# *Western Blotting*

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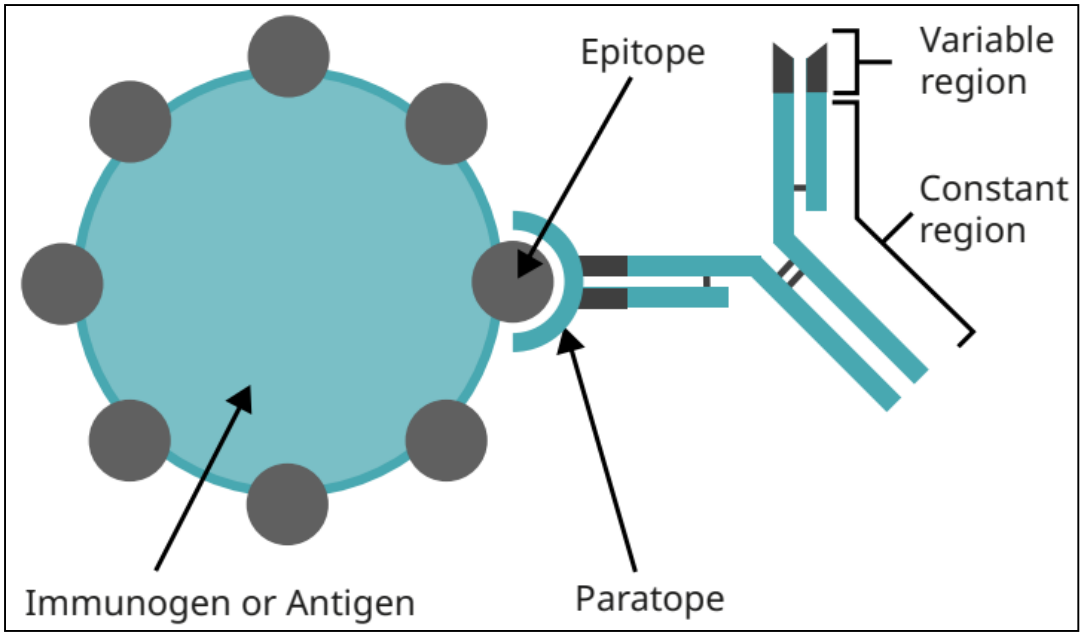
# ➢Immunoassays

**Immunoassays are tests that detect the presence of a specific molecule in a sample using antibody-antigen binding reactions.**

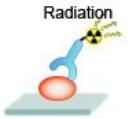
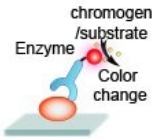
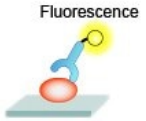
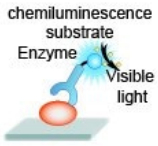
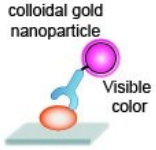
**Antibodies bind to the specific structure of a particular antigen (called epitope), making immunoassays highly specific: the antibody will only bind to a specific structure of a particular antigen.**

**As a result, immunoassays are a fundamental tool for hospitals, life science research and industry laboratories. Immunoassays come in a range of formats, and can be used to assess disease, track proteins, and detect environmental contamination.**



# ➢Types of Immunoassays

## According to detection methods:

| | Illustration | Label | Substrates | Signal | Sensitivity | Advantages | Drawbacks |
|---|---|---|---|---|---|---|---|
| Radioimmunoassay (RIA) |  | Radioactive Isotopes (I-125) | None | Radiation | Ultrahigh | 1. Sensitive and precise<br>2. Radio-label conjugation easy to prepare | 1. Radioisotopes must be utilized within a few weeks<br>2. Risk of radiation exposure |
| Enzyme Immunoassay (EIA) |  | Enzymes (HRP,AP) | Chromogen/ substrate | Color change | High | 1. Sensitive and safe<br>2. Quick and convenient, easy to automation<br>3. Widely used in variety of test | 1. Need monoclonal antibody<br>2. Non-specific absorption<br>3. Enzyme/substrate reaction is short term and must be read soon. |
| Fluoroimmunoassay (FIA) |  | Fluorogenic Reporters (Rhodamine) | None | Fluorescence | High | 1. Sensitive, specific and save<br>2. Limited background fluorescence<br>3. Easy to automation<br>4. multiple label can be used | 1. Rely on fluorescence detecting instrument<br>2. Photo bleaching |
| Chemiluminescence Immunoassay (CLIA) |  | Chemical probes (Acridinium Ester, luminol) | luminescence substrate | Visible light | Ultrahigh | 1. Excellent sensitivity and safe<br>2. Stable of reagent<br>3. Easy to automation, can be used in high throughput system | Need CCD equipment to capture the light signal |
| Colloidal Gold Immunochromatographic Assay (ICA) |  | Colloidal gold nanoparticle | None | Visible color | Low | 1. Rapid, easy to use<br>2. Low cost and safe<br>3. Visible, without any equipment facilities | Low sensitivity and specificity |

