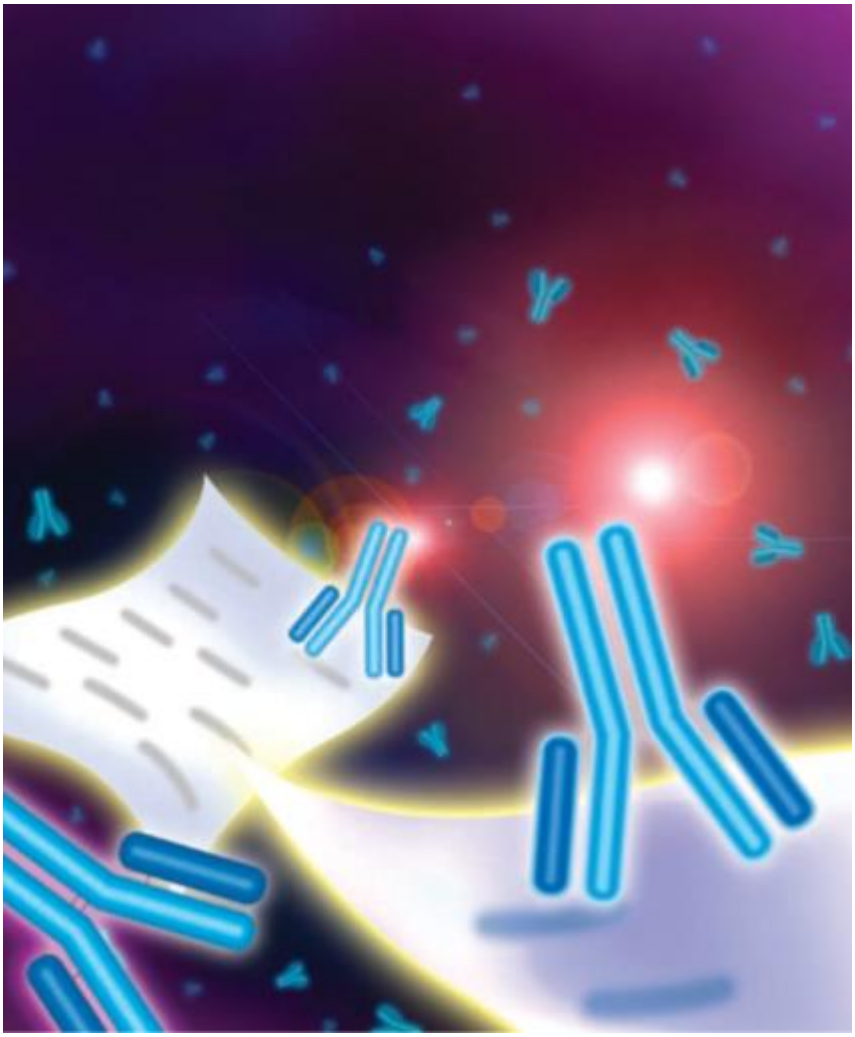

WESTREN BLOTTING

ABSTRACT
Western blotting is one of the most useful ways to know a lot about what our bodies contains, and it also has great importance in detecting diseases, we will see how does it work, and some of its applications.

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Intro and problematic

In the last two decades, the biological domain start growing rapidly for the humans good, the researchers start thinking in ways to know more and more about the human body, and they have succeeded in revealing a lot of secrets in the body, but one of the most important things that been discovered is western blotting, which is a procedure or an analysis to figure out the proteins that exist in the cell and their concentrations, you might say what is the use of knowing this things??, I will say: everything… it helps us to know what is happening in our bodies and what is wrong with them, like what are the diseases we have, what are the things that are not working correctly, and a lot of other important information.

We will talk in this research about the procedure of western blotting, from sample preparation until imaging the results, and we will mention some applications for it.

And in the end of this research you will be able to answer these questions:

- How we know what proteins exist in our cells???
- How we make this procedure??
- What is the applications of this procedure??
- Is there a certain way to do it, or it varies???

1.0 Sample preparation

Protein purification include total proteins extraction from a sample, and removal interfering or contaminating substance (sample preparation or clean-up).

Cell lysis is the first step in cell fractionation and protein purification and, as such, opens the door to a myriad of biological studies. Many techniques are available for the disruption of cells, including physical and detergent-based methods. Historically, physical lysis has been the method of choice for cell disruption; however, physical methods often require expensive equipment. In recent years, detergent based lysis has become very popular due to ease of use, low cost and efficient protocols. All cells have a plasma membrane, a protein-lipid bilayer that, in animal cells, forms a barrier separating cell contents from the extracellular environment. Plant cell walls are particularly strong, making them very difficult to disrupt mechanically or chemically. Until recently, efficient lysis of yeast cells required mechanical disruption using glass beads. Bacterial cell walls are the easiest to break compared to these other cell types. The lack of an extracellular wall in animal cells makes them relatively easy to lyse. Clearly, the technique chosen for the disruption of cells, whether physical or detergent-based, must take into consideration the origin of the cells or tissues being examined and the ease or difficulty in disrupting their outer layer(s). [1]

1.1cell lysis

1.1.1 Physical (traditional) methods

Several methods, including mechanical disruption, liquid homogenization, sonication, freeze/thaw cycles and manual grinding are commonly used to physically lyse cells. [2]

1.1.1.1Mechanical disruption

Mechanical methods rely on the use of rotating blades to grind and disperse large amounts of complex tissue, such as liver or muscle. [2]

1.1.1.2 Liquid homogenization

Liquid-based homogenization is the most widely used cell-disruption technique for small volumes and cultured cells. Cells are lysed by forcing the cell or tissue to go through a narrow space, thereby shearing the cell membranes. Three different types of homogenizers are in common use. A Dounce homogenizer consists of a round glass pestle that is manually driven into a glass tube. A Potter-Elvehjem homogenizer consists of a manually pestle coated with PTFE Material and shaped to fit a rounded or conical vessel.

A French press consists of a piston that is used to apply high pressure to a sample volume of 40 to 250 ml, forcing it through a tiny hole in the press. The equipment is expensive, but the French press is often the method of choice for breaking bacterial cells mechanically. [2]

1.1.1.3 Sonication

Sonication is the third class of physical disruption commonly used to break open cells. The method uses pulsed, high-frequency sound waves to agitate and lyse cells, bacteria, and spores. The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe initiates the formation of microscopic vapor bubbles that form momentarily and implode, causing shock waves to radiate through a sample. To prevent excessive

heating, ultrasonic treatment is applied in multiple short bursts to a sample immersed in an ice bath. Sonication is best suited for volumes < 100 ml. [2]

1.1.1.4 Freeze/Thaw cycle

The freeze/thaw method is commonly used to lyse bacterial and mammalian cells. This technique involves freezing a cell in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37°C. This method of lysis causes cells to swell and ultimately break as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, and the process can be quite lengthy. However, freeze/thaw has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and it is recommended for the lysis of mammalian cells in some protocols. [2]

1.1.1.5 Mortar and Pestle

Manual grinding is the most common method used to disrupt plant cells. Tissue is frozen in liquid nitrogen and then crushed using a mortar and pestle. Because of the tensile strength of the cellulose and other polysaccharides constituting the cell wall, this method was the fastest and most efficient way to access plant proteins and DNA. [2]

1.1.2The modern way

Detergent cell lysis is a milder and easier alternative to physical disruption of cell membranes, although it is often used in conjunction with homogenization and mechanical grinding.

