**Swayam Course - Analytical Techniques**

**Week: 9, Module 22 - Western Blotting Techniques**

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## 1. INTRODUCTION

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting is a technique which involves detection of a particular protein from a pool of total proteins. It is widely used nowadays in molecular biology and immunogenetics.

This technique was discovered by W. Neal Burnette and Harry Towbin in 1979. The term "western blotting" was coined by W. Neal Burnette in 1981. This has been named "western" with respect to southern blotting for DNA by Edwin Southern and because of the West Coast location of its invention.

W. Neal Burnette

Harry Towbin

## 2. OBJECTIVES

The following sections will be covered in this chapter:

- principle of western blotting
- detailed description of various steps involved in this technique
- buffers and reagents used in western blotting

- various applications of this technique in different fields

## 3. STEPS OF WESTERN BLOTTTING

1. Preparation of lysate from cells or tissues
2. Protein quantification
3. Separation of proteins on the basis of molecular weight (SDS-PAGE)
4. Transfer of proteins from gel to membrane
5. Blocking of membrane
6. Incubation with primary and secondary antibody
7. Detection of that particular protein by different staining methods

The first three steps have already been discussed in previous tutorials but here, we are re-emphasizing again in brief.

## 4. PREPARATION OF LYSATE FROM CELLS OR TISSUES

Lysate preparation is the first step in the process of western blotting. The detection of a particular protein involves extraction of total proteins from any source such as blood, cells or tissues. This extraction occurs by treatment with lysis buffer which solubilise proteins to facilitate their migration and separation. The lysis buffer contains sodium dodecyl sulfate (SDS) or other ionic detergents which breaks open the cells by destroying cell membrane and nuclear membrane. Detergent and salt also helps to keep proteins away from the DNA molecules for easy separation. Osmolarity has also been maintained by salt content. The most commonly used lysis buffer is RIPA (Radio Immuno Precipitation Assay) buffer.

### 4.1 Composition of RIPA Buffer

- 150 mM sodium chloride
- 1.0% Triton X-100
- 0.5% sodium deoxycholate
- 0.1% SDS (sodium dodecyl sulphate)
- 50 mM Tris, pH 8.0

RIPA buffer can be stored at 4˚C for several weeks to months.

Cell lysis by RIPA buffer exposes proteins to certain proteases which can degrade and denature proteins. This could be prevented by adding protease inhibitor cocktail in lysis buffer before adding to the cells which maintain the protein integrity.

### 4.2 Steps for lysate preparation

- The cells isolated from respective source (blood, tissues or cell lines).
- ~70-100µl of RIPA buffer containing protease inhibitor cocktail was added to cells.
- Kept at 4°C for 30 minutes with gentle pipeting after every 10 minutes.
- Cells were then centrifuged at 12000 rpm for 20 minutes.
- Supernatant (total cell lysate) was collected and stored at -80°C for future use.

## 5. PROTEIN QUANTIFICATION

The concentration of protein in the total cell lysate is to be determined to proceed further as equal amount of protein (in µg) should be loaded in electrophoresis as will be discussed in later section.

### 5.1 Bradford Method

This method employs coomassie brilliant blue G-250 which results in absorbance shift from red to blue colour when binds to protein. This dye forms a blue-coloured non-covalent and strong complex by binding to protein which shows absorbance maxima at 595 nm and hence, can be estimated. The absorbance is directly proportional to the amount of protein present in the sample. The standard normally used is Bovine Serum Albumin (BSA) whose different dilutions in an appropriate range were made. The absorbance of these dilutions was estimated and a standard curve was plotted between absorbance and concentration. The equation thus obtained was used to calculate the concentration in an unknown sample.