# ➢Types of Immunoassays

**According to Lab technique:**

**1- Western Blot**

**2- ELISA and Digital ELISA**

**3- Immunohistochemistry**

**4- Flow cytometry and FACS**

**5- Protein Arrays**

**6- Others (Immunoprecipitation, ……..)**

# ➢ Western Blot

**Western blot allow investigators to determine the molecular weight of a protein and to measure the relative amounts of this protein present in different samples.**

**Two Main Types of Westerns:**

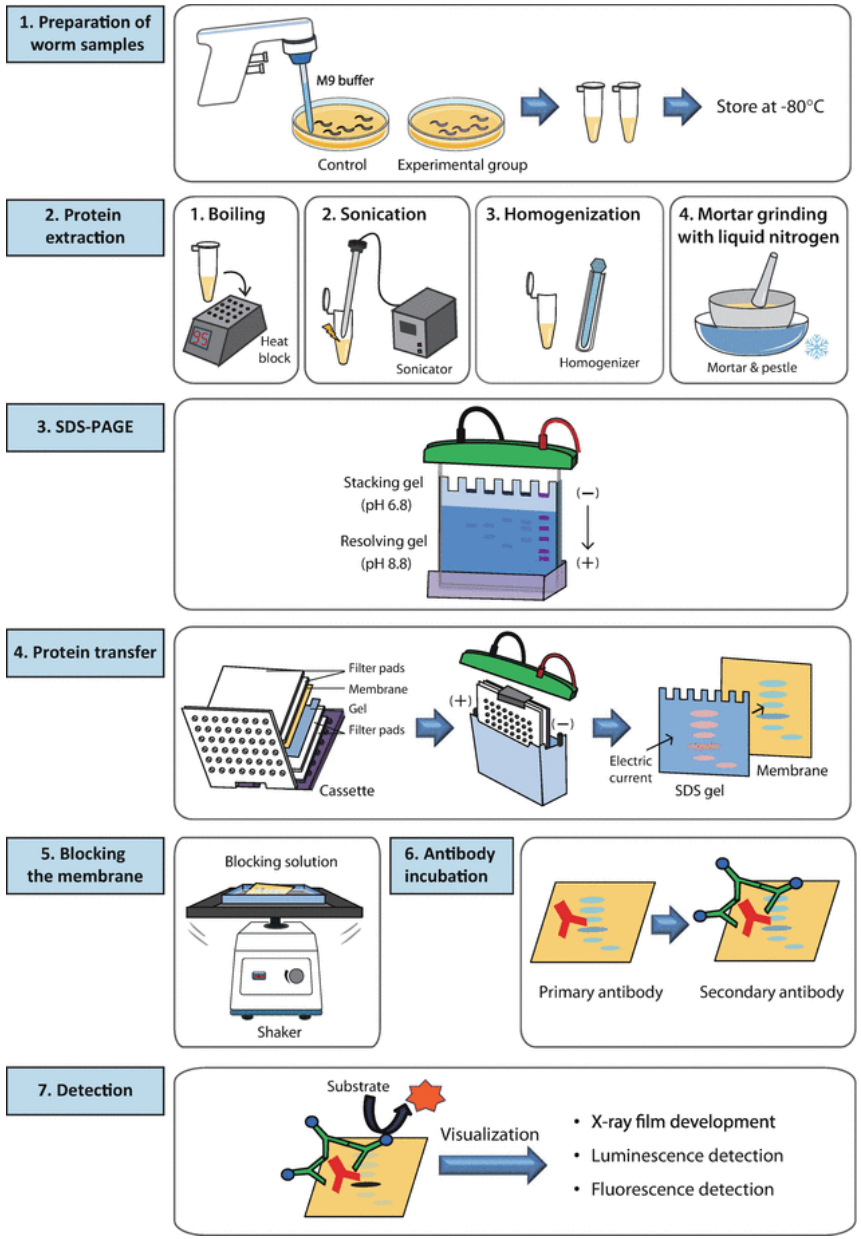
1. **Denaturing (Most Commonly Used)**
2. **Non-Denaturing (Native)**