Detergents break the lipid barrier surrounding cells by solubilizing proteins and disrupting lipid : lipid, protein : protein and protein : lipid interactions. Detergents, like lipids, self-associate and bind to hydrophobic surfaces. They are composed of a polar hydrophilic head group and a nonpolar hydrophobic tail and are categorized by the nature of the head group as either ionic (cationic or anionic), nonionic or zwitterionic. Their behavior depends on the properties of the head group and tail. there is no standard protocol available for selecting a detergent to use for membrane lysis. The ideal detergent will depend on the intended application. In general, nonionic and zwitterionic detergents are milder and less denaturing than ionic detergents and are used to solubilize membrane proteins when it is critical to maintain protein function and/or retain native protein: protein interactions for enzyme assays or immunoassays. In contrast, ionic detergents are strong solubilizing agents and tend to denature proteins, thereby destroying protein activity and function. Studies assessing protein levels strictly through gel electrophoresis and Western blotting typically use SDS to fully denature protein samples by boiling. There are a few commonly used ionic detergents that are only mildly denaturing, including sodium cholate and sodium deoxycholate. The choice of detergent for cell lysis also depends on sample type. Animal cells, bacteria and yeast all have differing requirements for optimal lysis due to the presence or absence of a cell wall. Because of the dense and complex nature of animal tissues, they require both detergent and mechanical lysis. In addition to the choice of detergent, other important considerations for optimal cell lysis include the buffer, pH, salt concentration and temperature. Consideration should be given to the compatibility of the chosen detergent with downstream applications. If the detergent used for lysis must be removed, then a dialyzable detergent should be selected. [1]

2.0 Transfer

The transfer step involves moving the proteins from a solution or gel and immobilizing them on a synthetic membrane support (blot). Transfer may be performed by dot-blotting of proteins that are in solution, or by electrophoretic transfer of proteins from a gel to a membrane. In performing a protein transfer, the researcher must choose the appropriate method, apparatus, membrane, buffer, and transfer conditions.

2.1 Electrophoresis

Electro refers to electron flow or current, Phoresis refers to movement. Thus Electrophoresis is movement under electric current. This technique therefore can separate molecules which can move in an electric field charged molecules. Charged molecules in an electric field behave in a predictable manner. Positively charged molecules will move towards the negative pole while negatively charged molecules move towards the positive pole. [3]

There are two types of gel electrophoresis:

One dimension.

Two dimensions.

2.1.1 One dimension

1.SDS-PAGE

2.Native-PAGE

3.IEF

Several forms of PAGE exist and can provide different types of information about the protein(s). SDS-PAGE, is the most widely used electrophoresis technique, separates proteins primarily by mass. Non denaturing PAGE, also called native PAGE, separates proteins according to their mass: charge ratio. Two –dimensional PAGE (2D-PAGE) separates proteins by isoelectric point in the first dimension and by mass in the second dimension. [4]

2.1.1.1 SDS-PAGE

the samples are treated with SDS (sodium dodecyl sulfate), an anionic detergent which denatures the protein by breaking the disulfide bonds and gives negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others. SDS linearizes the proteins so that they may be separated strictly by molecular weight. The SDS binds to the protein in a ratio of approximately 1.4 g SDS per 1.0 g giving an approximately uniform mass : charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye like bromophenol blue may be added to the protein solution to track the progress of the protein solution through the gel during the electrophoretic run. [5]

2.1.1.1.1 Gel

The gel used for SDS-PAGE is made out of acrylamide which form cross-linked polymers of polyacrylamide. Standard gels are typically composed of two layers, one top-most layer called the stacking gel and a lower layer called separating or resolving gel. The stacking layer contains a low percentage of acrylamide and has low pH, while the acrylamide concentration of the separating gel varies according to the samples to be run and has higher pH. The difference in pH and acrylamide concentration at the stacking and separating gel provides better resolution and sharper bands in the separating gel. [5]

2.1.1.1.1 Stacking gel

The stacking gel is a large pore PAG (4%T). This gel is prepared with Tris/HCl buffer pH 6.8 of about 2.0 pH units lower than that of electrophoresis buffer (Tris/Glycine). This gel is cast over the resolving gel. The height of the stacking gel region is always maintained more than double the height and the volume of the sample to be applied. [5]

2.1.1.1.2 Resolving gel

The resolving gel is a small pore polyacrylamide gel (3 -30% acrylamide monomer) typically made using a pH 8.8 Tris /HCl buffer. In the resolving gel, macromolecules separate according to their size. Resolving gels have an optimal range of separation that is based on the percent of monomer present in the polymerization reaction. What happens after denaturing the sample with SDS, the proteins denatured by SDS are applied to one end of a layer of polyacrylamide gel submerged in a buffer. Buffer provide uniform pH and ions for conducting electric potential. When an electric current is applied across the gel, the negatively-charged proteins migrate across the gel to the positive pole. Short proteins will more easily fit through the pores in the gel and move fast, while larger ones will have more difficulty. Due to differential migration based on their size, smaller proteins move farther down the gel, while larger ones stay closer to the point of origin. After a given period of time, proteins might have separated roughly according to their sizes. Proteins of known molecular weight (marker proteins) can be run in a separate lane in the gel to calibrate the gel. [5]

Uses of SDS-PAGE

- Determine molecular weight of a peptide or a protein.
- Identify protein.
- Determine sample purity.
- Identify existence of disulfide bonds.
- Quantify amounts of protein.
- Protein induced in response to environmental changes.

2.1.1.2 Native-PAGE

Native PAGE is used to separate proteins in their native states according to difference in their charge density. Native state of protein means proteins are in properly folded state, not denatured or unfolded state. There are no denaturants present in the gel and buffer in the gel maintains the protein in its native state. Many proteins are shown to be enzymatically active after separation by native PAGE. Thus, it is used for preparation of purified and active proteins. In native PAGE the mobility depends on both the protein's charge and its hydrodynamic size. The charge depends on the amino acid composition of the protein. Native PAGE can be carried out near neutral pH to avoid acid or alkaline denaturation to study conformation as well as self-association or aggregation, and the binding of other proteins or compounds. The apparatus is kept cool to minimize denaturation of proteins and proteolysis. [6]

2.1.1.3 Isoelectric Focusing (IEF)

Isoelectric focusing (IEF), also known as electro focusing, is a technique for separating different molecules by their electric charge differences. In Isoelectric focusing, proteins are separated by electrophoresis in a pH gradient based on their isoelectric point (pl). A pH gradient is generated in the gel and an electric potential is applied across the gel. At all pHs other their isoelectric point, proteins will be charged. Whatever the charge the proteins carry, he will move towards his isoelectric point, and at its isoelectric point, since the protein molecule carry no net charge it accumulates or focuses into a sharp band. [7]

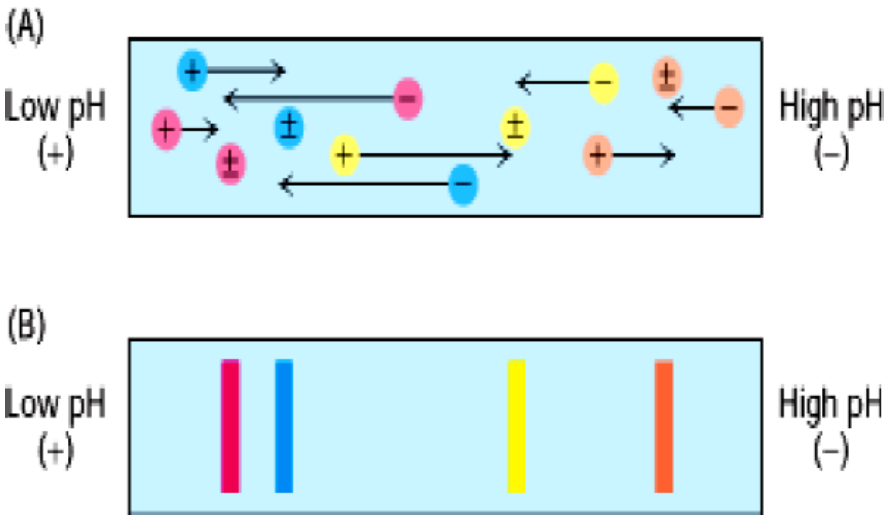

Figure 1 how IEF works.

