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Western Blotting Revisited: Critical Insights and Methodological Innovations

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Abstract: Western blotting, or immunoblotting, is a fundamental laboratory technique for protein detection, identification, and quantification. Since its development in the late 1970s, it has become a cornerstone in molecular biology, biochemistry, and biomedical research. The method involves sequential steps of protein separation by SDS-PAGE, transfer to a solid membrane, blocking of nonspecific binding sites, antibody incubation, and visualization using chemiluminescent, fluorescent, or colorimetric detection systems. Each stage requires careful optimization to minimize errors and ensure reproducibility. This guide provides a comprehensive overview of western blotting, covering essential materials, equipment, and protocols, as well as practical troubleshooting strategies for common issues such as weak signals, high background, or transfer inefficiency. Advanced applications—including multiplex assays, phospho-specific blots, and detection of post-translational modifications—demonstrate the versatility of the technique in both qualitative and quantitative analyses. Emphasis is also placed on rigorous data analysis, proper use of controls, and transparent reporting practices to improve reliability and reproducibility in published research. By integrating fundamental principles with modern innovations, this resource serves as a valuable reference for both novice and experienced researchers seeking to apply western blotting effectively in protein studies.

1. Introduction to Western Blotting

Western blotting, or immunoblotting, is one of the most widely used techniques in molecular and cellular biology laboratories. This relatively simple technique can provide valuable information regarding protein identification and quantification (Kroon et al., 2022). In more complex terms, a western blot detects and quantifies proteins based on their size and immunoreactivity. Western blots can be used to detect specific post-translational modifications and protein–protein interactions, in addition to protein identification and quantification.

Western blotting involves five basic steps: 1. Gel electrophoresis. A sample containing proteins is loaded onto a gel. An electric field is applied, causing the proteins to migrate through the gel matrix, where they are separated based on size (Mahmood & Yang, 2012). 2. Transferring. The proteins in the gel are transferred to a membrane. 3. Blocking. The membrane is incubated with a blocking buffer to prevent nonspecific binding. 4. Detection antibody. The membrane is incubated with a detection antibody that binds to the protein of interest. 5. Visualization. The bound detection antibody is visualized using a suitable method.

For optimal results, western blotting requires careful attention to detail and strict adherence to protocols. Troubleshooting tips are provided to assist in resolving common issues that may arise during western blotting.

1.1. Definition and Purpose

Western blotting or immunoblotting is a common laboratory method used to detect proteins and assess their expression levels in a sample. It is widely used in biology, biochemistry, and biomedical research. A protein of interest is identified based on its molecular weight and immunoreactivity with a specific antibody. In a typical western blot experiment, proteins in a sample are separated by gel electrophoresis and then blotted onto a solid support membrane. The membrane is probed with a specific antibody that binds to the protein of interest. The antibody is then detected with an enzyme-linked secondary antibody and a chemiluminescent substrate, producing a visible band on the blot that indicates the presence of the protein of interest. The intensity of the band indicates the relative expression level of the protein (Kroon et al., 2022).

Western blotting is highly sensitive and can detect proteins in low abundance, making it a versatile technique. However, it is also susceptible to artifacts and errors that can lead to misinterpretation of results. Small variations in how the western blotting steps are performed, such as protein quantification, sample preparation, gel running, electroblotting, strip-and-reprobe experiments, antibody validation, and imaging, can alter the quality of the blot, introduce blots or bands that are not representative of a real sample, or affect how experimental results are interpreted (Omondi et al., 2023). For these reasons, there are many “good practice” or “experimental design” guidelines available for western blotting, focusing on how to minimize the impact of variability.

1.2. Historical Background

Western Blotting (or Immunoblotting) is a popular technique used to detect and measure specific proteins in complex samples containing thousands of different proteins. The technique relies on the use of primary antibodies to bind specifically to the proteins of interest (antigens). Antibody-antigen complexes are then visualized and analyzed for their relative abundance in the sample.

Western blotting involves a multi-step process that employs gel electrophoresis and protein transfer to membranes, followed by antigen detection using specific antibodies (Kroon et al., 2022). The idea for the western blot technique started in 1979 when W. Neal Burnette, a postdoctoral researcher in the lab of Gary L. Nelsont at the University of California, San Diego, was working on developing a sensitive method to study the binding of proteins to DNA. Burnette combined several pre-existing techniques to create his version of the blotting procedure. Later that year, Frances L. M. (Frank) van de Ploeg, a postdoctoral researcher in the lab of David Baltimore at MIT, independently developed a similar method for studying proteins. In a competition for publication, Burnette's article was chosen over van de Ploeg's, leading to an ongoing debate over who invented the western blot technique.

2. Principles of Western Blotting

Western blotting, also known as immunoblotting, is a common laboratory method used to detect proteins and assess their expression levels. The target protein is identified based on its molecular weight and immunoreactivity with a specific antibody. Western blotting consists of a series of interrelated steps. A simple protein mixture is loaded into a well in a polyacrylamide gel. Gel electrophoresis is performed, and when current is applied, proteins are separated within the gel based on size. Small proteins migrate faster through the gel matrix than larger proteins. After gel electrophoresis is performed, a protein of interest can be detected based on its molecular weight and immunoreactivity with a specific antibody (Mahmood & Yang, 2012). The proteins in the gel are then transferred onto a membrane (normally nitrocellulose or PVDF). Each membrane has a unique chemical composition that holds proteins differently. After incubation in blocking buffer, the membrane is probed with primary antibodies that specifically bind to the protein of interest. Unbound primary antibody is washed off, and the membrane is incubated with secondary antibodies that bind the primary antibody. The secondary antibody is usually conjugated with a reporter enzyme that produces a detectable signal.

Western blotting is commonly used in research labs to separate and identify proteins. First, a simple mixture of proteins is separated based on molecular weight through gel electrophoresis. After gel

electrophoresis, the results are transferred to a membrane, and this step produces a band for each protein. The membrane is then incubated with labeled antibodies specific to the protein of interest. Unbound antibody is washed off, leaving only the antibody bound to the protein of interest. Finally, bound antibodies are detected by developing the film. Since only one band should be visible on the blot, that band corresponds to the amount of protein present.

2.1. Protein Separation by Electrophoresis

Western Blotting Techniques

The western blot technique is one of the most widely used techniques in research and diagnostic laboratories. The fundamental premise of western blotting is the recognition of antigens (proteins) by specific antibodies. A western blot can be done with as few as 10 cells; however, in many cases, thousands or millions of cells are used (Mahmood & Yang, 2012). Western blotting is a multi-step process that includes protein separation by electrophoresis, protein transfer, protein fixation, protein detection, and protein identification. Each step is crucial to the overall success of the experiment.

