



WESTERN BLOT GUIDE PROTOCOLS



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1. INTRODUCTION

A Western blot is a technique used to determine the presence or absence of selected proteins in a sample. First the proteins are separated on a basis of size by gel electrophoresis. Following this the protein is transferred to a membrane usually nitrocellulose or PVDF, through the use of an electrical current. The membrane is then stained with antibodies specific for the protein of interest, enabling the acquisition of qualitative or semi-quantitative information about the protein.

Western blot is advantageous over other antibody based detection assays such as ELISA as cross-reactivity with non-target proteins can be differentiated from the target antigen based on molecular weight.

2. SAMPLE PREPARATION – PROTEIN EXTRACTION

The first step of a Western blot protocol is protein extraction from cells or tissue. The protein of interest must be solubilized in order to migrate through the separating gel.

The choice of lysis buffer used will depend on the yield of protein required and the subcellular localisation of the protein. Lysis buffers containing sodium dodecyl sulfate (SDS) and other ionic detergents are considered to give the highest protein yield and are in turn the most damaging to the sample. It should also be noted that the lysis buffer used will affect antibody choice further on in the protocol with regards to the protein form it recognizes, either native or denatured.

Lysis buffers containing SDS have a denaturing effect on protein, whilst buffers without detergent or mild non-ionic detergents such as NP-40 and Triton X-100 should be used when the antibody will only recognise protein in its native structure. Information about the protein form your antibody recognises can be found on the data sheet supplied by the manufacturer. It is important to note that when preservation of protein-protein interactions is required a buffer without ionic and non-ionic detergents should be used.

PROTEIN LOCALISATION AND LYSIS BUFFER CHOICE

| PROTEIN LOCALISATION | RECOMMENDED BUFFER |
|----------------------------|--------------------|
| Cytoplasmic (Cytoskeletal) | Tris-Triton |
| Cytoplasmic (Soluble) | Tris-HCL |
| Membrane bound | NP-40/RIPA |
| Mitochondria | RIPA |
| Nuclear | RIPA |
| Whole Cell | NP-40/RIPA |

Table 1: Protein localisation and lysis buffer choice

2.1 RIPA BUFFER

RIPA (Radio Immuno Precipitation Assay) buffer is used to lyse and extract protein from cultured cells. RIPA buffer is an ideal cell lysis reagent for whole cell extracts and membrane-bound proteins. As RIPA buffer will disrupt protein-protein interactions it may not be ideal for immunoprecipitation assays.

2.1.1 SAMPLE RIPA BUFFER RECIPE

50mM Tris HCL pH 7.4, 50 mM NaCl, 2mM EDTA, 0.1% SDS. Plus freshly added proteinase inhibitors (Apoprotein, Leupeptin, DTT and PMSF).

2.2 NP-40 BUFFER

NP-40 buffer is widely used for the extraction of cytoplasmic, membrane-bound and whole cell extracts. NP-40 is considered a weaker buffer to RIPA buffer.

2.2.1 SAMPLE NP-40 BUFFER RECIPE

150 mM sodium chloride, 1.0% NP-40 (Triton X-100 can be substituted for NP-40), 50 mM Tris, pH 8.0.

2.3 TRIS-HCL BUFFER

20 mM Tris-HCl, pH 7.5.

2.4 TRIS-TRITON BUFFER

10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1% Triton X-100
10% glycerol
0.1% SDS
0.5% deoxycholate

*All buffers can be stored at 4°C for several weeks and at -20°C for up to a year.

2.5 PROTEASE AND PHOSPHATASE INHIBITORS

Once cell lysis occurs so too does protein degradation. To prevent proteolysis and dephosphorylation the samples must be kept on ice at all times. Proteinase and phosphatase inhibitors are added to the lysis buffer to slow this process down. These inhibitors must be added freshly each time to the lysis buffer.

| INHIBITOR | INHIBITOR TARGET |
|------------------|--|
| Aprotinin | Trypsin, Chymotrypsin, Plasmin |
| EDTA | Metalloproteases requiring Mg ⁺⁺ and MN ⁺⁺ |
| EGTA | Metalloproteases requiring Ca ⁺⁺ |
| Leupeptin | Lysosomal |
| Na Fluoride | Serine/Threonine phosphatases |
| Na Orthovanadate | Tyrosine phosphatases |
| Pepstatin A | Aspartic proteases |
| PMSF | Serine, Cysteine proteases |

Table 2: Protease and Phosphatase inhibitors and targets.

