning for each channel to 1 × 1 if necessary. Select Auto Exposure for each channel and run protocol to capture the images.

Imaging five different fluors requires at least two multichannel protocols (each protocol permits a maximum of three fluors).

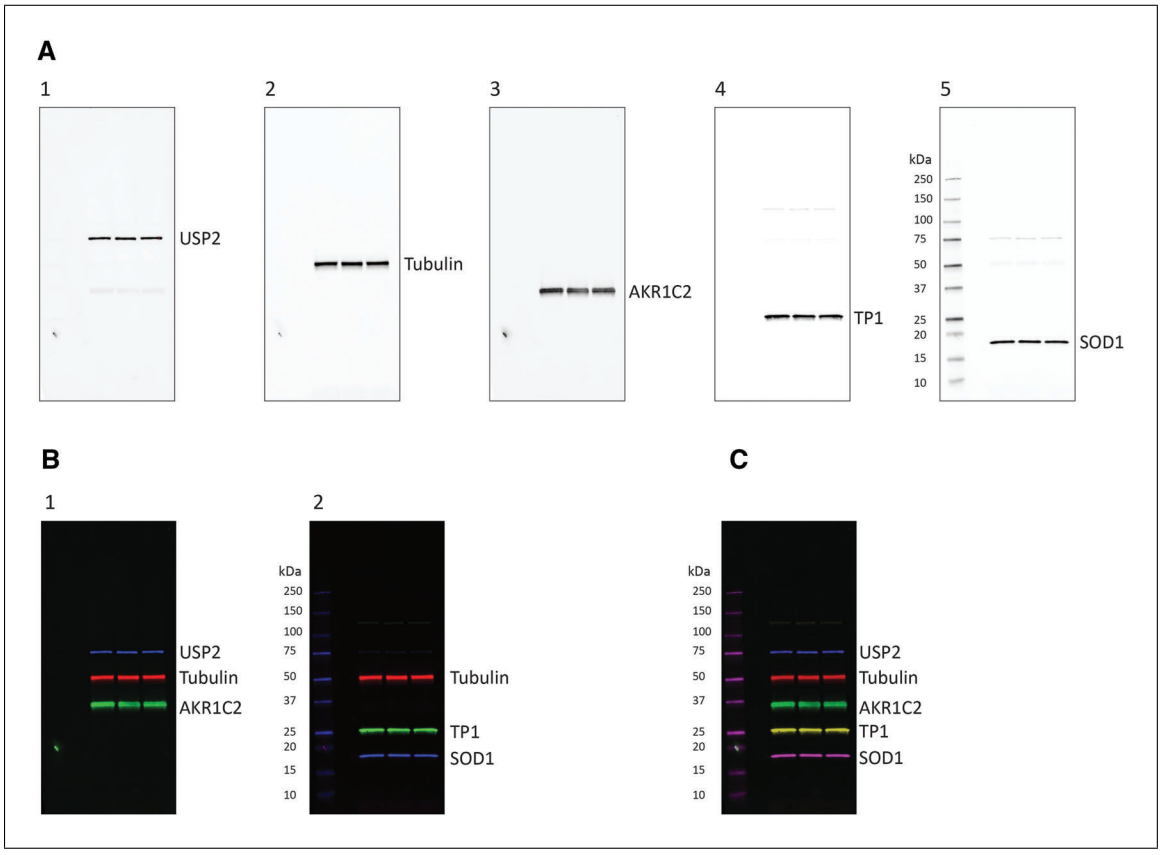

Figure 1 Results of 5-plex western blot. One lane was loaded with All Blue standards and three lanes were loaded with 20 µg of HeLa cell protein. (A) The images for each individual detection channel are shown separately. The channels are described in Table 2. (**1**) USP2 detected with rabbit anti-USP2 and StarBright Blue 700 goat anti-rabbit, 4-s exposure. (**2**) Tubulin detected with hFAB rhodamine anti-tubulin, 4-s exposure. (**3**) AKR1C2 detected with mouse anti-AKR1C2 and StarBright Blue 520 goat anti-mouse, 1-s exposure. (**4**) TP1 detected with biotinylated goat anti-TP1 and DyLight 800 streptavidin, 3.5-s exposure. (**5**) SOD1 detected with chicken anti-SOD1 and AlexaFluor 647 goat anti-chicken, 3-s exposure. All images were generated with 1 × 1 binning. (**B**) Composite three-color images generated with ImageLab software. (**1**) StarBright Blue 700–detected USP2 (blue), hFAB rhodamine-detected tubulin (red), and StarBright Blue 520-detected AKR1C2 (green). (**2**) hFAB rhodamine-detected tubulin (red), DyLight 800-detected TB1 (green), and AlexaFluor 647-detected SOD1 (blue). (**C**) Composite five-color images generated from the individual images using Adobe Photoshop. StarBright Blue 700-detected USP2 (blue), hFAB Rhodamine-detected tubulin (red), StarBright Blue 520-detected AKR1C2 (green), DyLight 800-detected TB1 (yellow), and AlexaFluor 647-detected SOD1 (lavender).