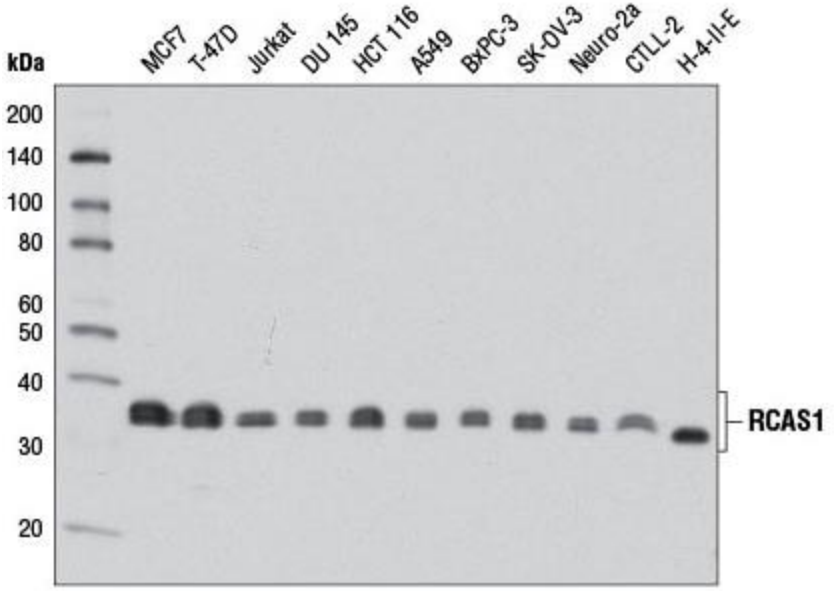
- **Antibodies typically recognize a small portion of the protein of interest (referred to as the epitope) and this domain may reside within the 3D conformation of the protein. To enable access of the antibody to this portion it is necessary to unfold the protein, i.e. denature it.**

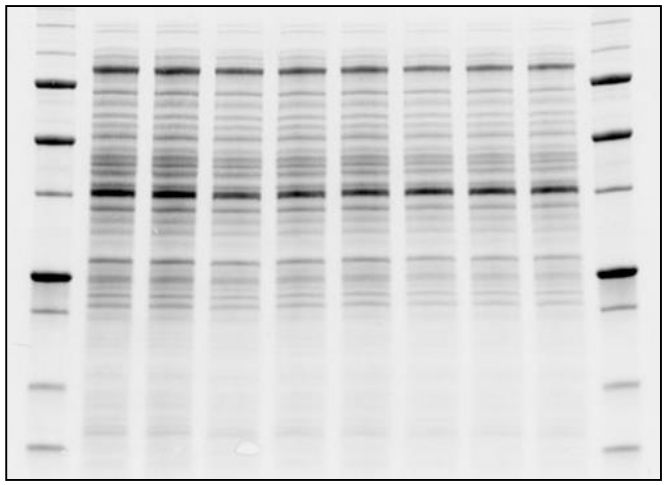
- **Alternatively, an antibody may recognize an epitope made up of non-contiguous amino acids. Although the amino acids of the epitope are separated from one another in the primary sequence, they are close to each other in the folded three-dimensional structure of the protein, and the antibody will only recognize the epitope as it exists on the the surface of the folded structure.**

- **Certain antibodies only recognize protein in its non-reduced form i.e. in an oxidized form (particularly on cysteine residues) and the reducing agents ß-mercaptoethanol and DTT must be left out of the loading buffer and migration buffer (non reducing conditions).**

# ➢Summary of Western Blot



# ➢A. Sample preparation

1. **Lysis buffers:**

- **To prepare samples for running on a gel, cells and tissues need to be lysed to release the proteins of interest. This solubilizes the proteins so they can migrate individually through a separating gel.**

- **There are many recipes for lysis buffers. They differ in their ability to solubilize proteins, with those containing sodium dodecyl sulfate (SDS) and other ionic detergents considered to be the harshest and therefore most likely to give the highest yield.**

- **It is important to note though that some antibodies will only recognize a protein in its native, non-denatured form and will not recognize a protein that has been extracted with a denaturing detergent (SDS, LDS, deoxycholate, and somewhat less denaturing, Triton X-100 and NP-40).**

# ➢A. Sample preparation

## 1. Lysis buffers (cont.):

### Protein Location And Lysis Buffer Choice

| Protein location | Buffer recommended |
|---|---|
| Whole Cell | NP-40 or RIPA |
| Cytoplasmic (soluble) | Tris-HCl |
| Cytoplasmic (cytoskeletal bound) | Tris-Triton |
| Membrane bound | NP-40 or RIPA |
| Nuclear | RIPA or use nuclear fraction protocol* |
| Mitochondria | RIPA or use mitochondrial fraction protocol* |

**RIPA buffer (R**adio **I**mmuno **P**recipitation **A**ssay buffer**)**

- **Cell lysis with detergent-free buffer is achieved by mechanical shearing, often with a Dounce homogenizer or by passing cells through a syringe tip. In these cases a simple Tris buffer will suffice, but as noted above, buffers with detergents are required to release membrane- or cytoskeleton- bound proteins.**