2.6 SAMPLE PROTOCOL- PREPARATION OF CELL LYSATE FROM CELL CULTURE USING RIPA BUFFER

1. Wash cells with ice cold PBS.
2. Aspirate PBS.
3. Add ice cold RIPA Buffer (~1 ml per 10⁷ cells).
4. Scrape adherent cells off the plate using a sterile pipette tip and collect in a 15 ml Eppendorf.
5. Centrifuge the sample – the centrifugation force and time may vary depending on cell type.
6. Remove from centrifuge and store on ice.
7. Aspirate the supernatant into a new tube and keep on ice, discard the pellet.
8. Determine protein concentration using a Bradford assay, a Lowry assay or a bicinchoninic acid (BCA) assay. BSA can be used a standard.
9. Once the protein concentration has been determined the samples can be frozen at -20°C / -80°C or prepared for loading.

2.7 BRADFORD ASSAY FOR PROTEIN DETERMINATION

1. Aliquot (2 ml) of each protein sample into separate wells of a 96 well plate.
2. Add Bradford reagent (100 ml) to each sample. Ensure that no bubbles are introduced.
3. Gently shake at room temperature for 5 min.
4. To calculate the protein concentration in each sample read the absorbance off a BSA standard curve, constructed as follows: prepare serial dilutions of BSA between 2 mg/ ml and 15 mg/ ml and add to 100 ml of Bradford reagent in a 96 well plate.
5. Measure absorbance at 595 nm, normalise to a reference measurement at 450 nm against the blank (2 ml lysis buffer, 100 ml dye reagent) on a microplate reader.
6. The protein sample concentration can be calculated against the standard curve.

2.8 PREPARATION OF SAMPLES FOR LOADING INTO GELS

In some cases, the antibody used will recognise the protein in native state, as the selected epitope may exist on the surface of the folded structure. When this is the case it is not necessary to denature the sample and therefore SDS should be left out. Furthermore, reducing agents such as β -mercaptoethanol and dithiothreitol (DTT) must be left out of the loading buffer and migration buffer.

Certain proteins will require denaturation for the antibody to work effectively. Heat denaturation will unfold the protein and enable the antibody to bind its corresponding epitope located within the 3D conformation of the protein. Protein denaturation can be achieved using a loading buffer containing a denaturing agent e.g. SDS which is heated at 95-100°C for 5 minutes.

The standard loading buffer for Western blot samples is 2x Laemmli buffer. Laemmli buffer contains beta-2-mercaptoethanol or dithiothreitol (DTT) which act to reduce disulphide bonds before they adopt a random-coil configuration and in turn denature the protein. Laemmli buffer also has a SDS component which provides the negative charge necessary for gel electrophoresis, in addition to glycerol to make the sample denser.

To enable visualization of the migration of proteins it is common to include small anionic dye molecule (e.g., bromophenol blue) in the loading buffer. As the dye is anionic and small it will migrate the fastest of any component in the mixture to be separated and provide a migration front to monitor the separation progress.

2.8.1 LAEMMLI BUFFER RECIPE

4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl. Check the pH and bring it to pH 6.8.

3. ELECTROPHORESIS

Once the sample has been lysed, the protein concentration determined and loading buffer added the sample can now be separated by gel electrophoresis.

There are 2 parts to an SDS-PAGE gel for a Western blot. The resolving and stacking gel. The bottom and larger portion of the gel is known as the resolving gel – this is poured first. Once the resolving gel has set (allow up an hour) the stacking gel can be added. The stacking gel is poured on top of the resolving gel – the combs are placed into this gel. The basic chemistry of these two gels is identical they only differ in their acrylamide concentration and pH.