Default binning may be set to something other than 1 × 1, which gives the highest-resolution image. Binning should be set to 1 × 1 unless this results in excessive exposure times.

Auto exposure will take a series of images for each channel to determine the optimal exposure time.

26. The exposure time can be fine-tuned by selecting Manual exposure. This might be necessary if bands of interest are saturated (indicated in red), and can be corrected by reducing the exposure time of the original image. The original exposure time can be determined by selecting the "i" information logo.

This experiment was designed to yield optimal exposure times in the range of a few seconds. Optimal exposure time can vary over a wide range, depending on the brightness of the fluor, the characteristics of the antibody employed, and the abundance of the target.

Additional analyses, such as band quantification and size estimation, can be performed through the ImageLab software.

Image data are displayed in Figure 1, with a summary of the results shown in Table 1.

Table 1 Multiplex Western Blot Results

Primary antibody	Fluorescent secondary detection reagent	Apparent MW of target band by SDS-PAGE
Rabbit anti-USP2	StarBright Blue 700 Goat Anti-Rabbit	80 kDa
	hFAB Rhodamine Anti-Tubulin[a]	50 kDa
Mouse anti-AKR1C2	StarBright Blue 520 Goat Anti-Rabbit	36 kDa
Goat anti-TP1 (biotinylated)	DyLight 800 streptavidin	26 kDa
Chicken anti-human SOD	AlexaFluor 647[b] Goat Anti-Chicken	17 kDa

[a]This recombinant Fab fragment is used to detect β-tubulin, an abundant housekeeping protein used as a loading control. It is directly labeled and included in the secondary incubation, as it requires no additional detection reagent for visualization.
[b]The All Blue size standards are visible in the channel used for imaging AlexaFluor 647.

Table 2 Imaging Channels Enabled on the ChemiDoc MP Imaging System

Fluors	Excitation light source	Emission filter (center wavelength/bandpass)
StarBright Blue 520 AlexaFluor 488 Cy2 DyLight 488 Fluorescein (FITC)	Blue LED	532 nm/28 nm
StarBright Blue 700	Blue LED	715 nm/30 nm
Rhodamine (TRITC, TAMRA) AlexaFluor 546 Cy3 DyLight 550	Green LED	602 nm/50 nm
AlexaFluor 647 Cy5 DyLight 650	Red LED	700 nm/50 nm
AlexaFluor 680 Cy5.5 DyLight 680 IRDye 680RD	Red LED	715 nm/30 nm
AlexaFluor 790 Cy7 DyLight 800 IRDye 800CW	Near-infrared LED	835 nm/50 nm

REAGENTS AND SOLUTIONS

HeLa cell lysis buffer

50 mM Tris·Cl, pH 7.4
150 mM NaCl
25 mM NaF
1 mM EDTA
0.2% (v/v) NP-40
1% (w/v) sodium deoxycholate

0.1% (w/v) sodium dodecyl sulfate
Store up to 6 months at 4°C

Tris glycine SDS running buffer, 1×

Dilute 10× Tris/Glycine/SDS (Bio-Rad, cat. no. 1610732) 10-fold with water. This solution may be stored at room temperature for up to 1 month.

TBS plus 0.05% Tween (TTBS wash buffer)

For 1 L, combine:
895 ml H_2O
100 ml 10× Tris-buffered saline (TBS; Bio-Rad, cat. no. 1706435)
5 ml 10% Tween 20 (Bio-Rad, cat. no. 1610781)

This solution may be stored at room temperature. A fresh solution should be prepared weekly, or when turbidity is observed.

