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Western Blot Handbook

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1. Introduction

Western blotting is a protein detection method using specific antibodies and involves two major processes: separation of soluble proteins into distinct bands and the subsequent transfer of those proteins onto a solid matrix for subsequent analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins based on polypeptide length. Further, the isolated proteins are transferred (blotted) onto a membrane matrix of nitrocellulose or Polyvinylidene Difluoride (PVDF), where they are detected with antibodies specific to target protein antigens. Typically, the primary antibody incubation is followed by a secondary antibody step, which is conjugated with a marker, used for signal visualisation.

The analysis of protein migration and the intensity of either chromogenic, chemiluminescent or fluorescent signals offer protein expression details from cells or tissue homogenates.

2. Principle

2.1. Protein Extraction and Quantitation

The first step in a Western Blot analysis involves the preparation of sample lysates for the subsequent protein separation. It is important to avoid sample degradation at this point, which can be prevented by keeping the samples on ice and adding protease and/or phosphatase inhibitors.
After protein extraction, the concentration of protein should be measured with commercially available protein concentration assays (e.g. Bicinchoninic Acid Protein Assay, Coomassie Protein Assay) to ensure that equal amounts are loaded onto the gel.

2.2. Protein Separation: Electrophoresis

The electrophoresis separates proteins according to their electrophoretic mobility, when moving through a gel matrix under the influence of an electric field, depending on the charge, size and structure of the molecules. The proteins are loaded onto polyacrylamide gels (PAGE), three-dimensional mesh polymers composed of cross-linked acrylamide, which are thermo-stable, transparent, strong, and can be prepared with a wide range of average pore sizes.

Native Proteins

The separation of native proteins relies on the sum of intrinsic charges and is accomplished through isoelectric focusing (IEF). IEF employs polyacrylamide gels containing a pH gradient, where a protein stops migrating under the influence of electric current at its isoelectric point (pI). A protein reaches its pI when it has no net charge. This pI may be variable for a given protein and indicative of the presence of post-translational modification such as sugars (glycosylation) or phosphates (phosphorylation). IEF separation may be accomplished by the addition of isophores to a polyacrylamide gel preparation, prior to crosslinking (casting gels), immobilization of pH gradients to a solid support (IPG strips), or using a solution phase technique.

Denatured Proteins

While native proteins still display their inter- and/or intra-chain bonds, creating secondary or tertiary structures, which may play a vital role in the activity of the protein, denatured proteins are most commonly used for Western blotting. The native protein structure might affect the migration of the protein through the pores created by the cross-linked polyacrylamide, creating artefacts. Also, many antibodies are generated against peptides and often better recognize protein in the denatured state.

Denaturation of proteins can be accomplished as follows:

- The anionic detergent sodium dodecyl sulfate (SDS) is used as a dena turing agent to break hydrogen bonds within and between molecules to unfold proteins and break up secondary and tertiary structures. The anionic (or negative) charge of SDS imparts a negative charge to linearized proteins that is proportional to its peptide length. In the presence of SDS, electro-phoretic mobility is largely based on molecule weight.
- Strong reducing agents such as β-mercaptoethanol (BME) and dithiothreitol (DTT) disrupt disulfide bonds between cysteine residues within or between protein chains.
- Heating the samples to 100°C promotes the protein denaturation and SDS binding, rod- shape formation, and negative charge adherence.

  The addition of loading buffers, containing tracking dyes such as bromophenol blue, allows the visualisation of the progress of the proteins through the gel during the electrophoretic separation.

Gel Percentage and Pore Size

The use of polyacrylamide gels allows the separation of proteins by size ranging from 5-2,000 kDa, due to the uniform pore size created during polymerization of acrylamide. Pore size is controlled via the ratios of acrylamide to bis- acrylamide in gel preparation process.
Typically polyacrylamide electrophoresis gels (PAGE) gels are cast with a stacking gel (5%) poured on top of a resolving gel. The stacking layer exhibits a lower acrylamide concentration to counteract the migration differential of protein samples as they first enter the separation matrix. At the interface of the two gel components, the proteins are “stacked” to optimize their separation during their migration through the resolving gel. Resolving gels are made in 5%, 8%, 10%, 12% or 20% formulations, each providing a range of protein separation, based on the size of the target protein. Higher percentage gels are used for separation of proteins of lower known or estimated molecular weight.