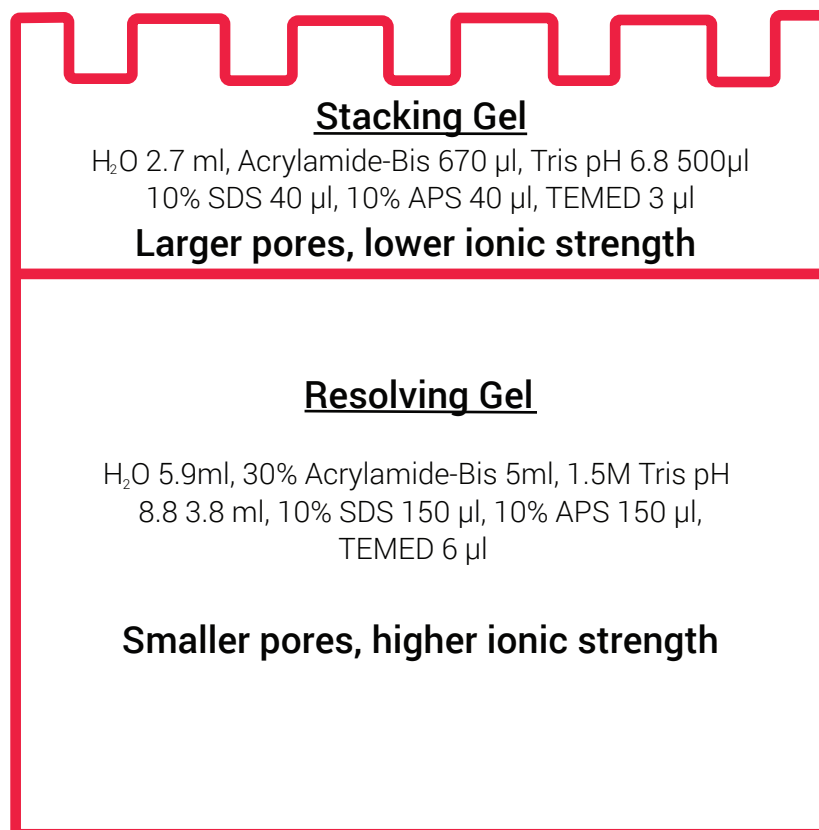


Figure 1: Western Blot Gel layout and recipes.

The percentage acrylamide used in the gel depends on the size of the protein of interest and the size of pore needed in the gel. Generally, for small proteins a high percentage gel should be used, for larger proteins a low percentage gel should be used. It should be noted that as the amount of acrylamide used increases the pore size decreases. The below table outlines the recommended percentage gels based on linear separation range.

| ACRYLAMIDE CONCENTRATION % | LINEAR RANGE OF SEPARATION (KDA) |
|----------------------------|----------------------------------|
| 5.0 | 57-211 |
| 7.5 | 36-94 |
| 10 | 16-68 |
| 15 | 12-43 |

Table 3: Acrylamide gel concentration % and protein molecular weight

Pre-made gels can be bought; however many research groups prefer to make their own. Below is a sample recipe for a 10% stacking gel and resolving gel.

| REAGENT | VOLUME |
|--------------------|--------|
| H ₂ O | 5.9 ml |
| 30% Acrylamide-Bis | 5 ml |
| 1.5M Tris pH 8.8 | 3.8 ml |
| 10% SDS | 150 µl |
| 10% APS | 150 µl |
| TEMED | 6 µl |

Table 4: Resolving Gel Recipe

3.1 10% RESOLVING GEL RECIPE

| REAGENT | VOLUME |
|------------------|--------|
| H ₂ O | 2.7 ml |
| Acrylamide-Bis | 670 µl |
| Tris pH 6.8 | 500 µl |
| 10% SDS | 40 µl |
| 10% APS | 40 µl |
| TEMED | 3 µl |

Table 5: Resolving Gel Recipe

3.2 STACKING GEL RECIPE

4. CONTROLS AND MOLECULAR WEIGHT MARKERS

As with all experiments a positive and negative control should be used to determine that the assay is accurate, sensitive and efficient. Loading controls should also be used to ensure that the gel has been evenly loaded.

Molecular weight markers

Using a range of molecular weight markers will enable the determination of the protein size (see below) and enable the electrophoretic run to be monitored.

| SAMPLE TYPE | PROTEIN | MW (kDa) |
|--|--------------------------|----------|
| Whole cell / cytoplasmic proteins | | |
| | Beta-Actin | 43 |
| | Alpha-Actin | 43 |
| | GAPDH | 30-40 |
| | BetaTubulin | 55 |
| | Alpha Tubulin | 55 |
| (high molecular weight) | Vinculin | 116 |
| Mitochondria | | |
| | VDCA1/porin | 31 |
| | Cytochrome C oxidase | 16 |
| Nuclear proteins | | |
| | Lamin B1 | 66 |
| | TATA binding protein TBP | 38 |
| | PCNA | 29 |
| | Histone H1 | |
| | Histone H3 | |
| Plant tissue | | |
| | LHCP | |
| | APX3 | |

Table 6: Molecular weight markers

5. SAMPLE PROTOCOL FOR LOADING SAMPLES AND RUNNING THE GEL

1. Separate proteins by SDS-PAGE on an acrylamide gel. The percentage of acrylamide used will depend on the molecular size of your protein of interest. In this sample protocol a 10% gel is described.
2. Pour the resolving gel between the plates and let set for 1 hr.
3. Level the gel with isopropanol. For this take 300 μ L of Isopropanol and pour carefully across the top of the gel.
4. Once your gel has set remove the Isopropanol by pouring out, wash with a gentle flow of water.
5. Pour the stacking gel and carefully place your comb into the gel, avoiding air bubbles. Allow to set for 1 hr.
6. Place the plate in the gel electrophoresis rig and add 400 ml of 1x SDS-PAGE running buffer (15.1 g TRIZMA , 94 g glycine , 50 ml of 10% w/v SDS , ddH₂O to 1 litre), first to the inside of the chamber and let over flow out to ensure no leakage.
7. Remove the combs and wash out the wells with running buffer if necessary.
8. Add 8 μ l of unstained molecular weight ladder and 20 μ l of the protein samples of interest to the selected well.
9. Run the gels at 25mA per gel for 1 hr 15 min.