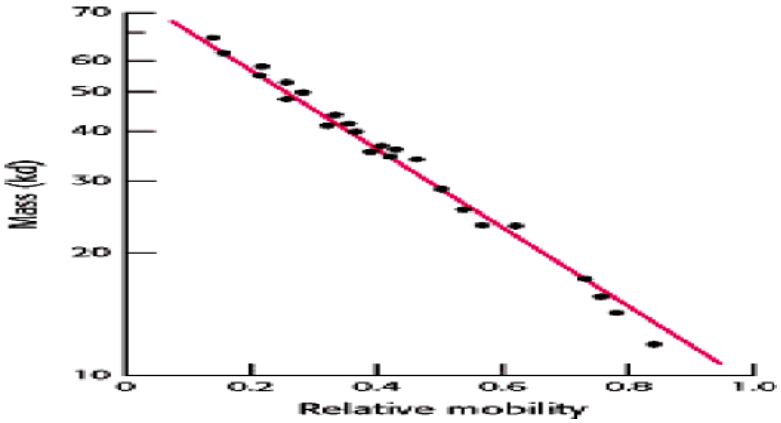

Figure 2 molecular weight determination.

Problems with SDS-PAGE and IEF

1. A limited number (20-30) proteins may be separated by SDS-PAGE or IEF.
2. Some proteins may have either same pI or same molecular weight.

But there is a solution for this problems, we can combine SDS-PAGE and IEF, and the produced technique is called 2-D electrophoresis. [7]

2.1.2 Two dimensions

First dimension is generally IEF, and the second dimension is generally SDS-PAGE. So in the first dimension the proteins are separated according to their isoelectric point and in the second dimension they are separated according to their molecular weight. [8]

Figure 3 two dimensions electrophoresis

2.1.2.1 Uses of two-dimensions PAGE

1. Analysis of complex mixture of proteins.
2. Comparison of different physiological states of the same tissue.
3. Partial characterization of proteins depending on their molecular weight and isoelectric point.
4. Purification protein for further analysis. [8]

2.2 microfiltration

Simple, bulk transfer of proteins that are in solution may be achieved by manual application (dotting) to a membrane from a pipet or syringe, or by vacuum-assisted microfiltration. Manual dot-blotting with a pipet or syringe is generally used for small sample volumes. Microfiltration devices, on the other hand, enable application of larger volumes, multiple assays with different probes, and quick, reproducible screening of a large number of samples. Microfiltration facilitates the determination of working conditions for a new blotting assay and is a convenient method in any other situation where the resolving power of gel electrophoresis is not needed. [8]

3.0 Membranes and Buffers

Selecting the appropriate membrane and transfer buffer is critical to successful protein transfer. The size and charge of the proteins, the transfer method, and the binding properties of the membrane must all be considered.

3.1 Membrane selection

Though nitrocellulose was once the only choice for protein blotting, advances in membrane chemistries have made a variety of membrane types available, each offering key attributes to suit particular experimental conditions. The physical properties and performance characteristics of a membrane should be evaluated when selecting the appropriate transfer conditions. Membranes are commonly available in two pore sizes: the 0.45 µm pore size membranes are recommended for most analytical blotting experiments, while the 0.2 µm pore size membranes are most suitable for transfer of low molecular weight proteins that might move through larger membrane pores. [9]

3.1.1 nitrocellulose and supported nitrocellulose membrane

Nitrocellulose was one of the first membranes used for western blotting and still a popular membrane for this procedure. Protein binding to nitrocellulose is instantaneous, nearly irreversible, and quantitative up to 80 to 100 µg/cm2. Nitrocellulose is easily wetted in water or transfer buffer and is compatible with a wide range of protein detection systems. Supported nitrocellulose is an inert support structure with nitrocellulose applied to it. The support structure gives the membrane increased strength and resilience. Supported nitrocellulose can withstand reprobing and autoclaving (121°C) and retains the ease of wetting and protein binding features of nitrocellulose. [9]

3.1.2 polyvinylidene Difluoride (PVDF) membrane

PVDF membrane is an ideal support for N-terminal sequencing, amino acid analysis, and immunoassay of blotted proteins. PVDF retains proteins during exposure to acidic or basic conditions and in the presence of organic solvents. Greater protein retention during sequencing manipulations enhances the likelihood of obtaining information from rare, low-abundance proteins by increased initial coupling and higher repetitive yields. In addition, PVDF membrane exhibits better binding efficiency of electro blotted material in the presence of SDS in the transfer buffer. PVDF membrane must be wetted in 100% methanol prior to use but may be used with a transfer buffer that contains no methanol. [9]

3.1.2.1 Sequi-Blot PVDF

Sequi-Blot PVDF membrane withstands the conditions of N-terminal sequencing while providing the binding capacity to sequence even low-abundance samples. [9]

3.1.2.2 Immun-Blot PVDF

Immun-Blot PVDF membrane is ideal for chemiluminescent and colorimetric western blots because it retains target protein but resists nonspecific protein binding that can obscure high-sensitivity detection. Immun-Blot PVDF has a strong binding capacity of 150–160 µg/cm2 (roughly twice that of nitrocellulose), will not crack or tear in common handling, and holds up under repeated stripping and reprobing. [9]

Membrane	Pore size	Binding Capacity(µg/cm^2)	Notes
Nitrocellulose	0.45 µm 0.2 µm	80-100	General-purpose protein blotting membrane.
Supported nitrocellulose	0.45 µm 0.2 µm	80-100	Pure nitrocellulose cast on an inert synthetic support; increased strength for easier handling and for reprobing.
Sequi-Blot PVDF	0.2 µm	170-200	High mechanical strength and chemical stability used for protein sequencing.
Immun-Blot PVDF	0.2 µm	150-160	High mechanical strength and chemical stability; recommended for western blotting.

Table 1 comparison between membranes.

3.2 Transfer buffer

Different gel types and blotting applications call for different transfer buffers, but in general, transfer buffer must enable both effective elution of proteins from the gel matrix and binding of the protein to the membrane. The choice of buffer will depend on the type of gel and membrane being used as well as the physical characteristics of the protein of interest. Transfer buffers contain a conductive, strong buffering agent (for example, Tris, CAPS, or carbonate) in order to maintain the conductivity and pH of the system during transfer. In addition, alcohol (for example, methanol or ethanol) may be included in the transfer buffer to promote binding of proteins to membranes, and SDS may be added to promote elution of proteins from gels.

Regardless of which transfer buffer is selected, transfer buffer should never be used more than once, since the buffer will likely lose its capacity to maintain a stable pH during transfer. Similarly, diluting transfer buffers is not advised, since this will decrease their buffering capacity. Lastly, adjusting the pH of transfer buffers when not indicated will result in increased buffer conductivity, manifested by higher initial current output and decreased resistance.