Protein Separation by Electrophoresis

The first step in a western blot experiment is protein separation by electrophoresis. In the simplest terms, electrophoresis is the movement of molecules through a gel matrix under the influence of an electric field. Molecules with an overall negative charge will move toward the anode (positive electrode), while positively charged molecules will migrate toward the cathode (negative electrode). Proteins generally have a net charge that depends on the pH of the surrounding buffer, which can be manipulated by using different buffer systems. At its isoelectric point (pI), a protein has no net charge and will not migrate in an electric field. On either side of the pI, the protein will have an overall positive (acidic pH) or negative charge (basic pH), and thus will migrate in an electric field.

2.2. Transfer of Proteins to Membrane

This section describes how proteins can be transferred from the gel to the membrane. First, see the transfer apparatus. It consists of two flat glass plates with a rectangular hole in the center and water-tight beading where the two plates touch. A tightening screw pushes the plates together, compressing the beads to create a seal. An inlet port allows the transfer buffer to be added, and an outlet port drains the buffer from the apparatus.

Cut the gel and place it in a beaker with transfer buffer and the gel-side-down membrane to equilibrate for about 5 minutes. Then carefully assemble the transfer sandwich inside the transfer apparatus. Although there are many ways to assemble the transfer sandwich, this method works very well. First keep a petri dish with transfer buffer. Then use a 10 ml serological pipette to transfer the stacking gel to the petri dish. Use a blade to cut off the bottom of the stacking gel close to the bottom of the tube. After discarding the excess gel, use a spatula to loosen the gel from the sides of the tube. Collect the gel into the petri dish. Then use a wide plastic spatula to transfer the gel (gel-side-down) into the apparatus. After carefully transferring the gel, add transfer buffer to one side of the apparatus, and then quickly cover that side with a plate to prevent air bubbles. Add transfer buffer to the other side, and then cover it with a plate as well. Make sure that the sandwich is covered with the buffer. Using the transfer apparatus, perform the transfer at 100 volts for 90 minutes. After the transfer is complete, remove the membrane from the apparatus. Wash the membrane with TBST for 5 minutes (Mahmood & Yang, 2012).

2.3. Blocking and Incubation with Primary Antibodies

Blocking is performed to prevent any non-specific binding of antibodies to the membrane. To do this, the membrane is incubated in a blocking buffer, usually consisting of protein diluted in a buffer containing a few detergents to lessen nonspecific binding. The membrane is probed with a primary antibody, which recognizes the target protein. A primary antibody can be obtained from a variety of sources, including polyclonal and monoclonal sources (Kroon et al., 2022). For a polyclonal antibody source, an animal is immunized with the target protein or a fragment of that protein. The animal produces serum containing antibodies against that protein, which can be purified to obtain

the polyclonal antibody. For a monoclonal source, an animal is immunized with the target protein, and its spleen cells producing antibodies are fused with immortal myeloma cells to create hybridomas. Each hybridoma produces one type of antibody. Hybridomas are cloned, and the resulting antibodies are screened to determine which ones recognize the target protein. This method results in a monoclonal source of antibodies, as each hybridoma is derived from a single cell producing a specific antibody. Antibodies can be directly conjugated to an enzyme or fluorophore or require a secondary antibody for detection. Directly conjugated antibodies only require one incubation step. However, because of the lower sensitivity, detection methods are not as versatile. Secondary antibodies recognize primary antibody targets and can be conjugated with different enzymes or fluorophores, allowing flexibility in detection methods and increasing sensitivity. However, they generally require two incubation steps.

2.4. Detection of Target Proteins

Western blotting is commonly used to identify and quantify protein expression levels. Initially, the membrane is pre-blocked to prevent non-specific binding of antibodies. Then the membrane is incubated with specific primary antibodies that bind to the target protein. After washing to remove unbound primary antibodies, the membrane is incubated with secondary antibodies that bind to the primary antibodies and are conjugated with an enzyme or fluorochrome. Unbound secondary antibodies are removed by washing, and the blot is developed using chemiluminescent substrate or other detection methods (Mahmood & Yang, 2012). Quantification is often done post-development by taking a digital image of the blot and using densitometry software to analyze band intensity.

3. Materials and Equipment

The general materials and equipment that are needed to perform a western blot experiment are as follows: • Equipment • Gel electrophoresis apparatus • Transferring device (semi-dry or wet transfer) • Incubator/rocker • Vacuum blotting apparatus (optional) • Imaging system (chemiluminescence, fluorescence, or colorimetric detection) • Materials • Precast polyacrylamide gel or acrylamide powder • Stacking gel • Transfer membrane (PVDF or nitrocellulose) • Membrane filter • Protein ladder • Protein sample • Electrophoresis running buffer • Transfer buffer • Blocking buffer • Washing buffer • Primary antibody • Secondary antibody • Chemiluminescent detection substrate • Reagents • Ammonium persulfate (APS) • TEMED • Acrylamide/Bis-acrylamide solution (30% w/v) • Tris base (1.5M pH 8.8 and 0.5M pH 6.8) • Sodium dodecyl sulfate (SDS) • Electroblotting transfer buffer (for wet transfer) • Methanol (20% for PVDF membrane incubation during transfer) A western blot experiment can be done in 1 day for one membrane, and can be done at the same time as other protocols. However, it is recommended to take 2 days for beginners to become more familiar with the procedure. A necessary equipment for western blot is an electrophoresis apparatus for resolving proteins. Precast gels can be purchased or a gel can be made by mixing acrylamide solutions with other reagents. Semi-dry blots require special equipment, while wet blots can be done using mini tanks. For chemiluminescence detection, a dark box with a camera system is required. Fluorescent detection requires an imaging system with specific light sources and filter sets. Low-end fluorescent imaging systems can also work with western blot, as they can be modified with 365 nm UV filter and prevent light exposure to western blots (Mahmood & Yang, 2012).

3.1. Gel Electrophoresis Systems

Western blotting is a widely used technique for the detection and quantification of specific proteins in complex samples. Advance in western blotting technology has led to microscale systems that reduce consumption of reagents yet maintain performance comparable to benchtop systems. Broadly speaking, microscale western blot systems implement gel electrophoresis, protein transfer, and immunodetection similar to benchtop western blot systems, but use different hardware and fluidic configurations to reduce the scale. All microscale western blot systems to date electrophoretically transfer proteins from gel to membrane similar to benchtop systems. Western blotting systems that have been engineered to the scale of microscope slides use diffusion-based transfer. At a size compatible with standard glass microscope slides, gelload micro-western blot

systems use capillarity to draw protein-laden gels into membrane sandwiches and transfer proteins from gel to membrane using a buffer with no current. Western blotting systems that use a glass capillary for gel electrophoresis transfer proteins from gel to membrane with a setup similar to benchtop systems, relying on the same buffer chemistry to create an electric field that transfers proteins from gel to membrane (O. Zadeh et al., 2022).

3.2. Transfer Systems

Once the proteins are separated by gel electrophoresis, they must be transferred to a solid matrix membrane in a procedure called “blotting.” There are several different systems that may be used to transfer proteins from the acrylamide gel to the membrane. It is important to realize that the transfer must effectively remove proteins from the gel without damaging them or denaturing them. Loss of proteins due to transfer malfunction will result in difficulty in detecting the protein of interest in an experiment. Also, if the proteins are damaged or denatured during transfer, they will not bind to antibodies and will be undetectable (Mahmood & Yang, 2012).