# ➢A. Sample preparation

## 2. Protease and phosphatase inhibitors:

**As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer.**
**Ready-to-use cocktails of inhibitors from various suppliers are available but you can make your own cocktail.**

| Inhibitor | Protease/phosphatase Inhibited | Final concentration in lysis buffer | Stock (store at -20°C) |
|---|---|---|---|
| Aprotinin | Trypsin, Chymotrypsin, Plasmin | 2 µg/ml | Dilute in water, 10 mg/ml. Do not re-use thawed aliquots |
| Leupeptin | Lysosomal | 5-10 µg/ml | Dilute in water. Do not re-use once defrosted. |
| Pepstatin A | Aspartic proteases | 1 µg/ml | Dilute in methanol, 1mM. |
| PMSF | Serine, Cysteine proteases | 1 mM | Dilute in ethanol. You can re-use the same aliquot. |
| EDTA | Metalloproteases that require Mg++ and Mn++ | 5 mM | Dilute in $dH_2O$, 0.5M. Adjust pH to 8.0. |
| EGTA | Metalloproteases that require Ca++ | 1 mM | Dilute in $dH_2O$, 0.5M. Adjust pH to 8.0. |
| Na Fluoride | Serine/Threonine phosphatases | 5-10 mM | Dilute in water. Do not re-use once defrosted. |
| Na Orthovanadate | Tyrosine phosphatases | 1 mM | Dilute in water. Do not re-use once defrosted. |

# ➢A. Sample preparation

## 3. Preparation of lysate (Ex. from tissues):

**1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.**

**2. Place the tissue in round-bottom microfuge tubes or Eppendorf tubes and immerse in liquid nitrogen to "snap freeze". Store samples at -80°C for later use or keep on ice for immediate homogenization.**


**Homogenize tissues with an electric homogenizer in a 1:10 w/v ratio (Ex. 10 mg tissue in 100 µl lysis buffer), using rounded bottom centrifugal tubes. Maintain constant agitation for 2 hours at 4°C (e.g place on an orbital shaker in the fridge). Volumes of lysis buffer must be determined in relation to the amount of tissue present (protein extract should not be too diluted to avoid loss of protein and large volumes of samples to be loaded onto gels. The minimum concentration is 0.1 mg/ml, optimal concentration is 1-5 mg/ml).**

**3. Centrifuge for 20 min at 12000 rpm at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice; discard the pellet.**

***The buffer (with inhibitors) should be ice-cold prior to homogenization.***

# ➢A. Sample preparation

## 4. Determination of protein concentration:

➢ Perform a Bradford assay, a Lowry assay or a BCA assay.
➢Bovine serum albumin (BSA) is a frequently-used protein standard.

➢ Once you have determined the concentration of each sample, you can freeze them at -20°C or -80°C for later use or prepare for loading onto a gel.

## 5. Preparation of samples for loading into gels:

➢ To denature proteins, use a loading buffer with SDS, and boil the mixture at 95-100°C for 5 minutes. Heating at 70°C for 5-10 minutes is also acceptable and may be preferable when studying multi-pass membrane proteins. These tend to aggregate when boiled and the aggregates may not enter the gel efficiently.

➢ The standard loading buffer is called 2X Laemmli buffer, to be mixed in a 1:1 ratio with the sample.

➢ When SDS is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. SDS denatures proteins by “wrapping around” the polypeptide backbone. SDS binds to proteins in a mass ratio of 1.4:1 conferring a negative charge to the polypeptide in proportion to its length.

# ➢A. Sample preparation

## 5. Preparation of samples for loading into gels (Cont.):

- **In denaturing SDS-PAGE separations, therefore, migration is determined by molecular weight.**

- **It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size by using ß-mercaptoethanol or dithiothreitol (DTT).**

- **Glycerol is added to the loading buffer to increase the density of the sample to be loaded and hence maintain the sample at the bottom of the well, restricting overflow and uneven gel loading.**

- **To enable visualization of the migration of proteins a small anionic dye molecule (e.g., bromophenol blue) is included in the loading buffer. Since 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.**

- **During protein sample treatment the sample should be mixed by vortexing before and after the heating step for best resolution.**

***Rule of thumb:*** ***Reduce and denature unless the datasheet specifies otherwise.***

# ➢B. Electrophoresis

➢ Electrophoresis can be one dimensional (i.e., one plane of separation) or two dimensional. One dimensional electrophoresis is used for most routine protein and nucleic acid separations. Two dimensional separation of proteins is used for finger-printing, and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell.

## 1. Preparation of PAGE gels:

➢ When separated on a polyacrylamide gel, the procedure is abbreviated as SDS-PAGE (for Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis). The technique is a standard means for separating proteins according to their molecular weight.

➢ Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N-methylenebisacrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with either DMAP or TEMED. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups.

➢The separation of molecules within a gel is determined by the relative size of the pores formed within the gel.

Acrylamide is a potent cumulative neurotoxin: wear gloves at all times.