SDS and alcohol play opposing roles in a transfer. SDS in the gel and in the SDS-protein complexes promote elution of the protein from the gel but inhibits binding of the protein to membranes. In cases where certain proteins are difficult to elute from the gel, SDS may be added to the transfer buffer to improve transfer. SDS in the transfer buffer decreases the binding efficiency of protein to nitrocellulose membrane; PVDF membrane can be substituted for nitrocellulose when SDS is used in the transfer buffer. Addition of SDS will increase the relative current, power, and heating during transfer, and may also affect the antigenicity of some proteins. Alcohol (methanol or ethanol), on the other hand, removes the SDS from SDS-protein complexes and improves the binding of protein to nitrocellulose membrane, but has some negative effects on the gel itself. Alcohol may cause a reduction in pore size, precipitation of some proteins, and some basic proteins to become positively charged or neutral. All of these factors will affect blotting efficiency. [9]

3.2.1 types of transfer buffers

3.2.1.1 Towbin and Bjerrum and Schafer-Nielsen buffers (Tris/Glycine Buffers)

The most common transfers are from SDS-PAGE gels using the buffer systems originally described by Towbin (1979). Standard Towbin buffer contains 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol and occasionally 0.025%-0.1% SDS. A buffer similar in composition to the standard Towbin is the Bjerrum and Schafer-Nielsen buffer (48 mM Tris, pH 9.2, 39 mM glycine, 20% methanol). Which is developed for other applications. [9]

3.2.1.2 CAPS Buffer

CAPS-based transfer buffers (10 mM CAPS, pH 11, 10% methanol) may be preferable for transfers of high molecular weight proteins and in cases where the glycine component of Towbin buffer may interfere with downstream protein sequencing applications. [9]

3.2.1.3 Dunn Carbonate Buffer

In some cases, using a carbonate buffer (10 mM NaHCO3, 3 mM Na2CO3, pH 9.9, 20% methanol) may produce higher efficiency transfers and improve the ability of antibodies to recognize and bind to proteins. Carbonate buffer has also been recommended for the transfer of basic proteins. [9]

3.2.1.4 Alternative Buffer Conditions

The mobility of proteins during electrophoretic transfer from native gels will depend on the size and pI of the protein of interest relative to the pH of the buffer used.

• If the pI of the protein is greater than the pH of the transfer buffer, the protein will carry a positive charge and will migrate toward the negative electrode.

• If the pI of the protein is close to the pH of the transfer buffer, the migration of the protein out of the gel will be decreased. A more basic or acidic buffer should be used to increase protein mobility Proteins in native gels as well as acidic and neutral proteins require buffers that do not contain methanol. Gels for isoelectric focusing, native PAGE, and those containing basic proteins or acid-urea may be transferred in 0.7% acetic acid. When using acetic acid for transfer, the proteins will be positively charged, so the membrane should be placed on the cathode side of the gel. [9]

Gel Type	Transfer Buffer	Membrane
SDS-PAGE	Towbin, with or without SDS, CAPS carbonate, Bjerrum Schafer-Nielsen.	Nitrocellulose or supported nitrocellulose. 0.45 or 0.2 µm, or PVDF.
Two-dimensional gel	CAPS, carbonate, Bjerrum Schafer-Nielsen.	Nitrocellulose or supported nitrocellulose, 0.45 or 0.2 µm, or PVDF.
Native, non-denaturing	Depends on pH of gel buffer and isoelectric point of protein of interest.	Nitrocellulose, 0.45 or 0.2 µm, or PVDF
Isoelectric focusing gel	0.7% acetic acid.	Nitrocellulose or supported nitrocellulose, 0.45 or 0.2 µm, or PVDF

Table 2 gel types and there transfer buffers.

4.0 Performing the transfer

This chapter provides an overview of the steps and protocols involved in performing protein transfer. Overall, the procedures and principles for semi-dry and tank transfers are the same. So we are going to talk about only tank transfer. Gels and membranes must be prewet and equilibrated with transfer buffer and the gel/membrane sandwich must be placed into the transfer apparatus in the correct orientation to ensure transfer of proteins to the membrane.

4.1 Performing a Tank Transfer

4.1.1 preparing the Transfer Buffer and Gels.

1. Prepare the transfer buffer in amounts that will suffice for gel equilibration, sandwich assembly, and electrophoresis.

Here is some useful information in performing a Tank Transfer:

- Use only high-quality, analytical grade methanol. Impure methanol can cause increased transfer buffer conductivity and poor transfer.
- Reusing the transfer buffer is not advised, since the buffer will likely lose its ability to maintain a stable pH during transfer. Diluting transfer buffers below their recommended levels is also not advised, since this will decrease their buffering capacity.
- Increased SDS in the transfer buffer leads to increased protein transfer from the gel, but decreased binding of the protein to nitrocellulose membrane. PVDF membrane can be substituted for nitrocellulose when SDS is used in the transfer buffer.
- Addition of SDS will increase the relative current, power, and heating during transfer, and may also affect antigenicity of some proteins.
- Increased methanol in the transfer buffer leads to decreased protein transfer from the gel and increased binding of the protein to nitrocellulose membrane.

2. Rinse gels briefly in H2O and equilibrate for 15 minute in transfer buffer. All gels should be equilibrated in transfer buffer to remove contaminating electrophoresis buffer salts. If salts are not removed, they will increase the conductivity of the transfer buffer and the amount of heat generated during the transfer. Also, gels will shrink or swell to various degrees in the transfer buffer depending on the acrylamide percentage and the buffer composition. Equilibration allows the gel to adjust to its final size prior to electrophoretic transfer. Equilibration is not necessary when the same buffer is used for both electrophoresis and transfer (for example, native gel transfers).
3. Place the transfer tank onto a magnetic stir plate and assemble the transfer tank with the anode and cathode cards in the correct position and orientation.
4. Add enough transfer buffer to the tank to fill it approximately halfway, add a stir bar, and begin stirring. The transfer buffer should be stirred during the course of all tank transfers. This will help to maintain uniform conductivity and temperature during electrophoretic transfer. Failure to properly control transfer buffer temperature results in poor transfer and poses a potential safety hazard. [6]

4.1.2 Assembling the Gel and Membrane Sandwich.

Each gel sandwich will contain the gel and membrane sandwiched between pieces of blot absorbent filter paper.

5. For each gel, cut one piece of membrane and two pieces of filter paper to the dimensions of the gel.
6. Equilibrate the membranes in transfer buffer. Membranes must be thoroughly wetted in transfer buffer prior to assembly of the gel and membrane sandwich. Nitrocellulose membranes may be

wetted directly with transfer buffer, while PVDF membranes must be thoroughly soaked in 100% methanol before being soaked in transfer buffer.

7. Place a fiber pad on top of the black side of the cassette, submerged in buffer. Push on the fiber pad with gloved fingertips to thoroughly wet the pad.
8. Place a piece of filter paper on top of the fiber pad (it should be wetted immediately).
9. Gently place the pre-equilibrated gel on top of the filter paper. Run a wet, gloved finger across the gel to remove any air bubbles that may be trapped underneath the gel.
10. Carefully place the pre-equilibrated membrane on top of the gel. Make sure the membrane is correctly positioned as it touches the gel. To avoid ghost prints and other artifacts, do not move the membrane after it is positioned. Use the roller to remove any air bubbles and to ensure proper contact between the gel and membrane.
11. Wet a second piece of filter paper in transfer buffer and place it on top of the membrane.
12. Soak a fiber pad in transfer buffer and place it on top of the filter paper. [6]

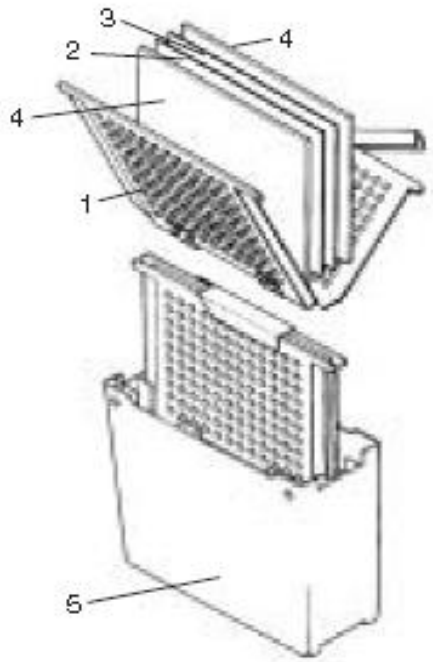

Figure 4 Transfer assembly for a tank transfer system

13. Once the cassette is closed and locked, insert it into the tank with the latch side up. Make sure the black cassette plate faces the black electrode plate.