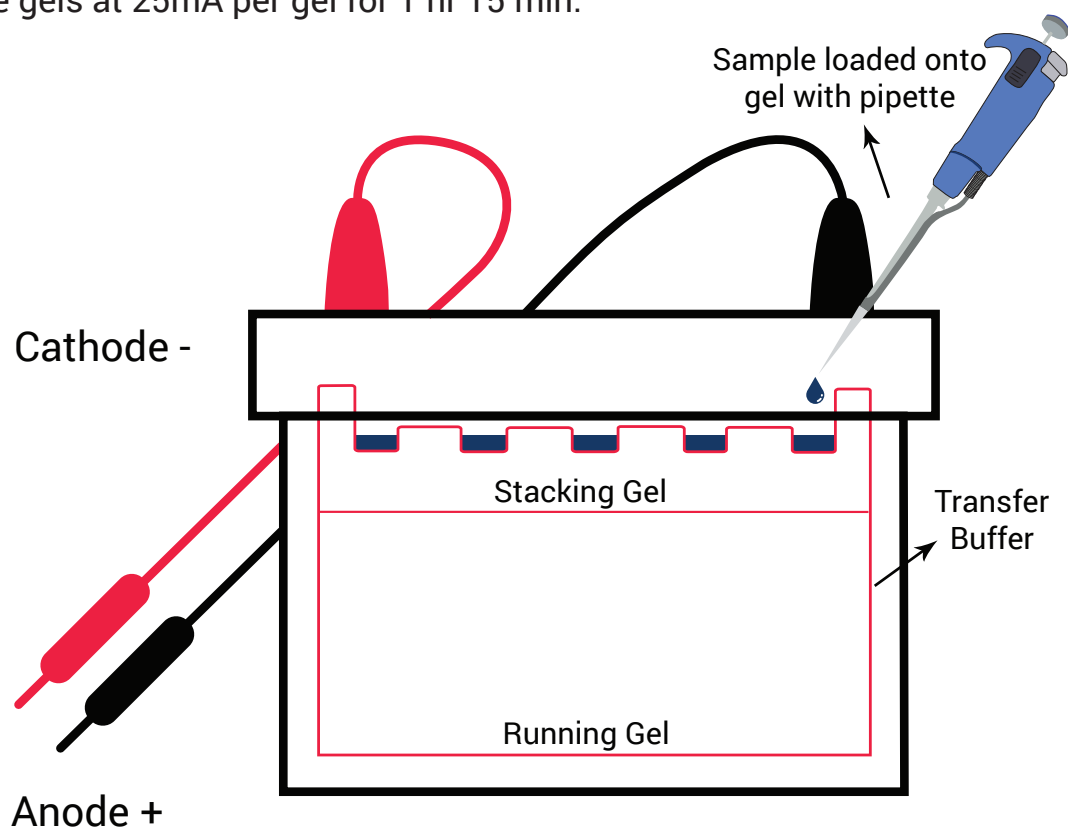


Figure 2: Western blot rig set up and well loading.

6. TRANSFER

Following electrophoresis, the protein must be transferred from the gel to a membrane, either nitrocellulose or PVDF. The most widely used transfer method is electroelution or electrophoretic transfer. This method relies on the hydrophobicity and electrostatic charges between the protein and the PVDF or nitrocellulose membrane, determining the efficacy of protein transfer.

The protein-containing gel is placed in direct contact with the transfer membrane and sandwiched between 2 electrodes submerged in a conducting solution. When an electric field is applied the proteins migrate from the gel and attach to the membrane. The membrane is now a copy of the protein pattern observed on the polyacrylamide gel.