COMMENTARY

Background Information

Since its introduction, Western blotting has become an indispensable biochemical tool (Burnette, 1981; Towbin, Staehelin, & Gordon, 1979). The technique combines the high resolving power of SDS-PAGE with the specificity of immunochemical detection, yielding a simple and accurate procedure for detecting and quantifying proteins in complex mixtures. A complex sample is separated by SDS-PAGE, followed by electrophoretic transfer and immobilization onto a membrane surface (blotting step). The membrane is exposed to a solution containing antibodies, which bind to bands containing the protein analyte(s) of interest. Detection and quantification of the analyte relies on the use of secondary detection reagents. These are typically antibodies with specificity toward the antibody used in the primary detection step. The secondary detection reagent is conjugated to an entity capable of generating a signal that can be quantitatively imaged, with the magnitude of the signal indicating the abundance of the detected protein. This was originally accomplished using radionuclide labels (Burnette, 1981; Towbin et al., 1979) and later with enzymes that catalyze chemiluminescent reactions (Alegria-Schaffer, Lodge, & Vattem, 2009; Leong, Milstein, & Pannell, 1986). While these detection methods were selected because of their high sensitivity, they suffer some limitations. Radionuclide labels are essentially obsolete because of safety concerns. As chemiluminescent labels generate signals that can be quite short in duration, they make it necessary to limit the time between blot processing and image acquisition. More recently, the increasing availability of bright fluorescent labels along with sensitive imaging instruments have made it possible to achieve a similar degree of sensitivity to older procedures using fluorescently labeled secondary reagents (Fradelizi, Friederich, Beckerle, & Golsteyn, 1999; Gingrich, Davis, & Nguyen, 2000; Park, Mabuchi, & Sharma, 2015). This detection method is easy to use, offers a stable signal, and can be quantitative if suitable controls are used to ensure that the relationship between fluorescence signal and target abundance is in a linear range (Taylor & Posch, 2014). The use of fluorescent detection reagents also offers the possibility of detecting multiple targets simultaneously. Instrumentation is available that allows detection in multiple channels, exploiting the differences in absorbance and emission characteristics among the different fluorescent labels. This approach greatly increases the amount of data generated by a single blot without the need for additional stripping and re-probing. This is especially valuable in situations where the amount of sample is limiting. The development of StarBright fluorescent labels (Rong et al., 2013) has further expanded the utility of multiplex fluorescent western blotting. These nanoparticle labels are intrinsically much brighter than conventional fluorescent reagents, thereby increasing the signal to background ratio and sensitivity in blue light excited channels.

Critical Parameters

When designing a multiplex fluorescent western blot experiment, it is important to consider the properties of the imaging instrumentation. Fluors for detection should be selected to fit the imaging channels available on the instrument that give minimal fluorescence spillover between channels. The ChemiDoc MP Imaging System is an example

Table 3 Troubleshooting Guide for Fluorescent Western Blotting

Problem	Possible cause	Potential resolution
Band of interest does not appear	Poor transfer	Select a longer transfer time
	Primary antibody does not recognize	Use more primary antibody (dilute less) or try another antibody
	Lack of recognition by secondary reagent	Ensure that the antibodies used are matched with the appropriate secondary detection reagent (e.g., goat anti-mouse when using a mouse primary)
Detection is not specific (multiple bands are detected)	Lack of antibody specificity	Try another antibody
	Insufficient blocking	Optimize blocking reagent
	Modification or degradation of target	Modify sample preparation procedure. Take steps to prevent degradation, such as keeping the sample cold and using protease inhibitors
High background	Insufficient blocking	Optimize blocking reagent
	Insufficient washing	Wash for a longer time or use additional wash steps. Use a sufficient volume of wash solution.
	Membrane autofluorescence	Use LF-PVDF membrane or select a different antibody-secondary combination for a channel with less membrane fluorescence. In general, the longer the excitation wavelength, the less autofluorescence observed.
Blotchy or uneven image appearance	Insufficient reagent volume or insufficient movement during processing	Use more antibody solution during processing. Ensure that shaking or rocking results in good mixing and that the blot is in motion during incubations.
	Poor assembly of membrane transfer stack	Be sure to assemble the membrane stack correctly. Do not move the gel after it has contacted the membrane, and make certain that air bubbles are removed by running the roller over the gel and membrane.

of an instrument where multiple LED light sources can be used in combination with multiple emission filters for several compatible imaging channels. The appropriate channel is selected when the fluors used are selected from a menu. While other instruments offer similar choices of fluors and channels, the full range of different channels is not available on all instruments.