# ➢B. Electrophoresis

## 1. Preparation of PAGE gels (cont.):

➢ The pore size of a gel is determined by two factors: the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases.

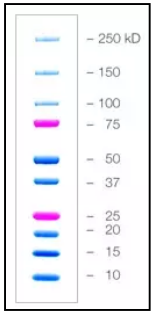
➢ Gels can be purchased ready-made (Ex. Nu-Page™ gels from Invitrogen), or produced in the laboratory (recipes can be found in laboratory handbooks). Either way, choose carefully the percentage of your gel as this will determine the rate of migration and degree of separation between proteins.

***Rule of thumb:*** ***The smaller the size of the protein of interest, the higher the percentage of mono/bis. The bigger the size of the protein of interest, the lower the percentage of mono/bis.***

| Protein size (kDa) | Gel percentage (%) |
|---|---|
| 4-40 | 20 |
| 12-45 | 15 |
| 10-70 | 12.5 |
| 15-100 | 10 |
| 25-200 | 8 |

# ➢B. Electrophoresis

## 2. Molecular weight markers:




➢ A range of molecular weight markers will enable the determination of the protein size and also to monitor the progress of an electrophoretic run.

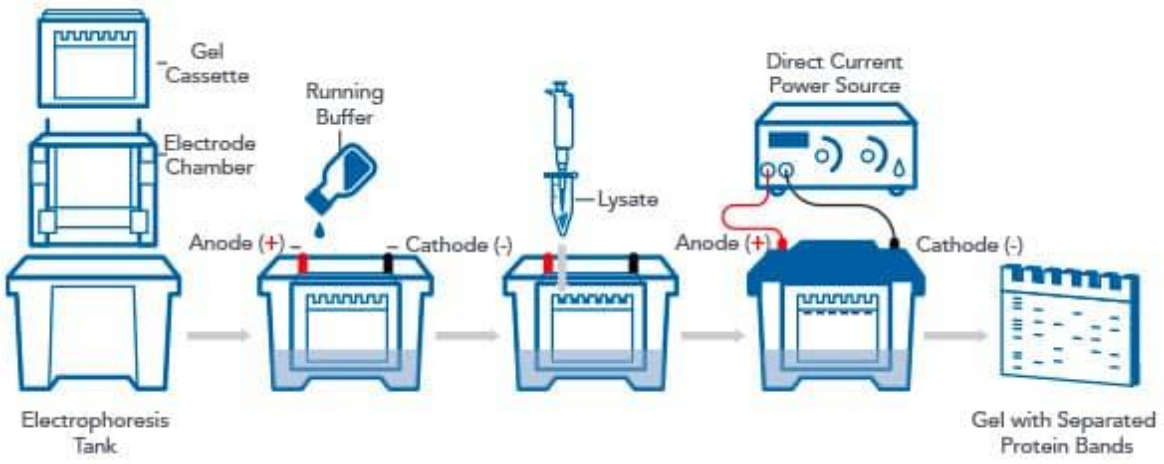
## 3. Loading samples and running the gel:

➢ Use special gel loading tips or a micro-syringe to load the complete sample in a narrow well. Take care not to poke the well bottom with the tip as this will create a distorted band.

➢ Never overfill wells. Load 20-40 µg total protein per mini-gel well.

➢ The gels will be submerged in migration/running buffer which normally contains SDS, except in native gel electrophoresis. Run the gel for the recommended time as instructed by the manufacturer; this can vary from machine to machine (1 hour to overnight depending on the voltage).

➢ When the dye molecule (the "migration front") reaches the bottom of the gel, the power is turned off. Proteins will slowly elute from the gel at this point, so do not store the gel; proceed immediately to transfer.

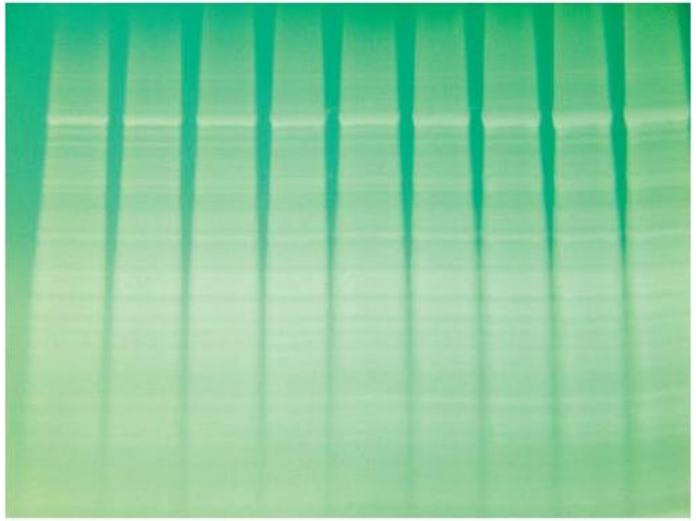
# ➢C. Transfer of proteins and staining (Western blotting)