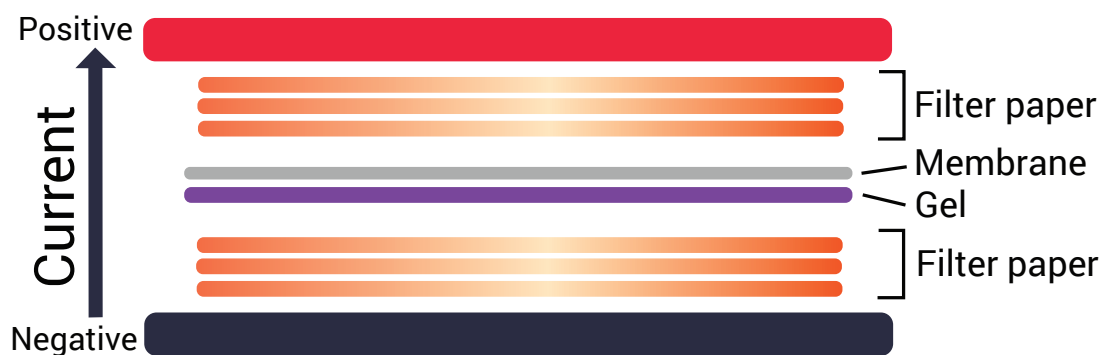


Figure 3: Transfer Sandwich set up

6.1 TRANSFER BUFFER RECIPE

5.8g Trizma base, 2.9g glycine, 0.37g SDS powder, 200 ml methanol ddH₂O to 1L

7. BLOCKING

Following transfer before the antibody is added it is necessary to block the membrane to prevent any nonspecific binding of the detection antibody. Blocking the membrane improves the sensitivity and reduces the signal-to-noise ratio of your assay. Milk and BSA are the most commonly used blocking agents.

The blocking agent used will depend on the antibody as some antibodies have been shown to non-specifically bind to milk. When probing with phosphorylation sensitive antibodies it is recommended to use BSA.

It is important to note that if your primary antibody was made against a horse, cow, goat or donkey protein that BSA or milk can't be used to block the membrane due to the potential for cross reaction or IgG contamination.

7.1 BLOCKING BUFFER RECIPE

5% dry milk powder in TBS-TWEEN (1X TBS with 0.1% v/v tween-20)

8. WASH BUFFERS

Wash steps in a Western blot are necessary to remove any unbound reagents and reduce background. Failure to wash properly can result in a high background noise, too much washing can elute the antigen from the blot.

The amount of detergent used in your wash buffer can vary depending on your assay with concentrations ranging from 0.05 to 0.5% for detergents like TWEEN 20. It is recommended to make fresh stocks of detergents e.g. TWEEN 20 as microbial growth can result in high background noise. Furthermore, as detergents contain peroxides it is essential to use high-purity detergents.

8.1 WASH BUFFER - TBS-T RECIPE

- 20 mM Tris, pH 7.5
- 150 mM NaCl
- 0.01% TWEEN 20

9. PRIMARY ANTIBODY INCUBATION

Once the membrane has been blocked to prevent non-specific binding it can now be probed with the primary antibody. A good primary antibody will recognise a specific protein or epitope on a group of proteins. The antibody used will depend on the protein under investigation.

When choosing a primary antibody, a number of considerations must be taken into account, is the antibody species specific as epitopes can vary between species, the antibody/protein interaction may be sensitive to denaturing conditions and post-translational modifications and finally not all primary antibodies are suitable for Western blotting and may need verification.

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Following incubation with primary antibody the membrane should be washed x5 times for 5 minutes with wash buffer.

*Note - Its best to always probe for your weakest antibody first, as handling and stripping can unstick your proteins of interest.

10. SECONDARY ANTIBODY INCUBATION

In general, as the primary antibody is not detectable a labelled secondary antibody must be used which binds the primary antibody and enables detection of the target antigen. The choice of secondary antibody used can depend on the species the primary antibody was raised in or the tag linked to the primary antibody e.g. biotin, histidine. For instance, if the primary antibody is an unmodified rabbit monoclonal antibody the secondary antibody used must be an anti-rabbit secondary antibody from a non-rabbit host.

As mentioned above secondary antibodies are conjugated to different developing molecules, for film detection and chemiluminescence HRP or AP is commonly used, whilst for laser capture a fluorophore is necessary.

11. ANTIBODY DILUTION

The dilution at which the antibody should be used at is commonly recommended by manufacturers. However, the optimal dilution of antibody can vary depending on the specifications of the assay. More strongly expressed proteins and assays of high sensitivity will require less antibody in comparison to more weakly expressed proteins and less sensitive assays. Using less antibody can decrease background noise and increase specificity. Antibody dilutions are normally made in wash buffer.

12. BLOT DEVELOPMENT

The type of label on the secondary antibody will determine the development system used. For enzymatically labelled antibodies e.g. HRP and AP a chemiluminescence method is needed. If a conjugated fluorophore was used a fluorescent scanning device can be used instantaneously.

12.1 CHEMILUMINESCENCE

HRP conjugated antibodies are the most widely used and considered superior to AP conjugates because of the specific activities of both enzyme and antibody due to the smaller size of the HRP enzyme and its compatibility with conjugation reactions. Furthermore, HRP substrates are widely available in addition to offering a high activity rate and stability.

AP although possessing a linear reaction rate, the increased reaction time required can often result in a high background signal and low signal-to-noise ratio.