- The cassette (1) holds the gel (2) and membrane (3) while fiber pads and filter paper (4) on both sides provide complete contact within the gel sandwich. The gel cassette is inserted vertically in the buffer tank (5).

4.1.3 Performing the Transfer

14. Add transfer buffer to the tank until the buffer level reaches the fill line.
15. Place the lid on top of the cell, making sure that the color-coded cables on the lid are attached to the electrode cards of the same color. Reversing the orientation of the cables will cause irreversible damage to the plate electrodes.
16. Upon completion of the run, remove the cassettes and disassemble the gel and membrane sandwich. Rinse the membrane briefly in H_2O to ensure that no residual gel pieces or sample adhere to the membrane. [6]

4.2 Microfiltration

Microfiltration involves presoaking the transfer membrane, assembly of the transfer apparatus, application of the vacuum, and application of the sample. Here is some advices to perform a good microfiltration:

4.2.1 Application of the vacuum

Apply the vacuum only until solutions are removed from the sample wells, then adjust the flow valve so that the unit is exposed only to atmospheric pressure and disconnect the vacuum. For best sample binding, the entire sample should be filtered by gravity flow without vacuum. During the assay, do not leave the unit with the vacuum on. This may dehydrate the membrane and may cause halos around the wells. [6]

4.2.2 Filtering or centrifugation of sample

For best results, filter or centrifuge sample to remove particular matter that might restrict the flow of solutions through the membrane. [6]

4.2.3 Air bubbles

Air bubbles trapped in the wells will prevent the sample from binding to the membrane. Air bubbles may be removed by gently pipetting the solution up and down. [6]

4.2.4 Membrane Removal

The best method for removing the membrane is to leave the vacuum on while loosening the screws and removing the sample template. Then, turn off the vacuum and remove the membrane. [6]

5.0 Detection and imaging

Once proteins have been transferred to a membrane, they can be visualized using a variety of specialized detection reagents. Protein standards are useful for monitoring transfer efficiency of the experiment and serve as molecular weight markers for calibration of blot patterns. Total protein stains allow visualization of the protein pattern on the blot and immunological detection methods, employing antibody or ligand conjugates, allow visualization of specific proteins of interest. We will talk about total protein stains, protein standards, and immunological detection methods available.

5.1 Protein standards

Protein standards are mixtures of well-characterized or recombinant proteins and are routinely used in electrophoresis and blotting applications. Protein standards:

• Provide a reference for determining the molecular weight of proteins identified by antibody or ligand probes.

• Are useful for monitoring transfer efficiency.

• Serve as controls to ensure proper location of transferred bands in repetitive screening Experiments.

Protein standards are available as either pre-stained, unstained, or biotinylated sets of purified or recombinant proteins. In general, pre-stained standards allow easy and direct visualization of their separation during electrophoresis and of their transfer to membranes. Though pre-stained standards can also be used for estimation of molecular weight of separated proteins, unstained or biotinylated standards are recommended for the most accurate molecular weight determination.

5.1.1 Pre-stained standards

The ability to visualize pre-stained standards during electrophoresis makes them ideal for monitoring protein separation during gel electrophoresis. The ease in transferring to the blot also makes them popular for monitoring transfer efficiency and the general location of antigens in repetitive screening assays. This, combined with recent improvements made in their design and manufacture, has made pre-stained standards an excellent choice for estimations of molecular weights on western blots. [10]

There are different types of pre-stained standards, we will mention some of them:

5. **Recombinant Pre-Stained Standards**

Advances in molecular biology and genetic engineering has led to the development of recombinant protein standards for electrophoresis. With recombinant technology, specific attributes, such as evenly spaced molecular weights or customization of proteins with affinity tags for easy detection, may be designed and engineered into sets of protein standards. [10]

6. **Precision Plus Protein Pre-Stained Standards**

Precision Plus Protein Pre-Stained standards are a blend of ten recombinant proteins and
provide a ten-band, broad range molecular weight ladder (10–250 kD) with single (all blue), dual (dual color), or multicolored (Kaleidoscope) protein bands, the colors allow easy band referencing and blot orientation. Because the proteins in the Precision Plus Protein standards are recombinant, and the staining technology is optimized. their molecular weight does not vary from lot to lot. Dye labeling can be controlled more effectively, delivering homogeneous staining and tight, sharp bands. All Precision Plus Protein Pre-Stained standards (all blue, dual color, Kaleidoscope) deliver the most linear standard curve available for Pre-Stained standards. As a result, these standards may be used for highly accurate estimation of molecular weight across a broad size range. [10]

7. **Natural Pre-Stained SDS-PAGE Standards**

Natural molecular weight standards are blended from naturally occurring proteins, often providing a familiar band pattern. Although very effective for monitoring gel separation and transfer efficiency, they have an inherent variability in the amount and location of dye that covalently binds to the protein. This

may produce broader bands than seen in recombinant Pre-Stained standards or in unstained standards, making them less desirable for molecular weight estimation. [10]

- **Kaleidoscope Standards**

Kaleidoscope Pre-Stained standards contain individually colored proteins that allow instant band recognition on western blots or gels. The molecular weights of the proteins in each lot are calibrated against unstained SDS-PAGE standards. The Kaleidoscope standards are available in broad or low molecular weight (polypeptide) formulations; polypeptide standards are designed for use with Tricine gels when resolving small proteins and peptides. [10]

- **Pre-Stained SDS-PAGE Standards**

Naturally occurring Pre-Stained SDS-PAGE standards are available in specific size ranges: low, high, and broad.

5.1.2 Unstained Standard for Western Blotting

Since unstained standards contain only the protein itself, they do not exhibit the variability in molecular weight that is often seen with Pre-Stained standards. Therefore, unstained standards, biotinylated standards, or standards with an affinity tag for blot detection deliver almost 100% molecular weight accuracy across a linear curve and are recommended for the most accurate molecular weight determinations for gels or blots. [11]

- **Precision Plus Protein Unstained Standards**

Precision Plus Protein unstained standards provide a recombinant ten-band, broad range molecular weight ladder (10–250 kD). These standards contain an affinity Strep-tag peptide that displays an intrinsic binding affinity towards StrepTactin, a genetically modified form of streptavidin. It is the high-affinity binding of the Strep-tag sequence to StrepTactin that allows convenient and simultaneous detection of both proteins and standards on western blots using either colorimetric or chemiluminescent methods. [11]

- **Biotinylated Standards**

Biotinylated protein standards have been developed specifically for accurate molecular weight determinations in blotting applications. These proteins have been treated with a limited amount of biotin. Since biotin is a relatively small molecule, the mobility of the standard proteins in SDS-PAGE gels are not altered. The biotinylated proteins form sharp, tight bands with well-defined molecular weights and are processed simultaneously with the immunostained antigens. Therefore, they provide fast, accurate, reproducible determinations of antigen molecular weights directly on the membrane. Biotinylated standards are easily detected using avidin or streptavidin conjugated with horseradish peroxidase or alkaline phosphatase. [11]

- **Unstained SDS-PAGE Standards**

The protein markers used for SDS-PAGE gels form very tight bands that transfer reproducibly to membranes. Molecular weight determination with these proteins, however, may require cutting the lane of standards from the membrane and using a separate total protein stain for identification. After visualization, the cut strip must be realigned with the probed membrane. Alternatively, the entire blot may be stained with a washable anionic total protein dye like Ponceau S and the positions of the standards on the blot marked with a pencil. SDS-PAGE standards are available in three molecular weight ranges. [11]

5.2 Total protein staining

Total protein staining of a membrane provides a visual image of the complete protein pattern.
This information is required for the full characterization of specific antigens detected in a complex protein mixture.