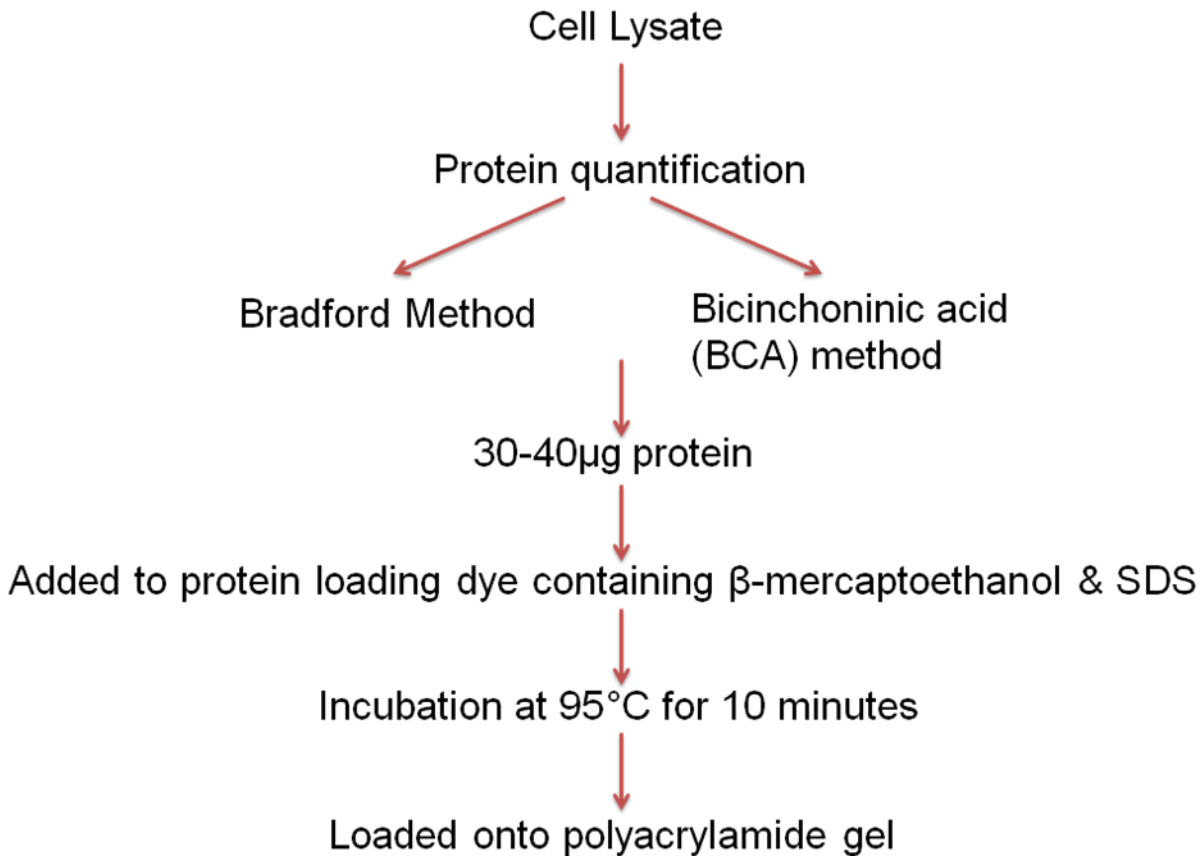
### 5.2 Bicinchoninic acid (BCA) Method

This method involves peptide bonds mediated reduction of $Cu^{2+}$ from the copper(II) sulfate to $Cu^{+}$ which is then chelated by bicinchoninic acid resulting in a purple-coloured complex which absorbs at a wavelength of 562 nm. The absorbance is directly proportional to the amount of protein. The standard was made and run as described in Bradford assay.

### 5.3 Lowry Protein Assay

The method involves the oxidation of aromatic amino acids in proteins resulting in the production of $Cu^{+}$ ions which then reacts with Folin–Ciocalteu reagent (a mixture of

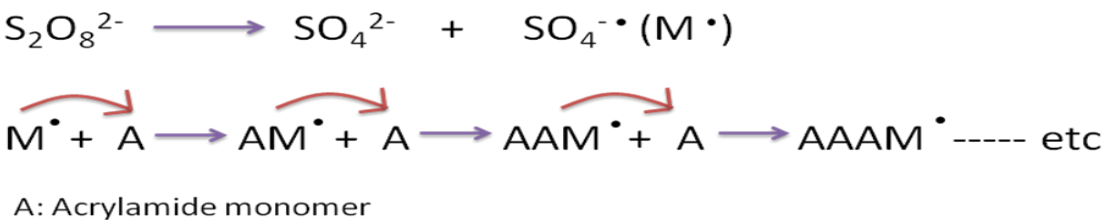
phosphotungstic acid and phosphomolybdic acid) to form a blue coloured product which absorbs at 660 nm. The absorbance is directly proportional to the concentration of proteins present in the sample.




## 6. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis refers to the migration of any charged particle under the influence of electric field. In case of proteins, gel containing polyacrylamide along with SDS is used for electrophoresis. Polyacrylamide gels are formed from the polymerisation of acrylamide monomer by cross-linker bis-acrylamide. This polymerisation is catalysed by a free-radical generator, TEMED (tetramethylene diamine).