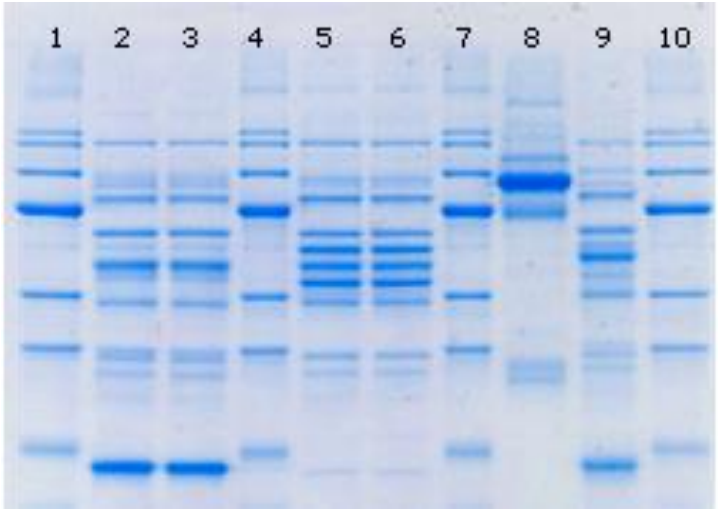
## 1. Visualization of proteins in gels:

➢ This visualization of protein at this stage is useful to determine if proteins have migrated uniformly and evenly.

➢Use the **copper stain** if you plan to transfer the separated proteins to a membrane, as the **Coomassie blue stain** is not reversible.

➢Only use the Coomasie stain on gels post-transfer to check the efficiency of the transfer, or if you have no plans to transfer and just want to observe the results of the SDS-PAGE separation.





# ➢C. Transfer of proteins and staining (Western blotting)

## 2. Transfer:

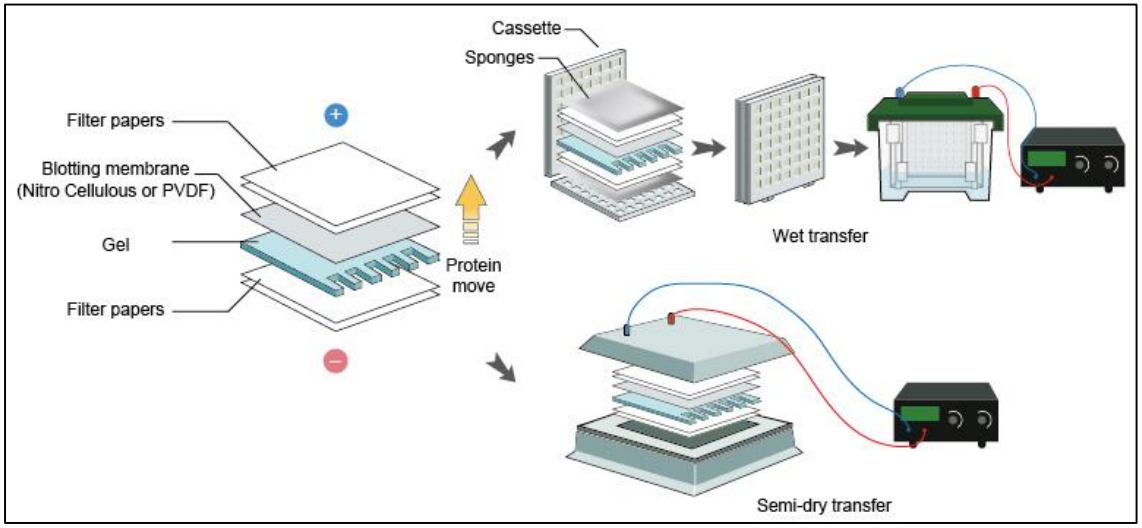
➢ Detailed instructions for the transfer process can be found on the websites of the manufacturers of transfer apparatus, and will vary depending on the system.

➢ Proteins with an electrical charge (provided by the SDS bound to them) can be transferred in an electrical field from the gel onto a sturdy support, a membrane that "blots" the proteins from the gel.

➢ Transfer can be done in wet or semi-dry conditions. Semi-dry transfer is generally faster but wet transfer is a less prone to failure due to drying of the membrane and is especially recommended for large proteins, >100 kD. For both kinds of transfer, the membrane is placed next to the gel. The two are sandwiched between absorbent materials, and the sandwich is clamped between solid supports to maintain tight contact between the gel and membrane.

➢ As for wet transfer, it is important that the membrane is closest to the positive electrode and the gel closest to the negative electrode.

Mini Trans-Blot cell.