5.2.1 Anionic dyes

The first techniques developed for total protein staining of blotted membranes used the same anionic dyes commonly used for staining proteins in polyacrylamide gels. Dyes that became popular for detecting proteins bound to membranes include Amido Black, Coomassie Brilliant Blue R-250, Ponceau S, and Fast Green FCF. Of the anionic dyes used, Amido Black and Ponceau S are the best choices because they destain rapidly in distilled water or low concentrations of methanol and produce very little background staining. Coomassie Brilliant Blue gives high background staining, even after long destaining procedures. Fast Green is not tightly bound to the proteins, so the dye can be easily removed after visualization to allow subsequent immunological probing. These dyes are easy to prepare and they stain proteins quickly, but they are relatively insensitive when compared to immunological detection assays. The stains that require alcohol-containing solutions (for example, Amido Black, Coomassie Brilliant Blue, Fast Green FCF) for solubility can shrink nitrocellulose membranes, making direct comparison of an immunologically detected antigen to the total protein on the stained membrane difficult. [10]

5.2.2 Colloidal Gold

An alternative to anionic dyes that provides detection sensitivities that rival those of immunological detection methods is colloidal gold. When a solution of colloidal gold particles is incubated with proteins bound to a nitrocellulose or PVDF membrane, the gold binds to the proteins through electrostatic adsorption. The resulting gold-protein complex produces a transient, reddish-pink color due to the optical properties of colloidal gold. This gold-protein interaction is the basis for total protein staining with colloidal gold as well as for specific, immune-gold detection. Silver enhancement of the colloidal gold signal produces a stable, dark brown reaction product and enhances sensitivity down to 10 pg of protein. The method of silver enhancement makes use of the fact that gold particles can reduce silver ions in solution; this reduction leads to deposition of the silver on top of the gold and the effective growth of the metallic particle. Since methanol is not required, colloidal gold with silver enhancement has the advantage over the anionic dyes of not shrinking the membrane during staining. [10]

5.2.3 Biotinylation

Biotinylation provides a sensitive total protein detection method that takes advantage of the high affinity binding of avidin to biotin. This method uses NHS-biotin to biotinylate all proteins on the membrane surface, followed by incubations with an avidin-horseradish peroxidase (avidin-HRP) conjugate and the HRP color-development reagent to detect the biotinylated proteins. The assay is 10–50 times more sensitive than the anionic stains, and does not require methanol. [10]

5.2.4 Fluorescence

Fluorescent protein stains like SYPRO Ruby, SYPRO Red, SYPRO Orange, and Deep Purple provide highly sensitive detection of proteins on blots as well as in gels. SYPRO Ruby protein blot stain allows detection as low as 2–8 ng. After staining, target proteins can be detected by colorimetric or chemiluminescent immunodetection methods, or analyzed by micro sequencing or mass spectrometry with no interference from the protein stain. [10]

5.3 Immunological Detection System

Immunological detection systems are used to identify specific proteins blotted to membranes. Though there are numerous systems available for immunological detection, the basic procedure for these assays varies very little. After the proteins have been transferred to the membrane, the membrane is blocked, incubated with a primary antibody, washed, incubated with a secondary antibody, and washed again. The primary antibody is specific for the protein of interest, and the secondary antibody enables its detection. The secondary antibody can be radiolabeled, labeled with a fluorescent compound or gold particles, or conjugated to an enzyme like AP or HRP. For many years, radiolabeled secondary antibodies were the method of choice for detection, but newer methods have evolved that are less hazardous and easier to use than radioactivity, yet maintain the same degree of sensitivity. We can summarize the steps of immunological detection system in this figure [11].

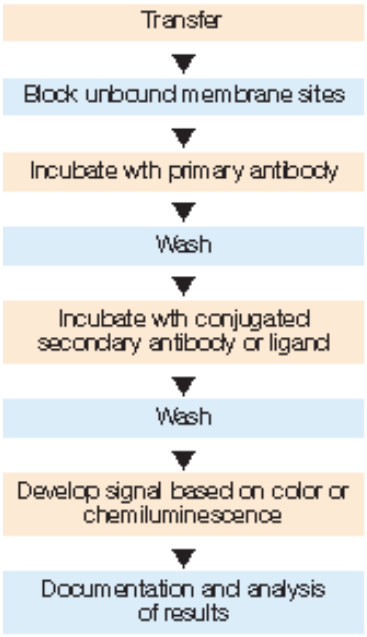

Figure 5 Basic immunological detection procedure.

5.3.1 Blocking Reagent

Following transfer, unoccupied binding sites on the membranes must be locked to prevent nonspecific binding if probes; failure to adequately saturate the membrane can lead to high background, since many probes are also proteins, and can also bind to the membrane. A variety of blocking reagents are available, including gelatin, nonfat milk, and bovine serum albumin (BSA). It is often useful to optimize the detection system for minimal background with no loss of signal by testing several blocking agents. The type of membrane will also affect the selection of blocker. [11]

5.3.2 Antibody Incubations

An antibody is a protein that is synthesized by an animal in response to exposure to a foreign substance, or antigen. Antibodies (also called immunoglobulins) have specific affinity for the antigens that elicited their synthesis. A typical experimental system utilizes two layers of antibody in the detection procedure. The primary antibody is directed against the target antigen; the antigen may be a ligand on a protein, the protein itself, a specific epitope on a protein, or a carbohydrate group. The secondary antibody is specific for the primary antibody; it is usually conjugated to an enzyme such as alkaline phosphatase (AP) or

horseradish peroxidase (HRP), and an enzyme-substrate reaction is part of the detection process. Antibody incubations are generally carried out in antibody buffer containing Tris-buffered saline with Tween (TTBS) and a blocking reagent. [11]

5.3.3 Washes

Between the two antibody incubations and prior to detection, the blot must be washed to remove excess antibody to prevent nonspecific binding. Though the washing solutions and times may vary, depending on the antibodies and detection systems used, washes generally utilize Tris-buffered saline (TBS) or TBS with additional detergent (Tween 20; TTBS). Note that the addition of detergent may inhibit certain detection reactions. [11]

5.3.4 visualization methods

Blotted proteins are generally detected using secondary antibodies that are labeled with radioisotopes or colloidal gold, or conjugated to fluorophores, biotin, or an enzyme like HRP or AP. Early blotting systems used 125I-labeled reagents similar to those used in radioimmunoassay. These systems provide sensitive results, but the special handling and disposal problems of 125I reagents have discouraged continued use of this technique. Instead, a number of enzyme systems and detection reagents evolved. By far, the most commonly used detection methods use secondary antibodies conjugated to HRP or AP. With these systems, when the enzyme substrate is added, either a colored precipitate is deposited on the blot (colorimetric detection) or a chemiluminescent or fluorescent product is formed (chemiluminescent and chemi-fluorescent detection) and the light signal is captured on film or with a CCD or fluorescence imager. [10]

5.3.4.1 Colorimetric Detection

Enzymes such as AP and HRP convert several substrates to a colored precipitate. As the precipitate accumulates on the blot, a colored signal develops that is visible on the blot. The enzyme reaction can be monitored and stopped when the desired signal over background is produced. Colorimetric detection is easier to use than any film-based detection method, which must be developed by trial and error, and uses costly materials such as X-ray film and darkroom chemicals. Colorimetric detection is typically considered a medium-sensitivity method, compared to radioactive or chemiluminescent detection. [10]

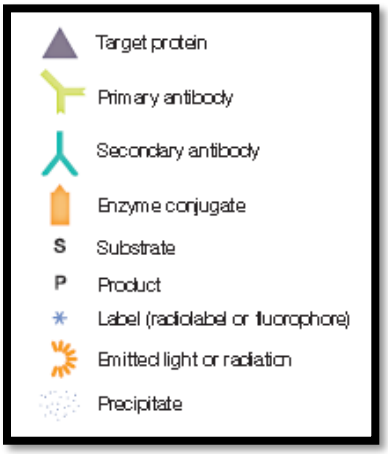

Figure 7 the shapes key.