The most common transfer system uses wet transfer with buffer soaked gel and membrane sandwiching electrodes. As current passes through the electrodes, proteins will migrate from the gel to the membrane. The rate of this transfer is determined by the size of the proteins being transferred, with smaller proteins moving more quickly than larger ones. Therefore, the efficiency of the transfer depends on the time length, gel composition, and buffer used for the transfer.

3.3. Membranes and Blocking Reagents

Membranes are the most important variable in the Western blotting procedure, affecting sensitivity, specificity, background noise and binding kinetics. The classic membrane material used for WBs is nitrocellulose and polyvinylidene fluoride (PVDF). Nitrocellulose membranes are widely used because of their low background and high protein binding capacity. Nitrocellulose membranes are available in different pore sizes. According to the manufacturer's instructions, 0.2µm nitrocellulose membranes are the best choice for WBs as they can trap all proteins with molecular weights larger than 15kDa. However, protein molecules smaller than 15kDa pass through the blot. The advantage of nitrocellulose membranes over PVDF membranes is that they are compatible with many common laboratory stains for protein visualization such as Ponceau-S red, amido black and Coomassie blue stain (Xu et al., 2019).

Polyvinylidene fluoride (PVDF) membranes were introduced to WBs. PVDF membranes are hydrophobic, necessitating the pre-activation of 20% methanol for five minutes prior to blotting. After pre-activation, PVDF membranes have a very high protein binding capacity, trapping even very small proteins that nitrocellulose membranes cannot trap. But to the low protein binding capacity of nitrocellulose membranes, for robustness, and to be the same as most staining methods used in histology, nitrocellulose membranes are preferred in the laboratory for WBs. However, in contrast to nitrocellulose membranes, PVDF membranes cannot be used with common laboratory stains used in protein visualization. Most staining methods for PVDF membranes use the same principle of intersection between the dye and the protein as WBs, dyeing the protein precipitated membranes. Thus, unlike nitrocellulose membranes, precipitated PVDF membranes cannot be stained after WBs.

3.4. Primary and Secondary Antibodies

The blotted membrane is ready for the incubation with antibodies. Antibodies are native or recombinant proteins, most commonly immunoglobulins (Ig) that have high affinity for a specific antigen. It is possible to use the native antibody or to produce a recombinant version of the antibody, known as an antibody fragment. The most commonly used antibodies in Western blotting are IgGs, which can be polyclonal or monoclonal. Secondary antibodies are used to decrease the background staining, and they are usually polyclonal.

Polyclonal antibodies are produced by immunizing animals with the target antigen. A mixture of different antibodies is produced from different B cells, which all recognize the same antigen but

different epitopes of that antigen. This polyclonal mixture displays a high signal detection but a high batch-to-batch variability. Monoclonal antibodies are produced by fusing a single B cell with a myeloma cell line. The immortalized hybridoma cell line secretes a single antibody clone that recognizes a particular epitope (Roncador et al., 2015). Monoclonal antibodies display a high specificity and low background, and they are more standardized and reproducible than polyclonal antibodies. However, monoclonal antibodies can take a long time to develop and usually have a lower signal detection than polyclonal antibodies.

4. Sample Preparation

Proper sample preparation is critical for the successful execution of Western blotting experiments (Kroon et al., 2022). While complex samples such as cell lysates, tissue homogenates or serum can be analyzed directly, Western blotting of purified proteins can make it easier to characterize proteins of interest. To avoid problems with the detection of low-abundance proteins, samples should ideally be run in parallel with a standard curve verification of dilution series containing known amounts of a target protein.

Protein Quantitation: The protein concentration of prepared samples should be determined using a colorimetric protein quantitation method such as the BCA, Bradford or Lowry assays. Except for the BCA assay, which is compatible with reducing agents, most other reagents contain ionic detergents that interfere with SDS-PAGE. Therefore, transferred membranes can be stained with Ponceau S, Amido Black or Coomassie Blue to enable quantitation of transferred protein loads (P Rybicki, 2015).

4.1. Cell Lysis and Protein Extraction

Cell lysates are the most common form of sample used for western blot. Protein extraction is perhaps the most crucial step in WB. Protein extraction attempts to collect all the proteins in the cell cytosol. Thus, all the steps in cell lysis and protein extraction must be done in a cold temperature, whether using a pre-chilled centrifuge or ice to chill down tubes and solutions. More importantly, protease inhibitors must be always added to the lysis buffer to prevent the denaturing of the proteins (Mahmood & Yang, 2012). It is important to note that tissue samples require a mechanical intervention, either homogenization in a dounce homogenizer or sonication, to destruct the tissue and extract the proteins. Cell samples do not require this since they can be simply trypsinized or exposed to mild EDTA to detach from culture plates, followed by a centrifugation step to pellet the cells. Media must be discarded prior to trypsinization to make sure the cells are rinsed of serum, which contains a high concentration of protein that could interfere with WB.

Once the protein is extracted, it is very important to know the concentration of the extract so that the samples can be compared on an equivalent basis. There are various ways to test for protein concentration. The simplest way is to use a spectrophotometer at 280 nm wavelength since proteins have an aromatic ring containing tryptophan and tyrosine, which absorbs UV light at this wavelength. This allows measuring the mass of the protein loaded into each well rather than the volume, which is crucial since different samples may have different viscosities. To prepare the sample, it is diluted into a loading buffer containing glycerol, which increases the density of the sample to allow easy sinking into the wells of the gel. A tracking dye is also present in the buffer to see how far the separation has progressed. In addition, the sample is heated at 95°C for 5 minutes to denature the higher order structure while retaining sulfide bridges. Denaturing is extremely important because during electrophoresis, the proteins have to move in an electric field, and this is only possible if they have a net charge. Thus, it is critical to understand that the higher order structure of proteins contains many interactions that stabilize the structure at neutral pH, and one of these interactions is the amino acid side chain forming ionic bonds with oppositely charged atoms. Hence, denaturing ensures that the negative charge of amino acids is not neutralized, enabling protein movement in an electric field during electrotransfer. It is very important to have both positive and negative controls for the sample. For a positive control, a known source of target protein is used, such as a purified protein or a control lysate, which contains a high concentration of

the protein of interest. A negative control is a null cell line, which normally does not express the target protein, used to confirm that the staining is not nonspecific.