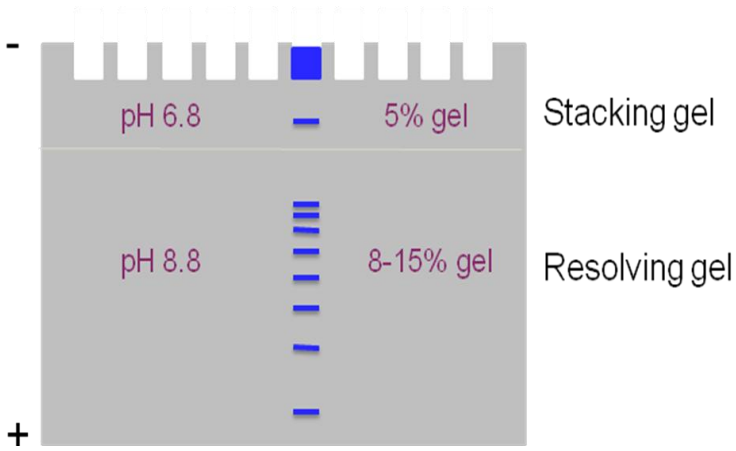
TEMED catalyses the decomposition of ammonium persulfate to give a free radical which then by free radical catalysis attack acrylamide monomer to form a chain. This chain is then cross-linked by bis-acrylamide.




This electrophoresis is discontinuous as two gels are used in a single set-up for better protein separation. Two gels are:

- Stacking gel: This is the upper part of polyacrylamide gel having 5% gel composition. This low percentage facilitate the movement of all the proteins in a regular manner, hence, stacking gel helps to concentrate all the proteins into a sharp band for uniform separation.

- Separating (or resolving gel): This part has percentage composition depending upon the molecular weight of the protein to separate, hence, resolving gel helps in separation of proteins on the basis of their size with smaller proteins moving fastest leaving behind the larger proteins.




Proteins being negatively charged due to SDS move towards positive charge and gets separated.

$$\text{Polyacrylamide percentage} \propto \text{Number of molecules} \propto \frac{1}{\text{Pore size}}$$

$$\uparrow \text{Molecular weight of protein} = \downarrow \text{Polyacrylamide percentage}$$

Markers with known molecular weight should be loaded everytime to determine the position (molecular weight) of target protein.

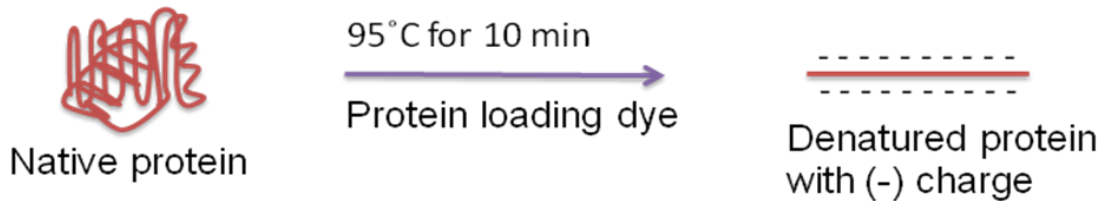
### 6.1 Protein loading dye

Detection of protein require antibody which bind to a specific portion of protein called epitope. This binding could be hindered in 3D protein conformation, hence, necessitates the denaturation of protein which is facilitated by protein loading dye containing β-mercaptoethanol and SDS

Protein loading dye has the following components:

1. β-mercaptoethanol: reducing agent which disrupts disulfide linkage between cysteine residues and distort the quaternary and tertiary structure of protein.
2. SDS: bind to amino acids of protein and impart negative charge leading to protein denaturation.
3. Glycerol: provide density to the sample so that it can be loaded into wells.
4. Bromophenol blue: being anionic and small molecule, it migrate fastest in the gel, hence, help to assess the migration front while separation of proteins.

**20-40 µg protein mixed with protein loading dye and incubated at 95°C for 10 minutes and then, loaded into the wells of polyacrylamide gel for separation.**