In general, fluors that are detected in channels that differ from each other in excitation light source and the emission filter will be compatible. StarBright Blue 520 and StarBright Blue 700 are compatible with each other despite using the same excitation source because the emitted light from each is restricted to regions of the spectrum that are distant from each other. The practical upper limit for multiplex detection with this instrument is five targets.

Unless the primary antibody is directly labeled with a fluor, it requires a specific fluorescent secondary detection reagent that exclusively recognizes that antibody in a mixture. This is accomplished by selecting antibodies from different species and using secondary antibodies with reactivity restricted to a single species. Commercial secondaries are cross-adsorbed and generally provide the necessary specificity. As a practical matter,

Table 4 Time Considerations for Fluorescent Western Blotting

Protocol step	Duration
Sample preparation and gel loading for SDS-PAGE	10 min
SDS-PAGE	30 min (hands-off)[a]
Preparation for transfer	5 min
Transfer to membrane	7 min (hands-off)[a]
Blot processing: Blocking	1 hr (hands-off)[a]
Blot processing: Primary incubation	Overnight (16 hr), or 1 hr at room temperature (hands-off)[a]
Blot processing: Wash 1	25 min
Blot processing: Secondary incubation	1 hr (hands-off)[a]
Blot processing: Wash 2	30 min
Imaging protocol set-up and image acquisition	20 min
Total	1 hr 52 min on day 1; 2 hr 15 min on day 2 Or: 5 hr 7 min within a single day

[a]These steps are incubations that do not require user intervention. Other tasks may be undertaken during these intervals.

most commercial antibodies are either from rabbit or mouse, limiting the number of detectable targets in a single experiment. However, there are antibodies from non-mouse, non-rabbit sources, such as the chicken antibody used in the experiment described in this article, that can be exploited. Other strategies may be used to provide specific secondary detection. An example from this experiment is the use of a biotinylated primary detected with fluorescently labeled streptavidin. Antibodies that are directly labeled with a fluor may also be employed as long as they do not interact with any of the secondary detection reagents. The rhodamine-labeled anti-tubulin Fab fragment used in the experiment detailed above is an example of this approach.

There are many other parameters that may be considered when optimizing a multiplex blotting experiment. Typically, there are several different antibodies available for a given target that vary widely in sensitivity and specificity. The choice of blocking reagent can influence the result, with some reagents, such as the TBS-casein used in the protocol described here, promoting high-stringency selective detection, and others promoting high sensitivity at the possible expense of nonspecific detection. There are many blocking reagents available, with the optimal choice being identified mainly through trial and error. The selection of membrane material is influential as well, with nitrocellulose offering convenience and sensitivity and LF-PVDF being useful in instances where fluorescence background is high.

Troubleshooting

Potential problems that may arise with the protocol in this article, along with their possible causes and potential solutions, are listed in Table 3.

Understanding Results

Shown in Figure 1 are western blot images from the five different detection channels used in this experiment. Each image represents detection of a different target. The reagents and conditions are highly optimized, resulting in prominent major bands and unambiguous identification. Some additional bands may, however, appear as well. In addition to the 80 kDa USP2 band visualized with StarBright Blue 700 (Fig 1. Panel A), a fainter band at 36 kDa can be seen. This is most likely spillover from StarBright Blue 520 fluorescence (Panel C), as the two fluors share the same blue light excitation. Likewise, the AlexaFluor 647 image (Panel E) shows a faint band at 80 kDa, which is probably spillover from StarBright Blue 700. These two fluors are both detected by emission in the far-red region of the spectrum, and use similar emission filters in their detection. In most cases, spillover can be minimized through optimization of channel and fluor selection, and the tolerance for spillover is dictated by the interpretability of the experimental result. In many cases,

a high degree of spillover can be tolerated because the targets are distinguishable on the basis of molecular weight. Detection with DyLight 800 streptavidin (Panel D) shows additional faint bands that are probably due to endogenous biotinylated proteins present in the sample. These bands are present whenever streptavidin is used as a secondary detection reagent. In this case, they are easily distinguished from the intended target.

Time Considerations

The entire procedure can be completed within 24 hr, or less, if the overnight primary incubation is performed at room temperature and shortened to 1 hr. See Table 4 for detailed time considerations.

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