4.2. Quantification of Protein Concentration

Before protein samples can be used for western blot analysis, it is important to determine the protein concentration of each sample. Without appropriate quantification of protein concentration, there may be too much or too little protein loaded into each lane. This results in low signal-to-noise ratio or signal saturation, both of which hinder accurate quantification of band intensity. In this protocol, three common quantification methods are examined: the BCA assay, the Bradford assay, and UV absorbance at 280 nm. Each method uses a different biochemistry principle to quantify protein concentration. The BCA and Bradford assays use color change to detect protein concentration. The BCA assay is based on the ability of proteins to reduce Cu2+ ions to Cu1+ ions under alkaline conditions. Although BCA is a widely used protein assay kit, the presence of SDS can interfere with quantification. The Bradford assay uses coomassie dye, which binds to protein in acidic conditions and shifts the absorbance maximum from 465 nm to 595 nm. Protein concentration may be determined by measuring the absorbance at 595 nm. Bradford is sensitive to personnel handling because this assay cannot be run in parallel with a western blot. Unlike the accuracy of colorimetric assays, the UV absorbance method is almost unaffected by experimental conditions, including salt concentration. Proteins containing aromatic residues absorb UV light at 280 nm. Protein concentration may be calculated by measuring the absorbance at 280 nm, although bandwidth and path length must be considered.

5. Gel Electrophoresis

Cell lysates are the most common form of sample used for western blot (Mahmood & Yang, 2012). Protein extraction attempts to collect all the proteins in the cell cytosol. This should be done in a cold temperature with protease inhibitors to prevent denaturing of the proteins. After extracting the protein, it is very important to have a good idea of the extract's concentration. Protein concentration is often measured using a spectrophotometer. After determining the appropriate volume of the sample, it is diluted into a loading buffer, which contains glycerol so that the samples sink easily into the wells of the gel. A tracking dye is also present in the buffer allowing the researcher to see how far the separation has progressed. The sample is heated after being diluted into a loading buffer, in order to denature the higher order structure, while retaining sulfide bridges. It is also very important to have positive and negative controls for the sample. For a positive control a known source of target protein, such as purified protein or a control lysate is used. A negative control is a null cell line, such as β-actin, is used as well to confirm that the staining is not nonspecific.

Western blot uses two different types of agarose gel: stacking and separating gel. The stacking gel has a lower percentage of acrylamide than the separating gel and is made at a pH of 6.8. The pH difference creates a channel that focuses the protein. After separation, the protein is thus separated by their size more so in this gel, as the smaller proteins travel more easily, and hence rapidly, than larger proteins. The proteins when loaded on the gel have a negative charge and will travel toward the positive electrode when a voltage is applied. The samples and a marker are loaded into the wells, and the empty wells are loaded with sample buffer.

5.1. SDS-PAGE Technique

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most widely used methods for separating proteins. SDS-PAGE separates proteins primarily based on size, thus denaturing proteins is crucial for SDS-PAGE. Sodium dodecyl sulfate (SDS) is an anionic detergent that denatures proteins to their primary structures (polypeptide backbone). SDS binds with proteins at a ratio of 1.4:1, conferring a net negative charge (on average exceeding −30,000) to each protein (Mahmood & Yang, 2012). Polyacrylamide gel is formed by the polymerization of acrylamide with N,N- methylenebisacrylamide (bis-acrylamide) in the presence of ammonium persulfate and tetramethylethylenediamine. Hydroxymethyl- bisphenone creates crosslinks between acrylamide polymers, forming a tightening mesh.

The pore size of the polyacrylamide gel is determined by the concentration of acrylamide. Stacking (4% acrylamide) and separating gel (10% / 12% acrylamide) are commonly used in Western blotting experiments. The stacking gel creates a narrow band as proteins enter the separating gel, allowing accurate size estimation. The separating gel should contain acrylamide concentrations that allow the detection of all proteins of interest. A protein ladder should always be included to determine the approximate size of the target bands.

5.2. Running the Gel and Staining

Once the gels have polymerized, they should be stacked in the gel apparatus and placed in the Running Buffer (0.025M Tris, 0.192M Glycine, 0.1% (w/v) SDS). Be careful to avoid trapping air bubbles between the gel and plate. Equal amounts of protein samples should be loaded into the wells and the gels should be run at a constant voltage, starting at low voltage for about 30 minutes and increase the voltage gradually afterward to about 200-250V (P Rybicki, 2015). Once the running is complete, remove the gel from the plate carefully and proceed with the staining method of choice. This protocol uses Coomassie Blue.

Prepare the Staining Solution (0.1% (w/v) Coomassie Blue R-250, 50% (v/v) Methanol, 10% (v/v) Acetic Acid, 40% ddH2O) in a Fume Hood and add the gel to the staining solution. Carefully agitate the solution by swirling to ensure even staining. The gel should be stained for at least 30 mins to overnight. Remove the Staining Solution (keep it for later reuse) and rinse the gel with the Water Rinse Solution (40% (v/v) Methanol, 10% (v/v) Acetic Acid, 50% ddH2O) for about 5-10 minutes. Then, move the gel to the Water Destaining Solution (10% (v/v) Methanol, 10% (v/v) Acetic Acid, 80% ddH2O) and destain until the background is clear (destaining may take several hours). Finally, move the gel to ddH2O until ready to image. Alternatively, protein bands in SDS-PAGE can be detected using silver stain (Chevallet et al., 2007). Silver stain is far more sensitive than Coomassie and is capable of detecting nanogram levels of protein, though it is also more tedious and time-consuming.

6. Transfer Techniques

Western blotting is a widely utilized method for detecting specific proteins in a sample. The steps involved in western blotting, including sample preparation, protein separation using SDS-PAGE, protein transfer onto a membrane, protein detection using antibodies, and membrane imaging, are discussed here. After separating the proteins by SDS-PAGE, they are transferred onto a membrane for subsequent detection using antibodies. Protein transfer can be done using capillary action, electroblotting, or vacuum blotting. Of these three methods, electroblotting is the most commonly used method for protein transfer in western blotting.

Protein transfer is done using a similar apparatus as that used for gel electrophoresis. In western blotting, after separating the proteins by gel electrophoresis, they are transferred onto a membrane. For protein transfer during electroblotting, a sandwich is prepared using a membrane, gel, and filter paper. The gel is placed in contact with the membrane with the side containing the running buffer facing the membrane. After the gel is placed in contact with the membrane, filter papers soaked in transfer buffer are placed on both sides of the gel-membrane sandwich. A blotting apparatus is used to complete the circuit, and an electric current is passed through the gel-membrane sandwich. Proteins in the gel migrate towards the anode. If a PVDF membrane is used, proteins can be transferred from the gel to the membrane. If NC is used, it must be noted that proteins will migrate towards the opposite side. Generally, a polyacrylamide gel is used to separate proteins of 10-250 kDa size. When using a 10% gel, the transfer is done for 1 hour at 350 mA. If a 4-20% gradient gel is used, transfer is done for 1-2 hours at 200 mA. If a PVDF membrane is used, it must be preactivated using methanol before use. The activation process should not exceed 10 minutes .