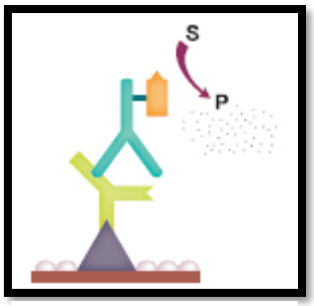

Figure 6 colorimetric Detection.

5.3.4.1.1 colorimetric HPR Systems

Colorimetric HRP systems were the first enzyme-conjugates used for immunological detection of blotted proteins. The advantage of HRP systems was that both the enzyme conjugate and colorimetric detection substrates were economical. The most common color substrates for HRP are 4-chloro-1-naphthol (4CN) and 3,3'-diaminobenzidine (DAB). HRP colorimetric detection systems are not as sensitive as AP colorimetric detection systems. Fading of blots upon exposure to light, inhibition of HRP activity by azide, and nonspecific color precipitation are additional limitations of HRP colorimetric detection systems. [10]

5.3.4.1.2 colorimetric AP Systems

Colorimetric AP systems use soluble 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitroblue Tetrazolium (NBT) as substrates to produce a stable reaction product that will not fade. AP can be easily inactivated by exposure to acidic solutions. Multiple probing of the same membrane with alternative antibody probes are easily performed using substrates that produce different colors, such as blue and red. And here is a comparison between AP and HPR systems: [10]

Detection method	substrate	Detection sensitivity	Signal color	Product options	Advantages	disadvantages
Colorimetric HRP	4CN DAB Opti-4CN Amplified Opti-4CN	500 pg 500 pg 100 pg 5 pg	Purple Brown Purple Purple	• Dry powder, liquid substrate, Immun-Blot kits •Dry powder. • Liquid substrate, Opti-4CN kit. • Amplified Opti-4CN kit.	• Fast color development, low cost, low background. • Fast color development, low background. • High sensitivity, nonfading color, low background. • Best sensitivity available, no extra materials (such as X-ray film) needed.	• Results fade over time; azide inhibits enzyme activity. • More safety precautions than for other substrates. • Azide inhibits enzyme activity. • More expensive than 4CN. • More steps than unamplified protocol.
Colorimetric AP.	BCIP/NBT. Amplified BCIP/NBT.	100 pg. 10 pg.	Purple. Purple.	• Dry powder, liquid substrate, Immun-Blot kits. • Amplified AP Immun-Blot kit.	• Stable storage of data. • High sensitivity.	• Detects endogenous phosphatase activity. • More steps than unamplified protocol.

Table 3 comparison between colorimetric HPR and AP.

5.3.4.2 Chemiluminescent Detection

Chemiluminescence is a chemical reaction in which a chemical substrate is catalyzed by an enzyme, such as AP or HRP, and produces detectable light as a by-product. The light signal can be captured on X-ray film, or by a charge-coupled device (CCD) imager. This technology is easily adapted to existing western blotting procedures because Chemiluminescence uses enzyme conjugated antibodies for the activation of the light signal. [10]

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Detection method	Substrate	Detection sensitivity	Product options	advantages	Disadvantages
Chemiluminescent HRP.	Luminol	1–3 pg	• Conjugates. • HRP substrate. • Immun-Blot kits	• Short (30 sec) exposure. • Signal duration 6–8 hours. • Compatible with PVDF and nitrocellulose. • Working solution stable for 24 hours at room temperature.	• Azide inhibits enzyme activity.
Chemiluminescent AP.	CDP-*Star*.	10 pg.	• Conjugates. • AP substrate. • Immun-Blot kits.	• 30 seconds to 5 min exposure. • Signal continues for 24 hours after activation. • Blot can be reactivated.	• Endogenous phosphatase activity may lead to false positives.

Table 4 comparison between chemiluminescent HPR and AP.

5.4 Imaging methods

Several methods are employed for the documentation of western blotting results: X-ray film or digital charge-coupled device (CCD) camera imaging for luminescent signals, CCD or laser-based imaging systems for the capture and documentation of fluorescent and colorimetric signals, and X-ray film or phosphor imagers for radiolabeled samples. [10]

5.4.1 Luminescent Detection

For chemiluminescent detection methods, CCD imaging is the easiest, most accurate, and rapid method. Traditionally, the chemiluminescent signal from blots has been detected by X-ray film. The popularity of Chemiluminescence has led to development of special films with enhanced sensitivity to the blue wavelengths of light emitted by the chemiluminescent substrates. Film is a sensitive medium to capture the chemiluminescent signal but suffers from a sigmoidal response to light that has a narrow linear region, which limits its dynamic range. To gather information from a blot, which has both intense and weak signals, it is necessary to perform multiple exposures to produce data for all samples in the linear range of the film. A process termed pre-flashing can improve linearity, but this requires extra equipment and effort. Additionally, quantitation of data collected by exposure to film requires digitization (that is, scanning of X-ray film with a densitometer). A CCD camera is capable of capturing data with a linear

response over a broad dynamic range. The linear dynamic range of CCD systems is 2–4.8 orders of magnitude, depending on the bit depth of the system. CCD systems also offer convenience by providing a digital record of experiments for data analysis, sharing, and archiving, and by eliminating the need to continually purchase consumables for film development. Another advantage of the CCD camera is its ability to approach the limit of signal detection in a relatively short time. For example, the VersaDoc 5000 imaging system can reach the limit of detection of a given experiment in less than one minute. For the same experiment, Kodak Bio-Max film requires about 30 min to reach the same limit of detection. [10]

5.4.2 Fluorescent, Chemifluorescent, and Colorimetric Detection

Fluorescent, chemifluorescent, and colorimetric detection all benefit from the advantages of digital imaging — convenience, digital records of experiments, sensitive limit of detection, and wide dynamic range. Fluorescent and chemifluorescent signals can be detected with a wide range of imaging systems including both CCD and laser-based technologies. For example, the VersaDoc and Molecular Imager FX Pro Plus systems can be used similarly to detect fluorescent and chemifluorescent signals. The decision to use one type of technology over another will depend on budget and requirements for limit of detection and resolution. CCD systems are generally less expensive than laser-based systems. While the dynamic range of CCD imaging systems varies from 2 to 4.8 orders of magnitude, laser-based systems do not provide a choice of dynamic ranges but do provide the widest dynamic range (4.8 orders of magnitude) available. The resolution of CCD and laser-based systems is similar, with the finest resolution settings generally being 50 µm or less. Another advantage of fluorescent and chemifluorescent detection is that CCD and laser-based detection limits generally far exceed the dynamic ranges of the fluorescent assays currently used for protein detection. [10]

5.5 screening apparatus

In some experiments, protein blots need to be screened for a number of different antigens or under a number of different conditions. Mini incubation trays allow screening of individual strips that have been cut from blots. The Mini-PROTEAN II multiscreen apparatus allows screening of a single blot with up to 40 different antibodies or sera without cutting the blot into individual strips. [10]

6.0 Applications of western blotting

In this chapter, we present a selection of applications in which Western blotting (and also in some instances, 1-D gel electrophoresis analysis) has played a central role, from confirmation of the presence of target proteins in a chromatography fraction to quantitative, multiplexed Western blotting.