If the molecular weight of the protein is unknown or if proteins of a variety of molecular weights are examined, a gradient gel, containing various concentrations of poly-acrylamide within the same gel, can be used. Protein samples are introduced into the gels via wells that define the lanes of protein migration. Changes to the buffer system of the gel can help to further resolve proteins of very small sizes.

Buffer Systems

A wide variety of buffer systems exist for the use with PAGE:

The most commonly used is the tris-glycine or “Laemmli” discontinuous buffer system, which uses a stacking gel prepared with buffer at pH 6.8 and a resolving gel at pH of ~8.3-9.0.

The disadvantages associated with this system include potential protein deamination and alkylation or disulfide bond formation between cysteine residues from the re-oxidation of cysteine due to non-migration of sample buffer reducing agents into the resolving gel. Additionally, different buffers are required for the cathode and anode ends of the gel. Advancements in the buffering technology (e.g., bis-Tris) overcome these pitfalls by casting and running gels under slightly acidic (pH ~6.5) conditions and include reducing agents (e.g. sodium bisulfite) that move into the gel ahead of the proteins to maintain a reducing environment. An additional benefit of using buffers with lower pH values is that the acrylamide gel is more stable at lower pH values and therefore gels can be stored for long periods of time before use. As voltage is applied to a Tris-glycine system, the negatively charged sample molecules (anions) migrate towards the positive electrode (anode) in the lower chamber. With the leading glycine buffers, proteins are compressed (stacked) into a micrometer thin layer between the stacking and the resolving gels. In the resolving gel, proteins with small molecular weight and hence greater negative charge per unit migrate faster than those with large molecular weight and therefore less negative charge per unit. The boundary moves through a pore gradient and the protein stack gradually disperses due to a frictional resistance increase of the gel matrix. For every protein at a different position, stacking and un-stacking occur continuously in the gradient gel.

2.3. Protein Transfer

Transfer Types

There are two main types of procedures used to perform electrophoretic transfer of proteins:

- **Semi-Dry Transfer**

The semi-dry protein transfer is a rapid, efficient and inexpensive method, which does not require buffer tanks or gel cassettes. A separation gel and a transfer membrane are equilibrated in transfer buffer and sandwiched between sheets of buffer-soaked filter paper and then clamped between two horizontal plate electrodes. To maximize the amount of buffer available during the transfer, extra thick filter paper is recommended.
In an electric field, the negatively charged proteins migrate towards the positive cathode, exiting the gel and depositing on the transfer membrane where they are held by hydrophobic interactions. Transfer times for semi-dry transfers are short due to the high electric field caused by the close proximity between the two electrodes and by the low buffer capacity of the saturated filter paper. High field strengths may cause some small proteins to be driven through the transfer membrane, while shortened run times may result in the inefficient transfer of some large proteins. Hence, semi-dry protein transfer methods are not considered to be reliable for quantitative analysis.

- **Wet Transfer**

The wet transfer process involves a gel-membrane-filter sandwich flanked by pads or sponges and placed vertically in a cassette or transfer tank filled with buffer. With this method, proteins also migrate from the gel to the membrane under the electric field produced by the parallel electrodes. The comparably large buffer capacities with the wet transfer procedures offer more flexibility for voltage settings, transfer times, and temperature conditions. Varying these parameters allows larger proteins to be transferred more effectively, while promoting efficient binding of lower molecular weight proteins. Although the wet transfer method involves more complex apparatus and higher buffer costs, the opportunities for optimization of the transfer conditions make the method a preferred choice when quantifying proteins of interest.

Transfer Membrane

Transfer membranes differ in their physical properties like the protein binding capacity and the mechanical strength, used with particular methods and conditions. Further downstream analysis such as re-probing or protein sequencing also have to be considered, when choosing a blotting membrane.