After the transfer process, to verify the efficiency of protein transfer, the gel can be stained using coomassie blue or Ponceau S. Coomassie blue is used for staining polyacrylamide gels. After soaking the gel in an adequate volume of coomassie blue solution, the excess solution is discarded, and the gel is destained using destaining solution until the gel becomes clear. The clear gel shows

that proteins have been transferred from the gel to the membrane. Ponceau S staining can be used for both NC and PVDF membranes. NC membranes can be stained by soaking the membrane in Ponceau S staining solution. After staining, the excess staining solution is washed off using PBS, and the membrane becomes clear, indicating efficient protein transfer. Ponceau S is reversible staining; proteins can be stripped off the membrane by washing the membrane using a washing buffer containing NaOH.

6.1. Tank Blotting

Electrophoretically separated protein bands can be transferred to a solid support membrane, such as nitrocellulose or polyvinylidene difluoride (PVDF) by tank blotting. Though the tank blotting technique generally requires gel and membrane sandwich, electrode fixers (or cassettes) are required to hold and completely ensure good contact between the gel and the membrane. A downward current flow is created by placing electrodes at the top and bottom of the tank. This technique is often preferred in laboratories for its high robustness and reliability. The standard blotting buffer is the transfer buffer used in the PAGE. In the absence of methanol for tank blotting, the transfer rate slows down, but more diffusion of proteins occurs in the air-interface on membranes (P Rybicki, 2015). Taking precautions to limit this loss, good quality blots have been obtained with minimization of background by extensively washing the membranes prior to blocking.

Tank blotting assembly consists of a gel and membrane holder (blotting cassette), a blotting tank, a power supply, and a temperature controller (optional). Gel and membrane holders come in various formats to accommodate different sizes and numbers of gels to be blotted at once. Generally these holders consist of some plastic molds with outer rubber gaskets to contain the blotting buffer and inner Mylar plates to hold the gels and membranes in place (Mahmood & Yang, 2012). These Mylar plates insert into grooves on the edges of the holders and prevents the gels and membranes from moving out of contact with each other during the transfer. Care should be taken not to scratch or damage the Mylar plates, as this could cause air bubbles to form between the gel and the membrane.

6.2. Semi-Dry and Dry Blotting

The western blotting protocol involves four major steps: protein sample preparation, gel electrophoresis, transfer to the membrane, and immunodetection. There are two types of protein transfer methods: wet transfer and semi-dry transfer. The semi-dry method is cost- and time-effective as it requires less buffer solution compared to the wet method and can produce satisfactory results within 30-60 minutes. Semi-dry blotting uses a piece of sponge instead of the classic wet blotting chamber (Mahmood & Yang, 2012). For semi-dry blotting apparatus, set it up in the order of the blotting membrane → gel → sponge → blotting apparatus anode plate (side with two holes) → cathode plate (side without holes), ensuring the gel and membrane are in contact with each other. Soak the sponges in pre-chilled transfer buffer for at least 10 minutes before use. For the dry transfer system, sandwich the gel and membrane between two blotting papers that have been soaked in transfer buffer and run the blotting at room temperature. After the gel is removed from the electrophoresis apparatus, soak the gel in transfer buffer for 5-10 minutes. Semi-dry transfer is done by assembling the membrane, gel, and sponges in the order of the membrane → gel → sponge → upper chamber → lower chamber containing anodes. To eradicate the background band problem, the blank PVDF membrane should be pre-run for 10 minutes at 25 V to remove all excess methanol. Turn on the power supply and allow it to run for 30-90 minutes at 20 V. For dry transfer, it is necessary to assemble the transfer sandwich in the order of the blotting apparatus base → blotting paper → membrane → gel → blotting paper → upper chamber → lower chamber containing cathode plates and turn on the power supply, running the system at 300 V for 1 hour or at 100 V overnight.

7. Blocking and Antibody Incubation

Blocking is essential for WBs to reduce non-specific binding of both primary and secondary antibodies. Therefore it is important to select an appropriate blocking agent and properly block the

membrane. Generally, common blocking agents include non-fat dry milk, bovine serum albumin (BSA), casein, and fish gelatin. Non-fat dry milk is widely used for detecting phospho-proteins, since it is often seen that BSA does not give strong signals for phosphorylated proteins. Blocking is typically performed for 1 h at room temperature or overnight at 4 °C with gentle shaking. Usually, to keep a low background, incubation with both antibodies is performed in the same blocking solution used for blocking the membrane. After incubation with primary antibodies, blots are typically washed in TBST three times for 10-15 min to reduce background due to unbound antibodies (Kroon et al., 2022).

Incubation with secondary antibodies is then performed in a similar manner to the primary antibodies, although the concentration of secondary antibodies is typically higher than that of primary antibodies; a final concentration of 0.1-1 µg/ml is usually recommended. This secondary incubation step will amplify the signal, since secondary antibodies recognize the Fc region of the IgG heavy chain of the primary antibody, resulting in each primary antibody being bound by several secondary antibodies. After the incubation with secondary antibodies, blots are again washed in TBST three times for 10-15 min. At this stage, the WB is ready for detection. Some secondary antibodies are conjugated with HRP, and HRP activity can be visualized using enhanced chemiluminescence (ECL) reagents.

7.1. Purpose of Blocking

Blocking is a vital step in preventing nonspecific binding of antibodies to the membrane. During the transfer, some proteins that are not the target protein may bind to the membrane. If these proteins are not blocked off, the antibodies may also bind to these accidentally, creating a background in the result. So, in the blocking step, the membrane is treated with a blocking solution that contains a certain concentration of protein. This protein will bind to all the sites on the membrane that are not occupied by the target protein, thus preventing the antibodies from binding anywhere except to the target protein (Mahmood & Yang, 2012).

There are several different blocking solutions that can be used, and the most common are either 5% BSA diluted in TBST or 5% nonfat dried milk diluted in TBST. The purpose of the milk or BSA is to reduce the background by blocking off the binding sites on the membrane. When using a detection label with HRP conjugated antibodies, it is often easier to use nonfat dried milk as the blocking solution. Milk is cheap and widely available, while BSA is more expensive. However, care must be taken that not all detection labels are compatible with milk proteins. For example, it is better to use BSA blocking solutions with biotin antibody labels, AP antibody labels, and antiphosphoprotein antibodies.

7.2. Optimization of Antibody Concentrations

Prepare the desired dilution(s) of primary antibody (e.g., 1:50, 1:100) in the provided Antibody Diluent 2. Antibodies are generally used at higher concentrations for capillary-based immunoassay than for traditional western blotting (M Nelson et al., 2017). The supplied secondary antibody is ready to use without dilution. First, secondary antibody concentration should be determined. Add 25µL of the desired dilution of secondary antibody to wells for testing (1:1000, 1:2500, 1:5000, 1:10000). Higher concentrations of antibodies generally improve sensitivity but may also increase background signal (E. Whyte et al., 2022).

8. Detection Methods

Western immunoblotting facilitates the interaction of immobilised proteins with specific polyclonal or monoclonal antibodies, identifying the corresponding immunoreactive protein. This technique is still widely used as a rapid and relatively straightforward method of protein identification. However, because of the relative simplicity of the procedure and the low amounts of protein needed for detection, western blotting is more widely used as a method for confirming the presence of a target protein following other experiments, e.g. transfections, purifications, degradations, etc. Western blots have also been used to assess enzymatic activity indirectly (M Donoghue et al., 2006).