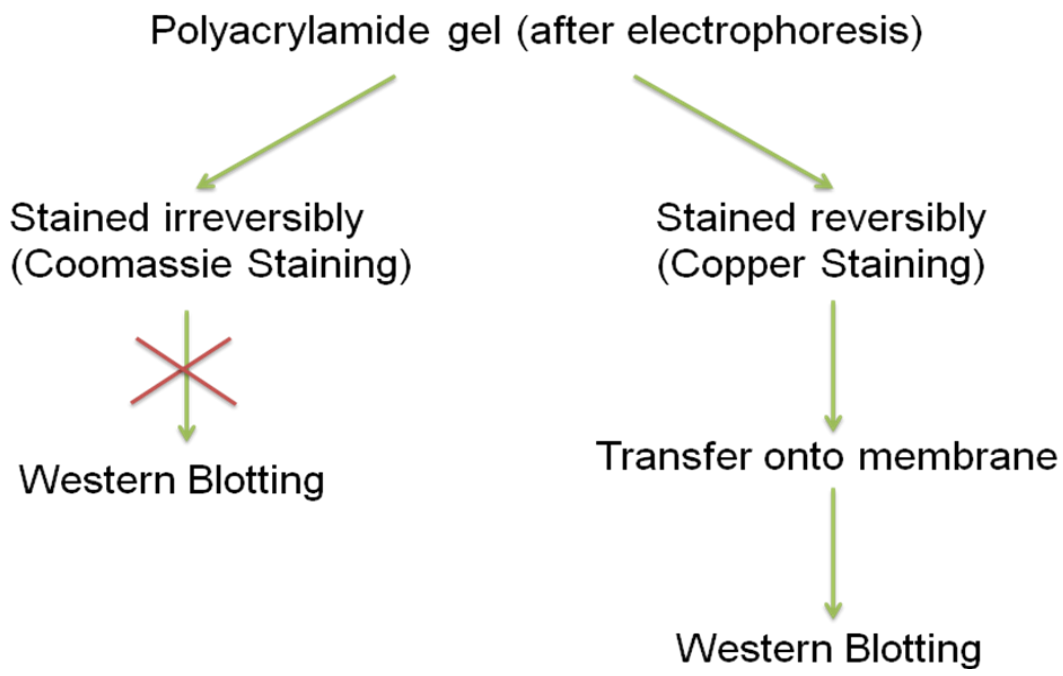



### 6.2 Electrophoresis buffer

- 25mM tris base
- 190mM glycine
- 0.1% SDS
- pH ~8.3

20-40µg lysate loaded into the wells. Voltage (50V-60V) applied in beginning and when samples enter separating gel, voltage can be increased upto 120V. It usually takes 2h to completely run the gel. The proteins on gel can then either be stained or transferred to the membrane.

**6.3 Visualisation of proteins on gel**




6.3.1 Coomassie staining

The principle is same as discussed in Bradford assay but for protein visualization, Coomassie Brilliant Blue R-250 is used. This is an irreversible stain, hence, proteins cannot be transferred to membrane.

6.3.2 Copper staining

The staining is based on the interaction of copper ions with polyacrylamide and proteins. Copper metal precipitate in the gel, resulting in an opaque blue/green gel but coating of SDS on the proteins inhibits copper ions from binding to the proteins. Hence, a negative image is produced; consisting of clear protein bands visualized against a semi-opaque blue/green polyacrylamide background. This staining can be reversed and proteins can be transferred to the membrane.

## 7. TRANSFER OF PROTEINS ONTO MEMBRANE

Proteins with negative charge due to bound SDS move towards positive charge under the influence of an electric field. On this basis, membrane is placed towards anode while gel towards cathode for transfer. There are mainly two conditions for transfer:

**Wet transfer**: Gel is sandwiched between paper and sponge & clamped tightly. The sandwich is submerged in transfer buffer and electric field is applied. This condition takes time to transfer but is advisable for transfer of larger proteins with molecular weight greater than 100kDa.

**Semi-dry transfer**: The sandwich is wetted in transfer buffer and placed directly to electrodes. This is faster but prone to failure due to drying of membrane.

### 7.1 Membranes for transfer

Two types of membranes commonly used for transfer are nitrocellulose and PVDF (polyvinylidene difluoride).

PVDF has higher protein binding capacity, offers high sensitivity than nitrocellulose membrane. The former is more durable, has higher chemical resistance, suitable for stripping or reprobing. PVDF needs to be charged prior to transfer as PVDF is very hydrophobic which does not allow aqueous buffers including transfer buffer to penetrate the membrane which could result in poor transfer and significantly reduced binding of proteins.

### 7.2 Transfer buffer

- 25mM tris base
- 190mM glycine
- 10-20% methanol

It usually takes 1h-2h to transfer smaller proteins but larger proteins need time. Proteins>100kDa require overnight wet transfer. The transferred proteins on membrane can be visualised by a reversible Ponceau S stain.

There are certain guidelines for transfer depending upon the molecular weight as discussed below:

Large proteins (>100 kD)

- Migration and transfer is slow, hence, ~ 8% gel can be used.
- Large proteins can precipitate in gel, might hinder transfer, hence, addition of SDS to 0.1% and reduction of methanol percentage to <10% will help against precipitation.

- Methanol is required only when using nitrocellulose and not PVDF.
- Choose wet transfer overnight at 4°C.