- **Nitrocellulose**

Nitrocellulose is inexpensive, has a high affinity for proteins, is easily prepared for blotting, and can be used with a variety of detection systems. Hence, nitrocellulose is considered a good choice for general purpose western blotting. However, due to its brittle nature nitrocellulose is not a preferred material for blots intended for workflows requiring repeated handling or manipulations. Some manufacturers offer nitrocellulose membranes with a synthetic backing providing increased strength while retaining the desirable characteristics of protein binding and ease of wetting. Nitrocellulose requires the use of methanol in the transfer buffer, when used as a transfer membrane to remove SDS bound to proteins and thus enabling hydrophobic interactions of protein with the transfer membrane. However, methanol causes shrinkage of the separation gel, reducing the size of pores in the gel matrix. The smaller pore size may influence the movement of some larger proteins, which has to be considered when selecting the transfer membranes and protocols.

- **Polyvinyl Difluoride (PVDF)**

PVDF membranes have some distinct advantages over nitrocellulose such as higher binding capacity (170-200 µg/cm2 vs. 80-100 µg/cm2) and durability. The higher binding capacity is advantageous when attempting to detect lowly expressed proteins, but may also lead to higher background noise during detection. Prior to use, PDVF membranes must be incubated in 100% methanol and further equilibrated in transfer buffer for at least 5 minutes.

PVDF membranes allow the re-probing of transferred proteins after a specific stripping procedure. Quantitative analysis of detected proteins on re-probed gels should be avoided as the stripping process may remove some target proteins from the membrane.

Membrane Pore Size

Blotting membranes are typically available at pore sizes of 0.2 µm, and 0.45 µm. For proteins of 20 kDa or smaller a pore size of 0.2 µm is recommended and 0.45 µm pore if your protein is greater than 20 kDa. For the quantitation of a target protein, or if the protein concentration is low, it is recommended to use the membrane with the smaller pore size.

Membrane Formats

- Pre-cut membranes are available for all of the most commonly used sizes of separation gels. Pre-cut and packaged by manufacturers, these membranes may provide more consistent protein transfers than membranes cut from rolls or large sheets.

- Rolls of membranes are available for cutting membranes to use with a variety of gel sizes. The lower cost of the membrane may be outweighed by the time expended to ensure that the size of the transfer membrane exactly matches that of the separation gel, to avoid introducing variability in the transfer analysis.

2.4. Membrane Blocking

Following the protein transfer unspecific sites on the membrane need to get blocked, using inert proteins and/or non-ionic detergent to reduce levels of nonspecific protein binding during the assay. Blocking buffers serve the purpose of occupying all unreacted sites without disrupting target protein-membrane interactions or affect epitope availability. Commonly used blocking solutions include non-fat dry milk, casein, gelatin, or Tween-20 in TBS and/or PBS buffers.
Whilst non-fat dry milk is the most economic choice, it should be avoided for blots

using biotin-conjugated antibodies, due to intrinsic amounts of glycoprotein and biotin present in milk. Furthermore, naturally present phosphatases may lead to protein dephosphorylation, interfering with target identification of phosphorylated proteins. BSA or casein in TBS are recommended for phosphorylated target analysis or when using alkaline phosphatase-based detection methods.

2.4 Primary Antibody Incubation

Following the transfer membrane blocking, a primary antibody specific to the target protein is incubated with the membrane. This procedure is typically carried out in a container allowing the membrane to sit horizontally, while immersed in buffer. Incubation is either performed overnight at 4°C or 1-2 hours at RT with mild agitation. For western blot analysis both polyclonal and monoclonal antibodies work well. Monoclonal antibodies recognize a single specific antigenic epitope, which contributes to low background levels of the analysis. However, target epitopes that are blocked or destroyed during the analysis may cause poor blot results when using monoclonal antibodies. On the other hand, polyclonal antibodies may recognize more epitopes on the target and they often have higher affinity, providing results for analysis when specific epitopes are not known or their identification is not required for the application. Further, polyclonal antibodies are generally found at lower cost than monoclonal antibodies.