# ➢C. Transfer of proteins and staining (Western blotting)

## 2. Transfer (cont.):

➢Two types of membranes are available: nitrocellulose and PVDF (positively-charged nylon). The choice is personal and both work very well.

➢The balance of SDS and methanol in the transfer buffer, protein size, and gel percentage can affect transfer efficiency.

➢Avoid touching the membrane with your fingers; use tweezers instead.

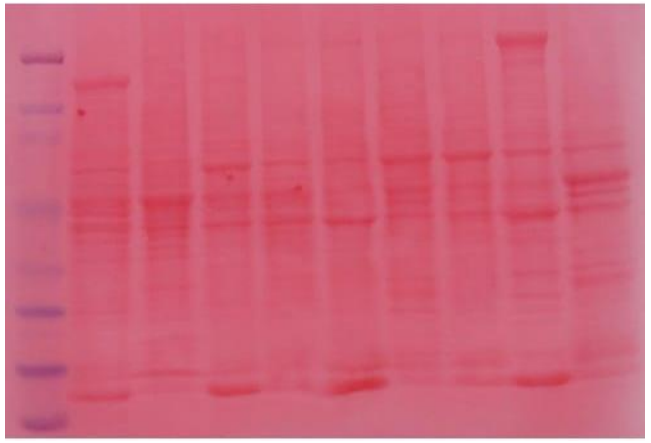
➢Remove air bubbles between the gel and membrane.

➢Make sure the paper and membrane are cut to the same size as the gel.

➢Chicken antibodies tend to bind PVDF and other nylon-based membranes, leading to high background. Switching to a nitrocellulose membrane should help reduce background staining.

# ➢C. Transfer of proteins and staining (Western blotting)

## 3. Visualization of proteins in membranes: Ponceau Red:



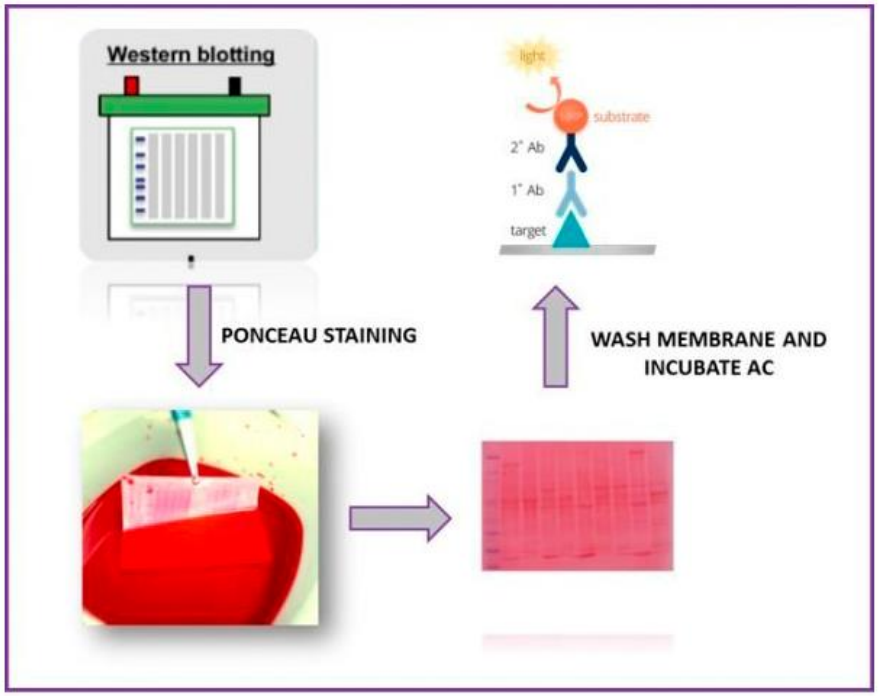
➢ To check for success of transfer, wash the membrane in TBST.

➢ Dilute the stock Ponceau Red 1:10. The stock is made of 2% Ponceau S in 30% trichloroacetic acid and 30% sulfosalicylic acid.

➢ Incubate on an agitator for 5 min. Wash extensively in water until the water is clear and the protein bands are well-defined.

➢ The membrane may be destained completely by repeated washing in TBST or water.

➢ When using a PVDF membrane, re-activate the membrane with methanol then wash again in TBST.



# ➢C. Transfer of proteins and staining (Western blotting)

## 4. Blocking the membrane:

➢**Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane (which has a high capacity at binding proteins and therefore antibodies).**

➢**Two blocking solutions are traditionally used: non-fat milk or BSA (Cohn fraction V). Milk is cheaper but is not recommended for studies of phospho-proteins (milk contains casein which is a phospho-protein; it causes high background because the phospho-specific antibody detects the casein present in the milk).**

➢**Some antibodies give a stronger signal on membranes blocked with BSA as opposed to milk for unknown reasons. Check the application notes on the datasheet in case there are specific instructions on how to block the membrane.**

➢**Incubate with blocking solution for 1 hour at 4°C under agitation. Rinse for 5 seconds in TBST after the incubation.**

# ➢C. Transfer of proteins and staining (Western blotting)

## 5. Incubation with the primary antibody:

### Incubation buffer:

➢ Dilute the antibody in TBST at the suggested dilution, if the datasheet does not have a recommended dilution try a range of dilutions (1:100-1:3000) and optimize the dilution according to the results. Too much antibody will result in non-specific bands.