Small proteins (<100 kD)

- Small proteins are much hindered from binding to membranes by SDS, hence, SDS can be removed from transfer buffer.
- Keep the methanol concentration at 20%.

### 7.3 Ponceau S Staining

Ponceau S Staining is used for the detection of proteins on nitrocellulose or PVDF membranes. It is a negative stain which binds to positively charged amino groups of proteins giving a sharp red colour band with a clear background. This stain being hydrophilic can easily be removed and membrane can be used for immunological detection. This is performed to assess whether transfer has been performed properly.

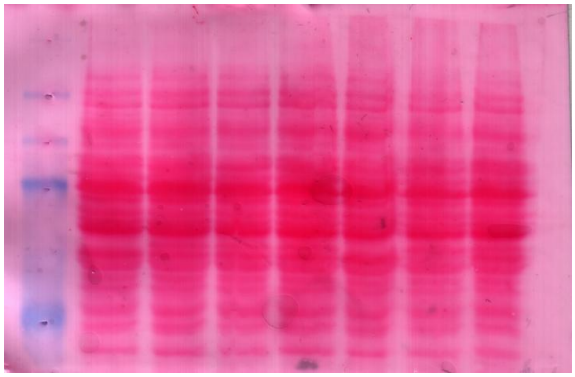
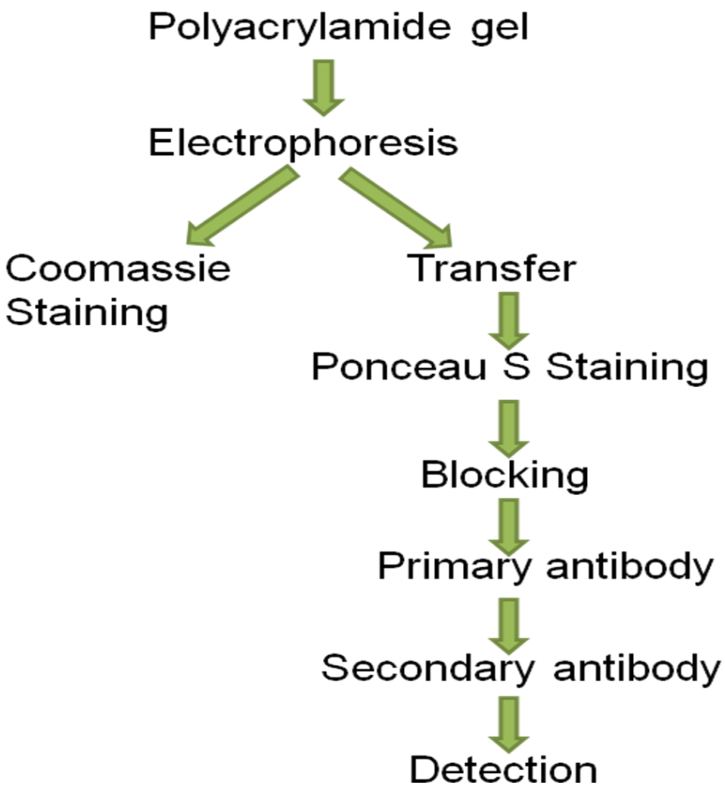


**Figure 1: The image of membrane (blot) after Ponceau S staining**




## 8. BLOCKING

Blocking the membrane prevents non-specific background binding of primary or secondary antibodies to the membrane. Two blocking solutions are used: non-fat milk (NFM) or Bovine Serum Albumin (BSA). NFM is a strong blocker, cheaper but is not recommended for phospho-proteins as milk contains casein which is a phospho-protein, thus, give high background. The blocking is done for 1 hour at room temperature under agitation followed by washing for 5 minutes in 1X Tris buffered saline tween (TBST) after the incubation.

### 8.1 Composition of 10X TBST

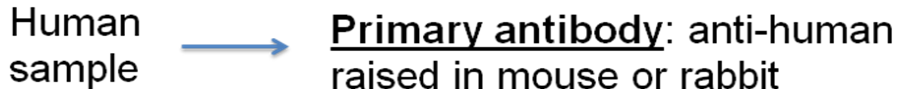
- 10X TBS (500ml)
- Tris base 12.1g
- Sodium Chloride 40.03g
- pH 7.6

1X TBST is prepared by diluting 10X stock to 10 times. Then, 0.1% tween 20 is added. TBS buffer is stored at room temperature for several weeks to months.