The Coomassie blue staining protocol in conjunction with western blotting produces a highly effective method of visualising total protein profiles for 1-D SDS-PAGEs. Using a gel to transfer proteins to a membrane makes it very easy to perform dual staining. A wide range of methods have been successfully used to stain membranes for total protein and these generally also work effectively with western blots, making it possible to simultaneously detect both specific immunoreactive proteins and total protein on the same membrane (P Rybicki, 2015). However, a number of these staining procedures are incompatible with the antibody probing steps of western blots. The most commonly used methods for detecting total protein on membranes following immunoblotting rely upon labelling proteins with a fluorescent dye or enzyme that generates a colour change.

8.1. Chemiluminescence

Chemiluminescence western blots are prepared by incubating membranes with primary antibodies conjugated with horseradish peroxidase (HRP) enzymes. Upon reaction with luminol, HRP enzymes cause light emission, which is detected on x-ray film or CCD cameras. The advantage of chemiluminescent systems is their high sensitivity due to the large signal amplification (up to 109 times) (A. Bonini et al., 1991). A large variety of one-shot chemiluminescent substrates are commercially available. These systems are very sensitive and detect protein concentrations in the femtogram range. In one-shot systems, the light emission lasts a few seconds, and the time for exposure must be carefully determined to optimize the signal/image ratio.

In some applications, the sensitivity of the one-shot chemiluminescent assays is not enough, and the best solution is to apply stable chemiluminescent systems that allow a prolonged emission of light. Stable chemiluminescent systems emit light continuously and detection can be performed several minutes or hours after the addition of the substrate (R. Viviani et al., 2021). Stable chemiluminescent systems usually incorporate chemiluminescent compounds whose chemiluminescent reactions are catalyzed by alkaline or acidic pH. These procedures produce a raised intensity of the emitted light and transform their time of emission from a flash to a constant glow.

8.2. Fluorescence

The fluorochromes used in fluorescence microscopy are chemical compounds that can be excited by absorption of light at appropriate wavelengths and emit light at longer wavelengths. Add either one or more defined fluorochromes to the sample. Quantify the emission from the various fluorochromes using either a wide-field fluorescence microscope, a confocal laser scanning microscope, or an imaging spectrograph. It should then be possible to reconstruct the spectral dimension of the sample. Given a good enough spectral separation, it is also possible to quantify the sample with an imaging microscope without any prior spectral separation steps. In wide-field fluorescence microscopy, the whole sample is illuminated with a defined light source and the emitted light is imaged directly onto the detector (R. Petty, 2007). The fluorescence of a sample results from the interaction of light and matter. A change in the energy state of the molecule generates a fluorescent event. To generate a fluorescent signal, the fluorochrome needs to be excited by a specific wavelength of light. After excitation, the fluorochrome goes through a series of high-energy vibrational states before returning to the ground state. The molecule emits a photon of light as it returns to the ground state. This emitted light has a longer wavelength than the excitation wavelength of the fluorochrome (C. Ishikawa-Ankerhold et al., 2012).

8.3. Colorimetric Detection

After the antibody-enzyme conjugate treatment of the blot, the colorimetric substrate solution is added directly to the blotting membrane. The blot is kept in the dark for the colorimetric reaction to occur. After the desired intensity of coloration is obtained, the reaction is arrested by washing the membrane twice with distilled water, or alternatively with deionized water (Gogalic et al., 2018). The colorimetric detection using alkaline phosphatase as the reporter enzyme results in a blue color on the blot. This procedure can be performed in capped plastic containers on the benchtop. Presto

Blue™ solution is used as a cell viability reagent and is generally added directly to culture plates. Upon entering the cells, it is reduced from a blue non-fluorescent state to a pink fluorescent state by reducing agents (Schüchner et al., 2016). Prior to coomassie staining, protein shifts due to western blotting were minimized by reducing the coomassie gel to the same size as the transfer membrane. After blocking, the blot was treated with anti-hu THRAP3 antibody at a dilution of 1:1000 in 5% BSA for one hour. A negative control Western blot was performed similarly, except that the anti-hu THRAP3 antibody was omitted.

9. Data Analysis

A western blot quantification analysis begins by estimating the background signal. For each blot, the user first selects the relevant picture. Then, the user identifies one or more regions of the blot with no bands. These areas should be small rectangles (or straight lines) and as large as possible without containing any band signal. The background estimate is based on the median signal in the selected areas, which is subtracted from all subsequent calculations. After defining the background, the user selects the type of quantification: full blot or band selection. For full blot analysis, the user selects the relevant images. The band detection algorithm requires at least one blot and one matched molecular weight marker image. Periodically, the user examines detected bands. For bands that are either not detected or incorrectly detected, the user can add, delete, or manually adjust the detection parameters (Kroon et al., 2022). The user can add or delete identifications of proteins used to calculate relative expression. For each protein of interest, a user selects all necessary parameters, including which bands correspond to the protein(s), whether a loading control is used, the type of normalisation, and mathematical transformations on the results. The user can choose to export underlying data at any point in the workflow.

9.1. Image Capture and Analysis Software

For optimal image capture, use a cooled charge-coupled device (CCD) camera or a scientific-grade camera, rather than a standard consumer camera. CCD cameras have higher quantum efficiency, which means they capture more light per exposure, resulting in better signal-to-noise ratio (SNR), lower limits of detection, and reduced risk of saturation. A cooled or thermoelectrically-cooled CCD camera reduces thermal noise. If using an analog system, capture images in the darkroom to reduce background noise. Western blot images are typically acquired using Chemiluminescence, a process based on the photo-excitation of a substrate-analyte complex where the substrate is catabolized by an enzyme (Kroon et al., 2022). During western blotting detection, a chemiluminescent substrate is oxidized by HRP, generating excited-state products that emit photons as the products return to the ground state. Consider exposure time thoroughly to ensure the maximal semi-quantitative range and avoid saturation. This is critical for images captured using CCD cameras, as the pixel intensity increases with exposure time. Analyze captured images to confirm that the lowest to highest intensity pixels cover a suitable range. If pixel intensity is outside this range, adjust exposure time and recapture the image. Determine whether an image is saturated by checking the maximum pixel intensity of the captured image. If the maximum intensity is the highest possible value for the image format, this pixel is saturated. It is crucial to prevent saturation in western blot images because the SNR is often poor for the strongest bands. When possible, capture multiple images at different exposure times to cover the entire semi-quantitative range. This can only effectively assess the dynamic range of the image. If a band is saturated, recapture the image with a shorter exposure time.