2.5 Secondary Antibody Incubation

After washing the membrane to remove unbound primary antibody, the membrane is exposed to an enzyme-conjugated secondary antibody, highly specific for a site on the primary antibody. The choice of secondary antibody depends upon the species of animal in which the primary antibody was raised. The most popular secondary antibodies are anti-mouse and anti-rabbit immunoglobulins as these animals are commonly used as host species for the production of primary antibodies. In turn, goats are used to raise anti-mouse and anti-rabbit polyclonal antibodies. Thus, goat anti-mouse and goat anti-rabbit immunoglobulins are the most commonly used secondary antibodies.

2.6. Signal Detection

An enzyme bound to a secondary antibody reacts with a substrate to generate a colorimetric precipitate, bound to the transfer membrane resulting in visible protein bands. The target protein levels in cells or tissues are evaluated though densitometry and location of the visible protein bands.

Chromogenic (Colorimetric)

Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes that are extensively used. As a result of alkaline phosphatase (AP) catalysis, the colourless substrate BCIP will be converted to a blue product. In the presence of H2O2, 3-amino-9- ethyl carbazole and 4-chlorine naphthol will be oxidized into brown and blue products, respectively.

Chemiluminescence

Enhanced chemiluminescence is another method that employs HRP detection. Using HRP as the light (luminesce). Luminiscence enhancers combined with this substrate will induce a 1000-fold increase in light intensity. The light is then detected and captured on a photographic film. Further, the film can be exposed for multiple time points to optimize the signal to noise ratio.

Fluorescence

The fluorescence detection uses fluorochrome conjugated primary or secondary antibodies to detect the protein of interest, which is captured using specialized fluorescent imaging systems. There are several advantages to this method including the elimination of the enzymatic step and the ability to multiplex. This method is also more quantitative than other methods.

2.7. Controls

Proper control design is essential to successful western blot analysis and will guarantee accurate and specific test results, identifying various problems quickly and precisely. Different types of controls are discussed in the following:

Positive Control

Positive controls are designed to verify working efficiency of the antibodies. Therefore, use a lysate from a cell line or tissue sample, known to express the protein of interest.

Negative Control (Primary Antibody Control)

Negative controls are used is to check the antibody specificity and identify nonspecific binding and false positive result. Hence, a lysate from a cell line or tissue sample known not to express the protein you are detecting.

Secondary Antibody Control

Signals produced by nonspecific binding may generate false positive results using the secondary antibody alone. In this case, a secondary antibody is incubated with the blot to check antibody specificity.

Blank Control

This analysis is performed to verify the transfer membrane itself does not cause a false positive result and is also used to check membrane blocking efficiency. For the blank control the primary and secondary antibodies are not incubated with the membrane.

Loading Control

Loading controls are used to check the sample quality and the performance of the secondary antibody system. Loading controls are antibodies specific for house-

keeping proteins or proteins that are expressed at equivalent levels in almost all tissues and cells. These are required to check that equivalent quantities of target protein have been loaded across gel lanes, especially when a comparison must be made between the expression levels of a protein in different samples.
In case uneven quantities of sample are loaded or transfers have not occurred, the loading control bands can be used to quantify the protein amounts in each lane. The use of a loading control is absolutely essential for publication-quality work. Some commonly used loading controls are:

Loading Control	Molecular Weight (kDa)	Sample Type
Beta-Actin	43	whole cell/cytoplasmic
GAPDH	30-40	whole cell/cytoplasmic
Tubulin	55	whole cell/cytoplasmic
VCDA1/Porin	31	mitochondrial
COXIV	16	mitochondrial
Lamin B1	16	nuclear (unsuitable for samples where nuclear envelope is removed)
TBP	38	nuclear (not suitable for samples where DNA is removed)

3. Recommended Protocol

3.1. General guidelines

- Use chemical safety glasses, nitrile gloves, fully buttoned front or back closing lab coat, and closed toe shoes.
- Prevent direct contact with membrane, film or reagents and use gloves and clean blunt-ended forceps where appropriate.
- All handling of chemicals should be done under a chemical fume hood.
- Note: acrylamide, as a monomer, is considered toxic, carcinogenic, and may cause CNS damage. It is readily absorbed through intact skin from aqueous solutions. However, once polymerized, the solid polyacrylamide is considered safe. However, PAGE gels should still be handled with gloves under the assumption that they may still contain unreacted monomer.