The signal generated from HRP/AP conjugates is transient and only persists as long as the enzyme-substrate reaction is occurring. A well optimised assay with sufficient substrate can produce light for 1 to 24hrs, allowing for documentation either with X-ray film or digital imaging equipment. X-ray film can be used to obtain semi-quantitative data but digital imaging allows for detection of a broad dynamic range and enables the retrieval of quantitative data for Western blots. In chemiluminescent blotting the signal obtained is variable and generally regarded as semi-quantitative.

Signal Detection in Western Blots

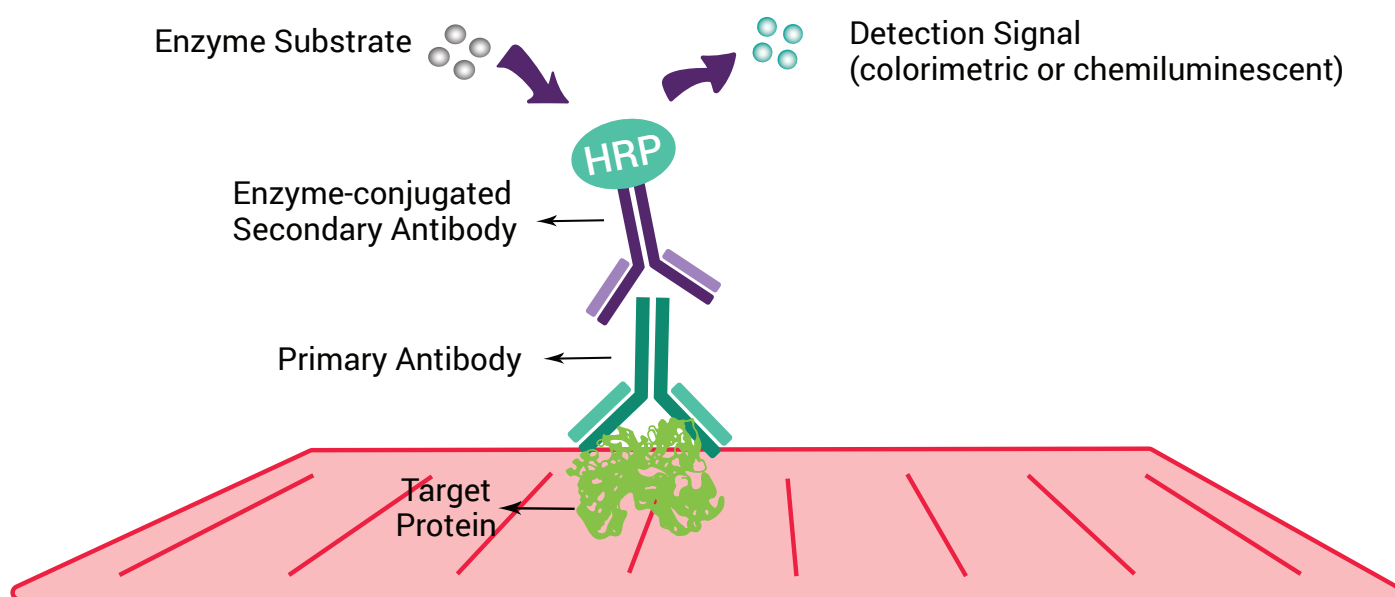


Figure 4: Western Blot detection principle. Target protein on PVDF or nitrocellulose membrane.

12.2 FLUORESCENT DETECTION

Fluorophore conjugated antibodies require fewer steps as no substrate is used, making the protocol much shorter. However, specialised equipment is required to detect the signal, requiring an excitation light source. The development of infrared, near-infrared and quantum dots has increased the sensitivity of fluorescent probed for Western blot analysis.

An advantage of using fluorescent detection over chemiluminescent detection is the ability to multiplex using more than one fluorophore. Fluorescent blotting also enables quantitative and consistent results across repeat experiments. Disadvantages of fluorescent detection can include a low signal to noise ratio as a result of auto - fluorescence and a failure to amplify lowly expressed proteins when using a charged-couple device camera.