9.2. Quantification of Bands

Relative quantities of proteins measured using western blots can be accurately assessed by densitometry on digital images for blots that follow appropriate design and analysis protocols (Omondi et al., 2023). Immunoblotting results should be assessed as they were acquired — as digital images of blots — instead of as numerical outputs of software processed data. To verify that immunoblotting data were generated and quantified appropriately, it is important to display raw data for all experimental conditions in the same format as for the representative data shown in published figures and provide detailed methods that allow others to replicate the analysis. Information about

the processed data for each figure panel is relevant for interpreting a figure and should always accompany processed data. Western blots displayed with inappropriate design or post-acquisition processing can unintentionally or deliberately obscure issues that mislead the reader.

To confirm that the appropriate procedures have been followed, data for published western blot figures should be checked and, if possible, data for all replicates for each experiment should be requested (Kroon et al., 2022). If the design cannot be confirmed from the raw data, any concerns about the interpretation should be resolved before acceptance. Western blot experiments should be designed and pre-registered so that all relevant data are generated, displayed, and clearly reported with each publication. Each blot containing multiple experimental conditions should show all relevant information concerning blots consisting of more than one figure panel. Each data figure should stand alone with all relevant detail and data displays without the need to refer elsewhere. After a decade of effort, similar problems persist in the design and reporting of immunohistochemistry experiments, and we hope that this approach to generating and presenting data from western blot experiments will be useful in addressing these issues.

10. Troubleshooting and Common Issues

Western blotting is widely used to quantitate specific proteins in samples and to evaluate protein modifications, interactions, and subcellular localization. Common problems during western blotting may lead to unreliable or inconclusive results. Therefore, it is essential to understand the processes involved in producing quality western blots and potential sources of error (Kroon et al., 2022). This chapter explores common issues encountered before, during, and after western blotting, and suggests practices for troubleshooting.

Determining Protein Concentration: Accurate determination of protein concentration in samples is essential for loading equal amounts of total protein per lane on the gel. If protein concentration is underestimated, detected bands will be faint or absent. Conversely, overestimation will lead to sample loading amounts exceeding the detection capacity of antibodies and membranes, resulting bands appearing compressed. Heterogeneous protein content in lysates from different sources can lead to sample variability, causing inconsistent detection of bands across blots. Use of high-bind plates during assays and staining of wells helps standardize sample handling and minimizes variability.

Sample Preparation: Sample homogenization, freeze-thaw cycles, protease inhibition, and buffer choice can all affect the quality of western blots. If protein samples are insufficiently homogenized, detected bands may appear as streaks, and some may be missed. Freeze-thaw cycles can precipitate proteins, particularly those with low solubility. If protease inhibitors are not included, protein degradation may compromise sample quality. On some blots, bands are detected and used as a loading control, but these bands vary with sample buffer choice.

10.1. High Background Noise

High background noise, either on a blot or in a band, is a common problem seen in initial and subsequent experiments. It can arise from various sources, primarily due to either detector saturation or excessive cross-reactivity. Fortunately, there are several easy steps that can be taken to reduce background noise. First, the simplest solution is to decrease exposure time when imaging fluorescent blots, ensuring the detector is not saturated. If this is insufficient, fluorescent blots can be scanned using different detection layers to find the one that produces the least background noise. Alternatively, adjustments can be made using software after acquisition. Most issues arise when imaging chemiluminescent blots that have been exposed to film. In these cases, excessive background noise cannot be resolved post-acquisition. As a result, blots that appear overexposed should be imaged using a different technique instead (J. Bass et al., 1970). The most common cause of a blot having a high background is excessive cross-reactivity of the detection antibodies. To ensure good western blotting results, primary antibodies should be thoroughly validated before use. This includes determining the optimal working concentration, identifying cross-reactivity with

related proteins, and testing whether the antibody requires any tags or modifications for detection (Kroon et al., 2022).

10.2. Weak Signal Intensity

In cases of faint bands, it is often advisable to show an entire gel and blot. Consider showing an unprocessed image of the blot, rather than the processed figure as it appears in the publication. Ensure that the methods include full details of the processing applied to the western blot figure, including what adjustments were made to brightness and contrast, as well as any cropping or splicing. If any adjustments were made to brightness or contrast, specify how these adjustments were applied (e.g., globally or to individual lanes).

If adjustments were made to brightness or contrast, provide representative raw data (ideally in unprocessed form) for the reader to independently evaluate the results. If the data cannot be provided, make every effort to characterize what processing was done, such as providing the processing history. Cropping can be problematic because it may conceal how signals have been selectively displayed. If lanes are cropped out of a blot, it is advisable to show an image of the entire blot. If any blots have been modified by splicing, provide evidence that such modifications were necessary (Kroon et al., 2022).

11. Applications of Western Blotting

Western blotting (or immunoblotting) is a common laboratory method used to detect proteins and assess their expression levels (Kroon et al., 2022). A protein of interest is identified based on its molecular weight and immunoreactivity with a specific antibody. Small variations in how these steps are performed can alter the quality of the blot, introduce errors, or affect the interpretation of experimental results. This has contributed to concerns about the reproducibility and reliability of western blot experiments. Some common issues include target protein not being detected, inappropriate loading control, differences observed in blots that should be similar, inappropriate controls, unreported methods, and unreported key details. Improving publication practices could have an important impact on reproducibility and trustworthiness. Informative figures and detailed methods sections help readers to identify well-executed experiments and potential sources of error, while providing information needed to replicate the experiment. Blots should include relevant controls, the accompanying methods section should describe controls and adjustments made to imaging systems in detail, and reporting a single example blot for each experimental condition should be avoided. Western blotting is a standard laboratory method that uses antibodies to detect target proteins in a sample.

11.1. Protein Expression Analysis

Western blot is widely used to examine protein expression, providing data on the relative abundance, presence, and size of specified proteins in a sample. In general, a western blot experiment involves sample preparation and determination of protein concentration, gel electrophoresis to separate proteins by size, transfer of proteins to a solid membrane support, membrane blocking to minimize non-specific binding, incubation with primary and secondary antibodies to visualize target proteins, and imaging detection of protein bands (Kroon et al., 2022). Small variations in how these steps are performed can alter the quality of the blot, introduce errors, or affect the interpretation of experimental results. Common and often unrecognized problems include the aberrant detection of a non-target protein that is not a specified isotype control, overexposure or underexposure of images, the use of inappropriate controls to show specificity, failure to account for differences in loading, or a failure to disclose all aspects of the methodology.

Measurements of the relative quantities of independent proteins in complex biological samples can be performed with a quantitative western blot (qWB) assay. Like other quantitative biomolecular assays, a qWB involves many procedural steps, each contributing experimental variability (Omondi et al., 2023). Methods to control for these variabilities normally yield relative comparisons of protein levels rather than absolute protein concentrations. Therefore, to increase the accuracy of inferences about protein levels from qWB data, it is paramount that the design of qWB experiments

and data analyses are rigorously conducted. A western blot is a gel electrophoresis-based technique used for the separation, transfer, and detection of proteins. Each lane of a blot is generally treated as a unique experimental unit, comparable to a well in a plate reader experiment. Across the life sciences, western blotting is one of the most common methodologies used to generate quantitative scientific data.