3.2. Sample preparation

Extraction from Cell Culture

- Culture cells in the cell culture dish to 80% confluency.
- Aspirate PBS and add ice-cold lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris- HCl and protease inhibitors).
- Digest cells with 0.05% trypsin and wash cells with PBS carefully to avoid inducing cell stress pathways.
- Centrifuge the cells at 3,000 rpm at 4°C for 2-3 min.
- Remove the supernatant and wash twice with ice-cold PBS buffer.
- Gently transfer the cell precipitate into a chilled tube.
- Add Lysis reagent (e.g. mammalian cell protein extraction reagent, from different companies) into the tube and re-suspend vigorously.
- If the above solution is turbid, sonicate for 10-15 sec to break up the proteins.
- Lyse the cells in RIPA lysate buffer on ice for 4-5 hours.
- Sonicate and lyse again if the cell solution remains turbid.
- Centrifuge at ~10,000 rpm and 4°C for 10 min. (Note: the centrifugation force and time might have to be adapted depending on the cell type)
- Discard lipid (at top) and cell debris (at bottom) by transferring the middle layer to a fresh tube
- Store at -20°C.

Extraction from Tissue

- Place surgically resected tissues in chilled normal saline.
- Wash off any blood remnants from the tissue and cut the tissue into small pieces (0.1 g to 1 g each).
- Add proteinase inhibitor reagent as recommended by the supplier.
- Add Mammal Tissue Protein Extraction Reagent according to the manufacturers guidelines.
- Reduce the tissue into small pieces and place the in a tissue homogenizer.
- Add chilled lysis buffer (add 300 µL of buffer for each 5 mg piece of tissue).

- Lyse the tissue homogenate on ice for 4-5 hours or at 4°C (high speed) for 5 min. If necessary, sonicate until no tissue chunks remain.
- Centrifuge at ~10,000 rpm and 4°C for 10 min. The centrifugation force and time can be adjusted to the sample type.
- Discard lipid (at top) and cell debris (at bottom) by transferring the middle layer to a fresh tube and keeping the tube at -20°C.

3.3. Protein Quantitation Assay

In order to assess the concentration of extracted protein commercially available assays like a Bradford, a Lowry or a BCA assay can be performed. Most assays use bovine serum albumin (BSA) as a protein standard.
At this stage the samples can be stored at -20°C or -80°C for later use or prepared for immunoprecipitation or for loading onto a gel.

3.4. Gel Preparation

First, determine the gel percentage based on the molecular weight of your protein sample:

Protein Size (kDa)	> 200	15-200	10-70	12-45	4-40
Gel Percentage	5%	8%	10%	12%	20%

If you are not sure of the size of your protein or are looking at proteins of a variety of molecular weights, a gradient gel may provide the best resolution.

A. Resolving Gel Preparation

Determine the final volume and percentage needed for the resolving gel and gently mix the ingredients (30% acrylamide, 10% SDS, 10% ammonium persulfate, TEMED, dH2O and 1.5 M Tris, pH 8.8).

- Pour the solution into your gel casting form.
- Layer the top of the gel with distilled water.
- Wait approx. 30 min for the gel to polymerize completely.
- Remove the water from the polymerized resolving gel (absorb excess water with paper towel).

A. Stacking Gel Preparation

- Determine the volume needed, gently mix the ingredients (30% acrylamide, 10% SDS, 10% ammonium persulfate, TEMED, dH2O, 1.0 M Tris, pH 6.8) and pour the stacking gel on top of the running gel.
- Insert the sample comb gently to avoid bubbles and allow 30 to 60 min for complete gel polymerization.

1.1. Pre-electrophoresis Sample Preparation

Mix the extracted protein sample with suitable protein loading buffer.
Protein Loading Buffer are designed to prevent protein degradation during sample heating prior to electrophoresis and is able to work against pH changes during SDS-PAGE run, as many proteins are sensitive to pH changes that result from temperature fluctuations of Tris buffers during electrophoresis. Protein loading dyes usually contain two tracking dyes for tracking the progress of electrophoresis and for monitoring of protein transfer to the membrane.