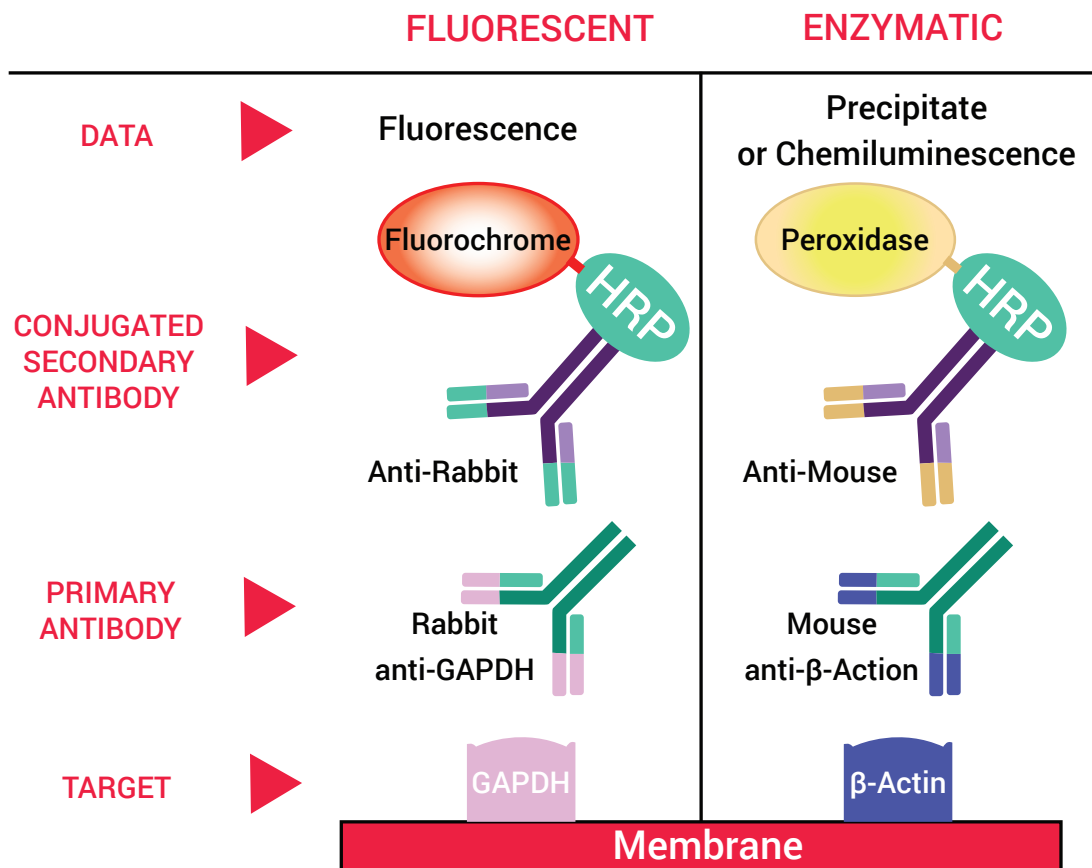


Figure 5: Principle and comparison of fluorescent and enzymatic detection methods.

12.3 DIFFERENCES BETWEEN CHEMILUMINESCENT DETECTION AND FLUORESCENT DETECTION

| | Chemiluminescent detection | Florescent Detection |
|---------------------------------|---|---|
| Principle | Secondary antibody labelled with enzyme (e.g. HRP or AP) | Secondary antibody labelled with fluorophore |
| Method of Detection | X-Ray film exposure/ Digital Imaging | Laser Scanning Imager |
| Ability to multiplex | No | Yes |
| Sensitivity | +++ | +++ |
| Strength of signal | Hours | Weeks to Months |
| Linear Dynamic Range | 15-fold (X-Ray Detection), 3,000-4,000-fold (Digital Imager) | >4,000-fold |
| Quantitative Measurement | Enzymatic signal is variable and only semi-quantitative | Fluorescent signal is static and quantitative |
| Substrate | Luminol | No substrate needed |

Table 7: Chemiluminescent Vs Fluorescent Detection

13. STRIPPING AND RE-PROBING OF BLOTS

The removal of primary and secondary antibodies from a Western blot membrane is known as stripping. Stripping enables the investigator to look at more than 1 protein on the blot e.g. the protein of interest and a loading control. Probing can also be used for multiple targets and is advantageous as it saves time and sample.

Stripping works best on a PVDF membrane as it has greater capabilities to keep the protein attached and not elute. Chemiluminescent reagents such as ECL are recommended for stripping as they don't stain the membrane which can interfere with the detection of targets of similar molecular weights. How many times a membrane can be stripped and stained depends on how optimised the stripping protocol is.

Following stripping it is important to thoroughly wash the membrane with wash buffer and block it before primary antibody incubation.

There are 2 different strengths of stripping buffer which can be used mild or harsh.

13.1 MILD STRIPPING BUFFER

A mild stripping buffer uses a low pH glycine solution to dissociate bound antibodies. The low pH acts to remove bound antibodies via structural alteration and deactivation of the active site.