11.2. Post-Translational Modification Studies

With the addition of new antibodies raised against post-translationally modified (PTM) regions, western blotting can be an excellent method for screening one or a few candidate proteins for such modifications following the appropriate treatment. However, when seeking new proteins undergoing a specific PTM, alternative methods are more effective because western blotting depends on the availability of highly specific antibodies. Two-dimensional gels run in the absence of denaturing conditions retain PTMs and can help detect and identify proteins undergoing such modifications. The simplest approach is to use one antibody raised against the unmodified sequence and run a second dimension in the presence of urea and thiourea to map the proteome and identify proteins that shift in pI or MW from the first dimension (Kusch et al., 2017). Still, this approach only identifies the overexpressed or abundant proteins in an immunoprecipitation experiment. A more sophisticated approach is first to partially immunoblot the 2D-gel onto a clean membrane to visualize only the proteins where a specific modification is detected by a specific antibody. The position of the proteomic shift can then be tracked back to the gel and excised for mass spectrometric identification.

Immunoblots can also be done with proteins run in 1D gels, but this strategy requires a specific antibody rather than a general detection method. After the first dimension, a conventional immunoblot is done to visualize the detection of the modification, and the second dimension can be a gel-staining procedure, or the gel can be run in the absence of PTM denaturing conditions to keep the modification intact (Emenike et al., 2022). The latter is a more sophisticated approach, allowing the use of quantification methods to assess the extent of the PTM. If the gel is stained with Coomassie blue prior to destaining, the proteome can be visualized, and a mass spectrometric identification of a protein with a specific PTM can then be done. However, to trace back the modification, mass spectrometric identification is done before gel-staining, and either method can map to the same gel. Again, as in the 2D-gel approach, proteins need to be excised from the gel and in-gel digested prior to identification by mass spectrometry.

12. Advanced Techniques and Variations

There are several advanced techniques and variations on the basic protocols that can enhance Western blotting results in terms of sensitivity, quantitative results, optimization for targets such as small or hydrophobic proteins, and simplification or multiplexing of procedures. Dot blots are a simple variant of Western blots in which sample is applied directly to the membrane instead of using gel electrophoresis. No size separation occurs, so the antibody must be able to bind in the same conformation as the native state. In contrast to Western blots, which use gel membranes like nitrocellulose or PVDF membranes that allow diffusion of the protein for size separation, dot blots can use any solid support such as poly-l-lysine coated slides, nylon membranes, or nitrocellulose membranes. A straightforward method utilizes poly-l-lysine coated glass slides, where the protein is covalently cross-linked to the surface prior to antibody incubation (Kroon et al., 2022).

To multiplex dot blots requires two or more different antibodies that should not cross-react with one another. One approach is to use tandems, where one primary antibody has two different epitope tags, allowing one antibody to recognize different species in different fluorescence channels. Alternately two different primary antibodies may be recognized by secondary antibodies conjugated to different fluorophores, for example, Alexa Fluor 605 and 488. Dot blots have applications in concanavalin A-affinity blotting, cytokine detection, and studying protein–protein interactions. To quantify protein levels, dot blots can use infrared dyes and an imaging system similar to that used for Western blots. Other techniques can analyze samples loaded onto the dot blot, such as matrix-

assisted laser desorption/ionization-time-of-flight mass spectrometry analysis or protein sequencing by Edman degradation.

12.1. Multiplex Western Blotting

Ninety-seven different cancer and normal human cell lines were screened for the expression of the novel 17 determinates SULF1, SULF2, HER3, MST1R, FLT3, PDGFRB, SELE, MMP19, CXCR4, MMP14, FN1, SNX19, THBS1, LAMC2, CTGF, ANX2, IGFBP5, and PROS1 using multiplex Western blotting with ProEx™ antibodies in combination with a two-step CyDye immunoblotting detection method. With the multiplex Western blotting approach it is possible to use a mixture of antibodies for the simultaneous detection of multiple different proteins in a single membrane (M Donoghue et al., 2006). This greatly reduces the amount of sample and time required compared to multiple Western blotting experiments with a single antibody. In contrast to the multiplex fluorescent Western blotting method using infrared dyes, this approach can employ any antibody that is detected with a label that fluoresces in the visible range. This is a considerable advantage given the wider availability and lower cost of the proposed CyDye immunoblotting detection system. The multiplex Western blotting proved to be a robust method. Even with 17 antibodies as a proof of principle, the electrophoresis, transfer, and detection steps could all be performed together without any changes in the procedure compared to a standard singleplex Western blotting assay (Schüchner et al., 2016). As expected, a slight increase in background fluorescence was observed, but this did not adversely affect the detection of the targets of interest.

12.2. Phospho-Specific Western Blotting

Phosphorylation is a reversible post-translational modification that regulates cellular processes, such as signal transduction, growth, and cell cycle progression. Understanding the roles of phosphorylated proteins in health and disease is important for drug discovery. Phospho-specific antibodies allow the detection and enrichment of phosphorylated proteins. Here, information about the preparation and quality control of phospho-antigen samples for phospho-specific antibody production is provided, and a screening method to preliminarily determine the quality of commercial phospho-specific antibodies is described. Phosphorylation regulates protein conformation and localization, thus playing important roles in cellular processes such as signal transduction, growth, and cell cycle progression. Abnormal phosphorylation is associated with diseases, such as cancer, neurodegenerative disorders, and diabetes. Understanding the roles of phosphorylated proteins in health and disease is important for drug discovery (Garaguso & Borlak, 2012).

13. Future Directions in Western Blotting

Easy-to-use and affordable commercial systems are available for rapid, consistent, and sensitive detection of a range of targets on blots, allowing less experienced users superior results from the beginning, but also encouraging expert users to experiment with more challenging targets. Concerns regarding quantifying large number of targets on a single blot from a single sample, preventing interference from loading control targets, and ensuring the data is reproducible across experiments are highlighted. A drastic reduction in the availability of major and even minor target validation reagents poses additional challenges for high trust experiments. Concerns around reproducibility and trust in biomedicine experiments are relative to non-validated reagents and inadequate detail in publication methods and experimental pipeline design (Kroon et al., 2022). Efforts on techniques and methodology improvements must be complimented with equal efforts on external validations, experimental pipeline transparency, and consideration of how data is shared, displayed, and interpreted to ensure not just accuracy of measurements but high trust throughout the scientific community.

Conclusion

Western blotting remains a cornerstone technique for protein detection and analysis in molecular biology and biomedical research. Its ability to combine size-based separation with antibody specificity provides valuable insights into protein expression, modifications, and interactions. While

technical variability and reproducibility challenges persist, careful optimization, use of proper controls, and transparent reporting significantly enhance data reliability. With ongoing advances in multiplexing, phospho-specific detection, and automation, western blotting continues to evolve, maintaining its essential role in both basic research and clinical applications.

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