6.1 detecting or characterizing Protein Expression

One simple and common use for Western blotting is to identify whether a particular protein is present or absent in a sample by looking for a band of the correct size on a Western blot. This routine type of Western blot is used to either test for endogenous cellular expression of a target protein, or to examine transfected cell lines to see if expression has been conferred by the introduction of a DNA construct. It is also used with techniques such as protein purification and cellular fractionation to identify which samples have the target protein, and thus is an aid to deciding which samples to combine or discard. After immunodetection, a band corresponding to the size of the target protein should become visible in positive test samples and the positive control, in comparison to a known null cellular sample. In the figure below, HEK293 cells transfected with three isoforms of the Homer 1 protein and are compared to rat cortical extract as a positive control. The blot shows that the antibody used, AbD Serotec's AHP737, recognizes Homer isoform 1c, but not 2b or 1a. The presence of untransfected cells (Lane 2) confirm that the signal is specific to transfection with the appropriate construct. The faint band seen in lane 4, is actually a small amount of spill over from lane 5.

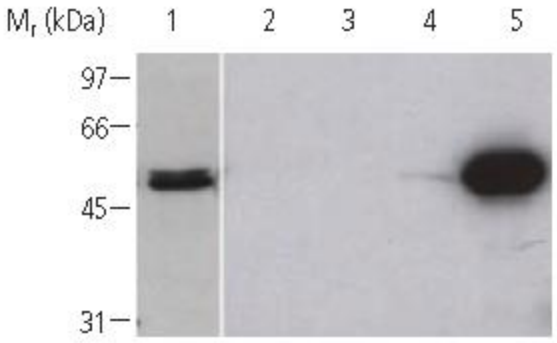

Figure 8 Detection of Rat Homer Protein Isoform.

- Western blot of Homer isoforms probed with anti-Homer [177-366] (AHP737) which detects the control and isoform 1c. Lane 1: control, 30 mg rat cortical extract. Lane 2: untransfected control. Lane 3: Isoform 1a. Lane 4: Isoform 2b. Lane 5: Isoform 1c.

6.2 Prion disease

Prion disease is a rare, progressive, neurodegenerative disorder that affects humans, cows, sheep (Scrapie), and deer. It leads to characteristic brain lesions and a rapid loss in neurological functions after a long latency period. The accumulation of an altered isoform of a normal cellular protein appears to be instrumental in the development of prion disease. There are several ways to identify the presence of the abnormal prion protein - a bioassay, immunohistochemistry on diseased tissue samples, and with a faster, well-characterized, and sensitive Western blot.

The Western blot below shows Scrapie infected sheep brain lysates (Lanes 3&4) compared to normal sheep brain lysates (Lanes 1&2). The key to this particular Western assay is the use of Proteinase (PK) treatment on parallel sets of samples. Since the Scrapie associated form of the prion protein is resistant to digestion, it is possible to distinguish between normal cellular forms and abnormal prion protein in a sample based upon their sensitivity to PK. Thus, it is possible to discriminate diseased samples from normal ones quickly and unambiguously on a Western blot. The absence of bands in lane 2 indicates that the normal form is present as compared to the bands visible in lane 4, which indicate the presence of the pathogenic form.[12]

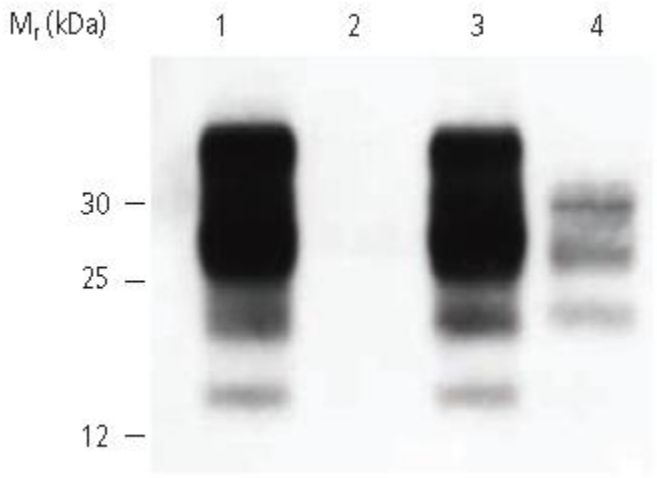

Figure 9 Detection of Scrapie infected sheep brain.

- Bands in lane 4 indicate that the protease resistant form of the prion protein is present in the sample as compared to normal tissue in lane 2. Lanes 1&2: Uninfected sheep brain homogenate. Lanes 3&4: Scrapie-infected sheep brain homogenate. Lanes 1&3: No PK added. Lanes 2&4: Digested with PK.

6.3 Confirmation of HIV

In the diagnosis of HIV infection in patients, an ELISA is used first because it demonstrates 99.5% specificity and is quick and easy to perform on a large number of samples. Western blotting is used as a supplementary assay because the ELISA is subject to false positives. In order to perform this assay, patient serum samples are used as the source of antibodies for immunodetection, and blots containing HIV

antigens, proteins, viral lysates, or peptides are used as the source of target antigen on the Western blot. If the HIV target proteins on the blot are detected by the patient serum, then it indicates that the patient sample is a true positive for HIV infection, since an immune response has been mounted by the patient. A similar strategy is also used in tests for Lyme disease and autoimmune disease.

Conclusion

As we saw, Western blotting is a very useful procedure in providing information about the sample, like what are the proteins that exist in it, and to verify if there is a certain protein(s) in the sample.

This information is very important in the medical field, because by knowing this information it can be decided if the disease exist or not in the sample.

Although there are very different ways to perform this procedure, there are some main steps to do:

- Sample preparation.
- Transforming the sample to a membrane.
- Detecting the proteins(s).
- Analyzing the results.

But the Varity comes from the differences in the sample type and condition, the protein(s) needed to detected, the information needed to know, and a lot of other variables related to the sample.

The most important thing in western blotting procedure is the good choose of the apparatus for it, and by choosing them we will get whatever information needed.

Choosing the right apparatus also depends on the way of performing the procedure, which varies as mentioned before.

So we can say that western blotting is a very important procedure in biological fields and it provides us a lot of information, and without it we wouldn't be able to know all this information about the human cells, or any other cell on earth.

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References

1. al, C.Y.e., *Sample preperation*. 2008.
2. S, G., *Mechanical/physical methods of cell disruption and tissue homogenization*. 2008. 3-22.
3. DP, G. and C. TE, *Gel elecrophoresis in studies of protein conformation and folding*. 1984.
4. DE, G., *One-dimensional gel electrophoresis* 2009.
5. N, B.W., *electrophoresis transfer of proteins from sodium dodecyl sulfate-polyacrylamid gels to unmodified nitrocellulose and radiographic detection with antibody and readioiodinated protein*. 1981. 195-203.
6. H, T., *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications*. 1979. 4350-4354.
7. R, W., *Isoelectric focusing*. 2004.
8. PH, O.F., *High resolution two-dimensional electrophoresis of proteins*. 1975.
9. Evans, F.a.W.H., *Biological Membranes: A Practical Approach*. 1987.
10. TH, S., *Protein gel staining methods: an introduce and overview*. 2009.
11. CR, M., *Detection of proteins seperated by electrophoresis.* 1987.
12. Wadsworth, J.D., *Tissue distirbution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay*. 2001.