1.2. Loading Samples and Running Electrophoresis

- Place the gel in the electrophoresis apparatus and fill both buffer chambers with SDS PAGE Electrophoresis Buffer (25mMTrisbase,190mM glycine and 0.1% SDS; pH 8.3).
- Carefully remove the comb from the gel and rinse the wells with electrophoresis buffer.

- Pipet your samples (for a well with maximum 30 µL, load 20 to 25 µL of 1 µg/µL sample per well), controls and/or molecular weight standards (load 10 µL each) into separate wells carefully and proceed quickly to the next step to prevent possible sample diffusion inside the well).

- Connect the anode and cathode of the electrophoresis chamber appropriately to the power supply. Turn on the power to run the electrophoresis at 100V/130V* (the applied voltage should be adjusted according to the gel thickness, power supply used and resolution desired) until the bromophenol blue dye reaches the gel bottom, which can take between 1.5 to 3 hours. You should observe fine bubbles rising from the gel apparatus bottom, as this observation indicates sufficient electric current is generated.

- Turn off the power when the protein samples have finished migrating in the gel.

Note: in a discontinuous system, the electrophoresis voltage for the stacking gel is lower than that for the resolving gel to ensure that proteins are concentrated on the same level before running into the resolving gel. The applied voltage should be adjusted according to the gel thickness, power supply used and resolution desired.

3.7. Protein Transfer to the Membrane

- **Gel Staining (Optional)**

Note: stained gel cannot be used in the subsequent protein transfer procedure.

- **Wet Transfer**

a. Blotting Membrane Preparation

- Cut the blotting membrane (NC or PVDF) according to the size of your gel (its recommended to cut a good supply of membranes in advance and store in a cool, dry place).

- Cut the blotting membrane (NC or PVDF) according to the size of your gel (its recommended to cut a good supply of membranes in advance and store in a cool, dry place).
- Carefully mark the membrane orientation by cutting one corner or marking it with a pencil.
- Soak the membrane in methanol for 1 min.
- Immerse the membrane in with 1X transfer buffer (25 mM Tris base, 190 mM glycine and 20% methanol; pH 8.3) for 5 min and rock the membrane gently until it sinks and water no longer beads up on the surface.

b. Transfer Cassette

Based on a sandwich model, install the electric transfer cassette in the following order: Foam Pad ‣ Filter Paper ‣ Gel ‣ Membrane ‣ Filter Paper ‣ Foam Pad

- Soak two filter papers with the same transfer buffer in a separate container.
- Open the transfer cassette with a spatula and make sure to loosen the cassette holder all the way around, before carefully pulling apart the two halves.
- Cut the gel according to the size of the membrane with a blade and then cut the corner on the side of the gel with lane #1, to keep track of the orientation.
- Immerse the gel in 1x transfer buffer for 15-30 min.
- Place the grey or black plate of the transfer cassette on a clean surface.
- Place a dampened foam pad on the grey side of cassette and cover with a also dampened sheet of filter paper on top of the foam pad.
- Carefully peel the gel off of the remaining half of the gel cassette and place onto the filter paper. In case bubbles formed between the gel and the filter paper, dampen the gel with transfer buffer and use a serologically clean pipette or a Falcon tube, to roll the air bubbles out of the membrane.
- Place the membrane onto the gel with the corners matching up.

Notice: once the membrane contacts the gel, it should not be moved or “ghost bands” can occur.

- Complete the sandwich by placing a piece of filter paper onto the membrane.
- Place the second foam pad on top of the filter paper.
- Lock the transfer cassette firmly with the white latch. Be careful not to move the gel and filter paper sandwich and make sure that the foam pads, filter papers and membrane are thoroughly immersed in the transfer buffer.