## 9. INCUBATION WITH ANTIBODY FOR DETECTION

### 9.1 Primary Antibody

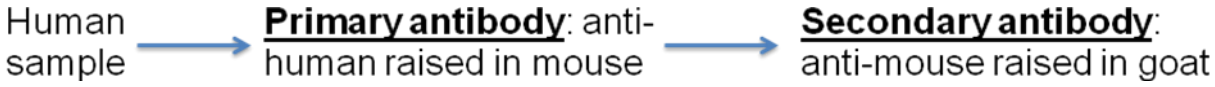
Detection of protein requires binding of an antibody. Antibody should be against the sample source while raised in another.




Antibody is diluted in 1X TBST and dilutions vary with the antibody. 1% blocking agent can also be used for dilution. The incubation with primary antibody is usually performed at 4°C for overnight. Following incubation, washing with 1X TBST is done thrice.

### 9.2 Secondary Antibody

Secondary antibody labelled with horse radish peroxidase (HRP) or alkaline phosphatase (ALP) is used for western blotting. Secondary antibody should be against the source of primary antibody while raised in another.




Antibody is diluted in 1X TBST. 1% blocking agent can also be used for dilution. The membrane is incubated with secondary antibody at room temperature for 2h followed by washing with 1X TBST thrice.

## 10. DETECTION METHODS

The region on blot or membrane where primary and secondary antibodies bind are detected by various methods as shown below:

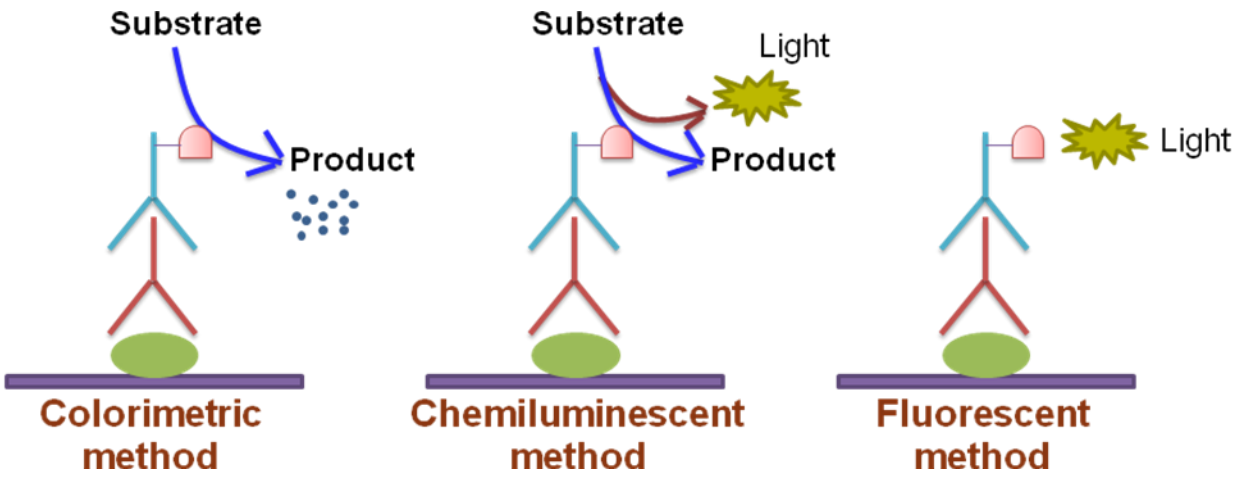



**Figure 2: Various methods of detection in western blotting**

### 10.1 Colorimetric method

Enzymes such as HRP and ALP convert several substrates to a colored precipitate which accumulates on blot and a colored signal develops which is visible by naked eye. The most common substrate for colorimetric HRP is 3, 3'-diaminobenzidine (DAB). There are some limitations of colorimetric detection systems which includes decreased sensitivity, fading of blots upon exposure to light, and nonspecific color precipitation.

### 10.2 Chemiluminescent method

Chemiluminescence occurs when a chemical substrate is catalyzed by an enzyme, such as ALP or HRP, and produces light as a by-product. The light signal can be captured on X-ray film or by a charge-coupled device (CCD) imager such as Chemi-documentation. This method is more sensitive than colorimetric system of detection as femtogram amount of protein can be detected by chemiluminescence.

### 10.3 Fluorescent method

In fluorescence detection, a primary or secondary antibody labeled with a fluorophore is used during immunodetection. A light source excites the fluorophore and the emitted fluorescent signal is captured by a camera to produce the final image.

## 11. APPLICATIONS OF WESTERN BLOTTING

- **Medicine**: This technique is widely used in the diagnosis of various diseases such as HIV, hepatitis, herpes simplex virus (HSV-2) infection. For example, proteins from known HIV infected patient is isolated and run and anti-HIV antibody present in the serum sample to be tested is detected by using serum sample in place of primary antibody in western blotting. Similarly, HBsAg protein is detected in serum sample by western blotting for diagnosis of hepatitis B infection.