13.2 MILD STRIPPING BUFFER RECIPE

15 g glycine
1 g SDS
10 ml TWEEN 20
Dissolve in 800 ml distilled water
Adjust pH to 2.2
Bring volume up to 1 L with distilled water

13.3 HARSH STRIPPING BUFFER

A harsh stripping is primarily used on blots with a high signal strength. Harsh stripping buffers also rely on a low pH to alter antibody structure and release the target protein. In harsh buffers this activity is achieved through the use of a neutral Tris-HCl solution containing reducing agents such as beta-mercaptoethanol and SDS. This solution is heated with the blot at 50–80°C for up to 45 minutes with agitation. It should be noted that the activities of SDS and beta-mercaptoethanol can't be reversed and the stripped antibody recovered. Furthermore, thorough washing is required following the use of a harsh buffer to prevent denaturation of the re-probing antibody.

13.4 HARSH STRIPPING BUFFER RECIPE

62.5 mM Tris-HCL,pH 7.8, 100 mM -mercaptoethanol, 2% (w/v) SDS.

13.5 SAMPLE STRIPING PROTOCOL

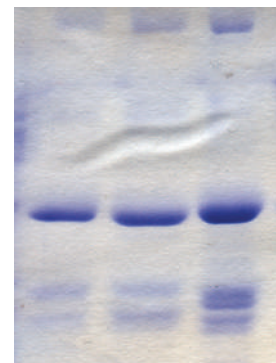
1. Incubate the PVDF Membrane with stripping buffer (62.5 mM Tris-HCL,pH 7.8, 100 mM B-mercaptoethanol, 2% (w/v) SDS) for 30 min at 50 °C.
2. Wash the membrane 2 X 5 min, 1 X 10 min, 2 X 5 min at room temperature with agitation on a platform rocker.
3. Block by incubation with gentle agitation in blocking solution for 1 h at room temperature.
4. Washed 2 X 5 min, followed by incubation with the appropriate primary and secondary antibody.

14. PROTEIN GEL VISUALISATION

The separation of protein by gel electrophoresis can be visualised in 2 ways: Coomassie staining or copper staining. The choice of staining used depends largely on the downstream applications of the protein.

14.1 COOMASSIE STAINING

Coomassie staining is used to determine whether the protein has migrated uniformly and evenly. A coomassie stain should only be used on gel if the aim is not to transfer but to observe the results of SDS-Page separation as coomassie staining is not reversible.



14.2 COOMASSIE STAINING SAMPLE PROTOCOL

1. Following gel electrophoresis treat the gel with 40% distilled water, 10% acetic acid, and 50% methanol, this will allow the proteins to precipitate.
2. Place the gel in the same solution as used in step 1 but with the addition of 0.25% by weight Coomassie blue stain.
3. Incubate for 4-24 hrs at room temperature on a shaker.
4. Transfer the gel to a mixture of 67.5% distilled water, 7.5% acetic acid, and 25%, methanol on shaker to rinse
5. Replace with fresh rinse buffer once the excess dye has been removed.
6. The stain will not bind to the acrylamide, and will wash out (leaving a clear gel).
7. The stain binds strongly to the proteins in the gel and give off a blue colour.

14.3 COPPER STAINING

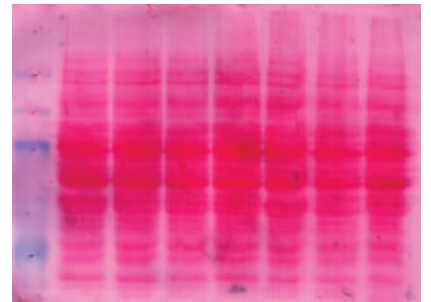
Copper staining should be used if you wish to transfer the protein following visualization. Copper staining is said to be faster and more sensitive than coomassie staining.

14.4 COPPER STAINING SAMPLE PROTOCOL

1. Briefly rinse the gel in ddH₂O following electrophoresis.
2. Transfer to a solution of 0.3 M CuCl₂ for 5–15 min.
3. Wash the gel in ddh₂O.
4. View against a dark field background.
5. The protein can be noted as clear zone in a translucent blue background.
6. Following visualisation gels can be de-stained by washing in 0.1–0.25 M Tris/0.25 M EDTA pH 8.0.
7. Place the gel in transfer buffer and proceed with the transfer.

15. PROTEIN MEMBRANE VISUALISATION

The efficiency and success of the transfer can be assessed by staining the PVDF or nitrocellulose membrane with Ponceau Red. Staining with ponceau red is easily reversed with washing and therefore enables subsequent antibody probing.



15.1 PONCEAU RED SAMPLE PROTOCOL

1. Dilute Ponceau stock 1:100.
2. Incubate on an agitator for 5 min.
3. Wash with ddH₂O until the water is clear and the protein bands visible.
4. Destain the membrane by washing with TBST.
5. If using a PVDF membrane, re-activate the membrane with methanol then wash again in TBST.