3.8. Protein transfer

- Fill the transfer tank with an adequate amount of 1X transfer buffer.
- Firmly insert transfer cassette into the slot of the transfer apparatus.
- Place the lid on top of the transfer tank and make sure the electrodes are lined correctly: the gel should be closer to the cathode, the membrane should be closer to the anode and thereby negatively charged proteins will migrate towards the anode.
- Set power source to constant voltage and operate at 25 V for 30 min*.
- Check the protein transfer efficiency by membrane staining: therefore place the membrane in Ponceau S staining (0.2% w/v Ponceau S; 5% glacial acetic acid) for 5-10 min. In case of efficient protein transfer a visible red band will appear, which can be de-stained completely by repeatedly washing in wash buffer.

Note: The transfer can be completed overnight at a lower voltage (for example, 10 V). Transfer time and voltage should be optimized according to the gel concentration. Higher gel concentration requires additional time.

3.9. Membrane Blocking

- Rinse the blotting membrane 3 times with TBS Wash Buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 0.05% Tween 20) at room temperature for 10 min each time.
- After rinsing, immerse the blotting membrane in TBS Blocking Buffer (5% non-fat dry milk in buffer of 20 mM Tris, pH 7.5; 150 mM NaCl) and incubate for 1.5 - 2 hours at room temperature (or overnight 4°C) shaking.

Alternatively, buffer containing non-fat dried milk, gelatin, or BSA can be used. For use with biotin systems or detection of phosphor-proteins, non-fat dried milk is not recommended.

3.10. Antibody Incubation

After blocking, the membrane is incubated with a primary antibody binding to the target protein, followed by the detection with a HRP- or AP-conjugated secondary antibody.

- Dilute the primary antibody with the TBS Wash Buffer according to the manufacturer's recommendation and incubate the primary antibody with the membrane at 4°C overnight or for 1-2 hours at room temperature. For the best results, incubation time and antibody concentration may need to be optimized.
- Wash the membrane 3x with the TBS Wash Buffer for 10 min each to remove unbound antibody.
- Dilute the secondary antibody with the TBS Blocking Buffer according to the manufacturer's recommendation and incubate the secondary antibody and the membrane at 4°C overnight or 1-2 hours at room temperature on a shaker.
- Wash the membrane 3x with the TBS Wash Buffer for 10 min each to remove unbound antibody.

3.11. Signal Detection

In this section, we provide the protocols for the Enhanced Chemiluminescence Detection (ECL) and colorimetric detection (DAB or BCIP/NBT) methods. Use the method that suits your application and preferences.

- **Enhanced Chemiluminescence Detection (ECL)**

a. ECL Substrate Preparation

For the detection of HRP-conjugated antibodies commercial ECL kits arw available from various manufacturers. Choose the right kit according to the species that the primary antibody is raised.

- Thoroughly cover the membrane with the substrate solution
- Incubate the membrane at room temperature until bands appear (usually 1-5 min; incubation time can be estimated in dark room).
- Gently blot the edge of the membrane on a piece of paper to remove excess any substrate solution.
- Put a clear preservative film or transparent glass paper over the membrane and remove any air bubbles observed.

b. Film Development and Fixing

Develop and fix the film in a dark room immediately using commercially available WB Developing and Fixing Kits; alternatively use fluorescence CCD scan, a digital imager or a luminometer.

- Put the X-ray film over the membrane.
- Develop the film by immersing it in developing solution for 10 sec to 10 mins (determine the exposure time required by observing under red light and stop developing once the film achieves the experimental purpose; Multiple exposures may be necessary for the optimal signal to noise ratio)
- Wash the film with clean water to remove the developing solution completely and stop washing when bands appear.
- Immerse the film in fixing solution for 3-5 min.
- Wash the film with clean water to remove the fixing solution.

c. Colorimetric Detection

Prepare DAB (for HRP-conjugated secondary antibodies) or BCIP/NBT (for AP-conjugated secondary antibodies) substrate solution according to the manufacturers guidelines.

d. Membrane treatment

- Thoroughly cover the membrane with the substrate solution.
- Incubate the membrane at room temperature until bands appear (usually 10-30 min); note that the incubation for BCIP/NBT should be performed in the dark.
- Wash the membrane in distilled water to stop the reaction.
- Observe the bands and image.

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