- **Research**: Any research question could not be answered appropriately without incorporation of western blotting. It has numerous applications in research but to list, this technique is used to determine the expression of any protein in physiology or in various disease states. The elucidation of any downstream signaling pathway for any mechanism is performed by this method. In addition, western blotting is used in genetic engineering to determine over-expression of any cloned protein.

- **Sports**: Blood doping is used to increase red blood cell mass by erythropoietin or blood transfusion to increase stamina and performance which is strictly prohibited by World anti-doping agency (WADA). Hence, western blotting was used during 2014 FIFA World Cup in the anti-doping campaign.

## 11. SUMMARY

Western blotting is an immunological technique which helps in identification of a particular protein. It employs the basis of antigen-antibody interaction for detection. Total proteins are first isolated from cells or tissues and then quantified to determine the concentration of proteins in sample. 20-40µg proteins are then run on polyacrylamide gel followed by transfer onto a membrane (blot). This blot is then blocked and incubated with respective primary or secondary antibodies and then appropriate detection method is used for detection. This technique has applications in various fields like medicine, research, sports, etc. This chapter has therefore discussed each and every steps of western blotting in detail, its various reagents & buffers and applicability in various areas. This technique has a great potential for future outcomings in various fields.

The complete process of western blotting is shown below:

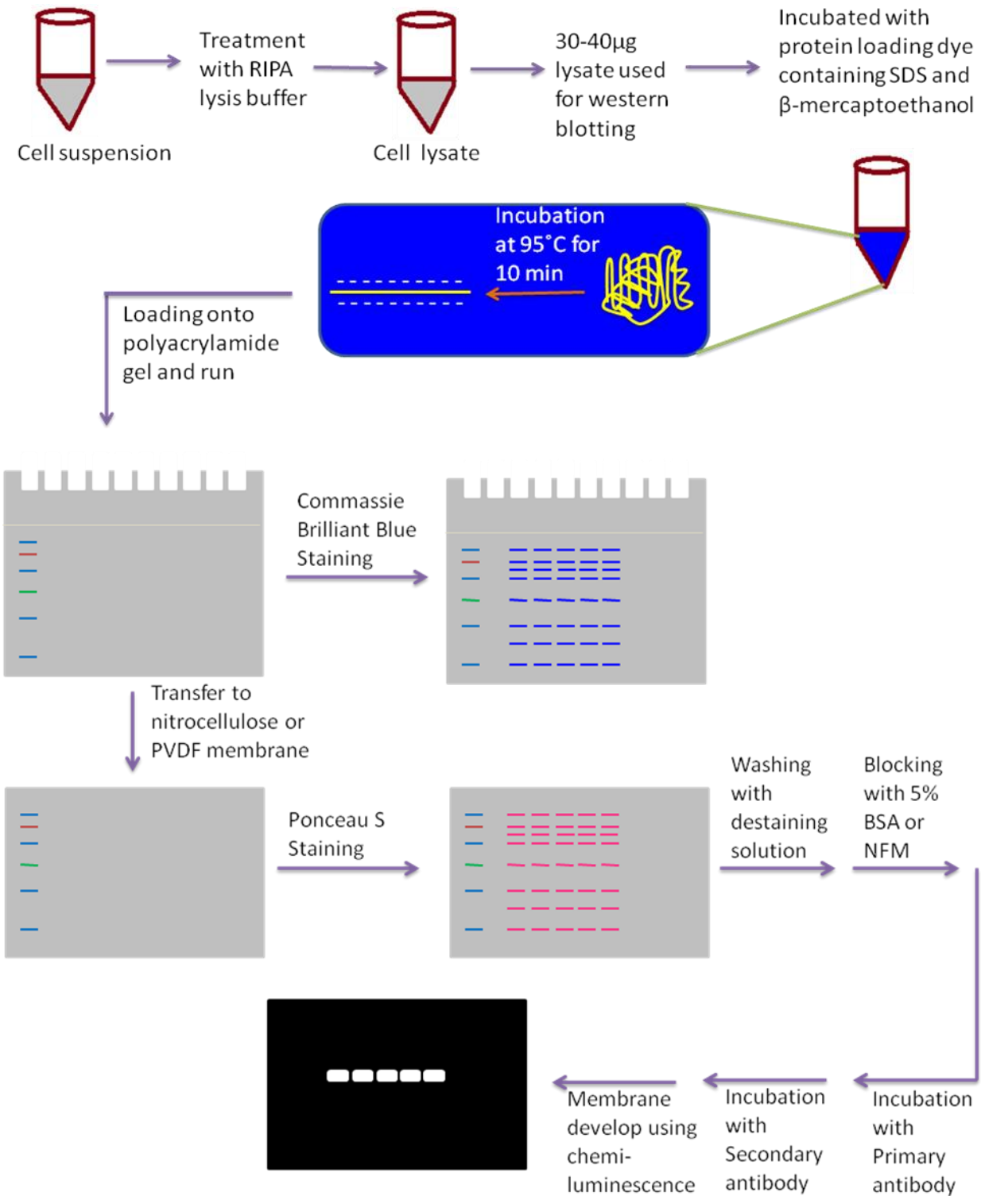



**Figure 3: The pictorial representation of the complete process of western blotting**

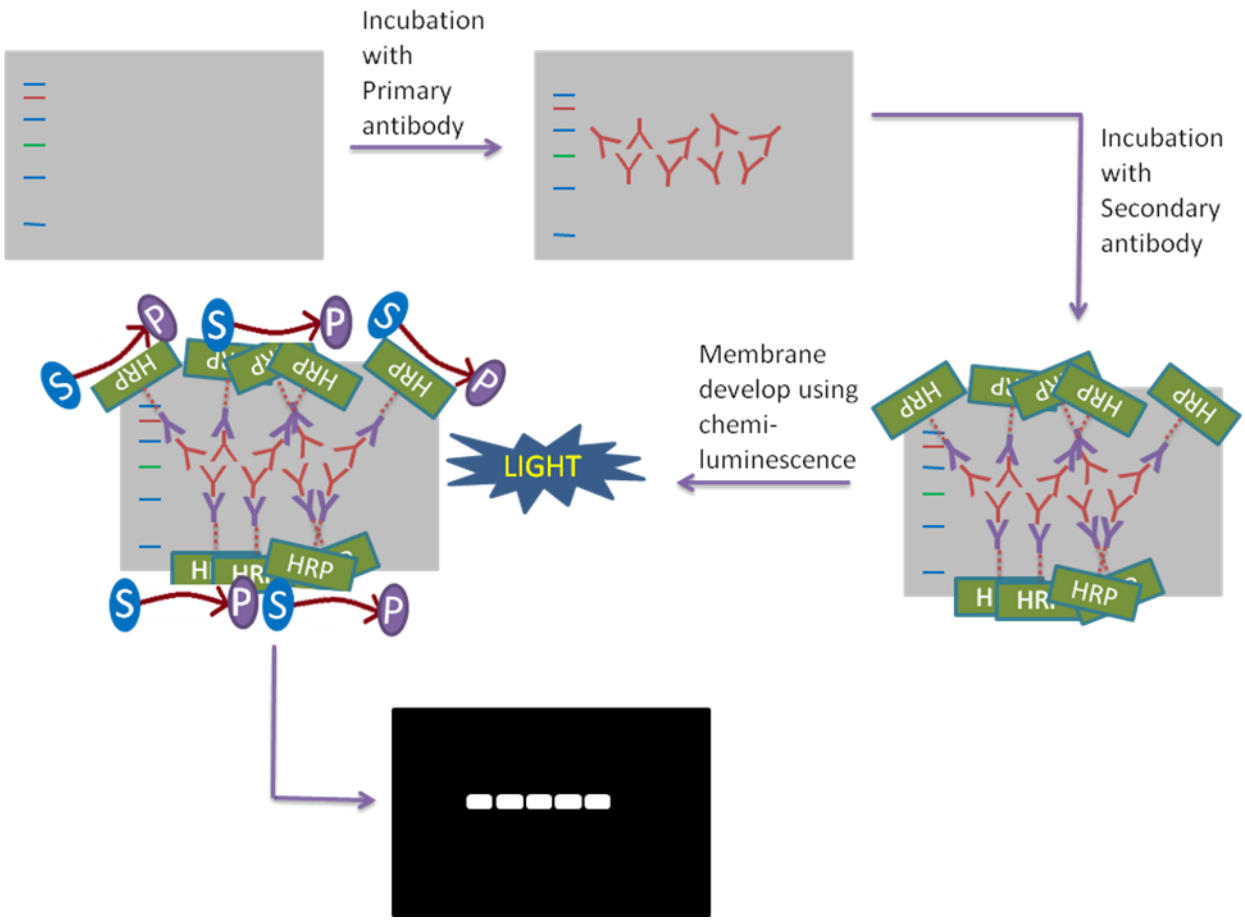



**Figure 4: The formation of immune complex while western blotting**