➢ If high background is not an issue, some antibodies produce a much stronger signal if diluted in buffer with low concentrations (0.5 – 0.25%) of milk or BSA, or none at all.

### Incubation Time at 4°C with contineous agitation:

➢ The time can vary between a few hours and overnight (rarely more than 18 hours), and is dependent on the binding affinity of the antibody for the protein and the abundance of protein.

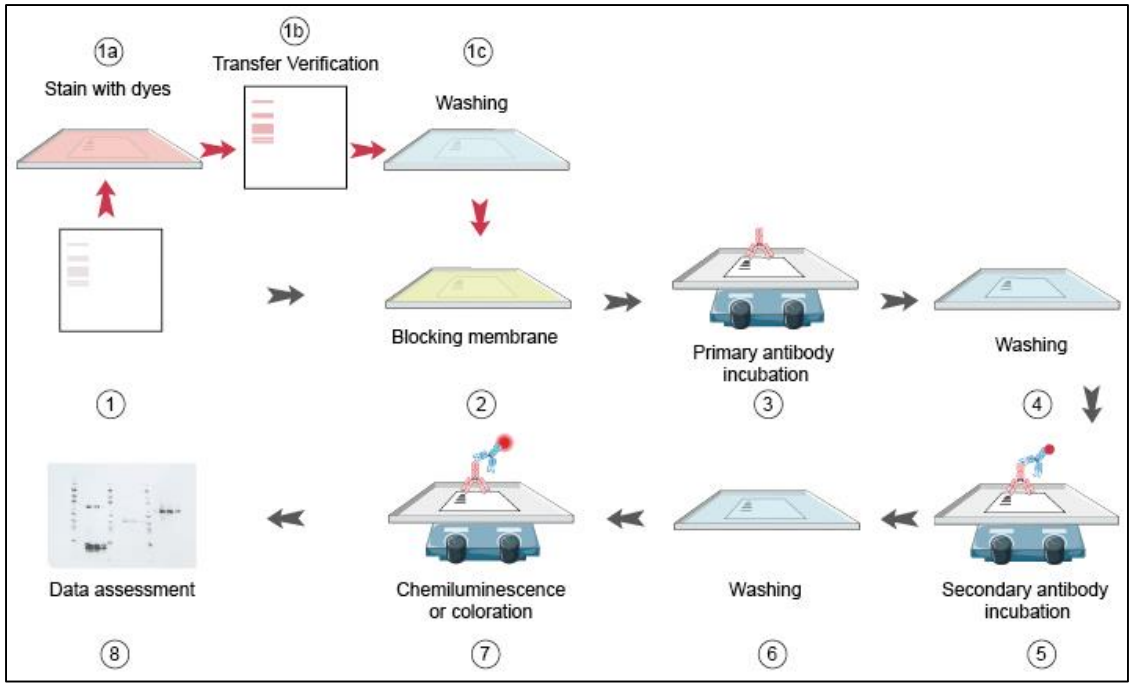
# ➢C. Transfer of proteins and staining (Western blotting)

## 6. Incubation with secondary antibody:

➢ Wash the membrane several times in TBST while agitating, 5 minutes or more per wash, to remove residual primary antibody.

➢ Dilute the antibody in TBST at the suggested dilution. If the datasheet does not have a recommended dilution, try a range of dilutions (1:1000- 1:20,000) and optimize the dilution according to the results. Too much antibody will result in non-specific bands.

Incubate with HRP-conjugated secondary antibodies at room temperature with continuous agitation for 1-2 hours.



# ➢C. Transfer of proteins and staining (Western blotting)

## 7. Development methods Detection kits:


**For HRP-conjugated antibodies: Use enhanced chemiluminescence (ECL) and ECL+ kits (home made or commercially available).**

**X-ray films:**
**Manual or automated film development in a dark room is traditionally used. Can control the incubation time of the x-ray film, but over-exposure may occur**


**Digital imagers to detect the chemiluminescence :**
**Using a CCD camera or fluorescence emanating from the membrane, transforming the signal into a digital image for rapid analysis with software provided with the detection machine. Ex. Li-cor image system**


**Digital imagers to detect the fluorescence :**
**Ex. The Odyssey Infrared Imaging System detects infrared fluorescence.**

# ➢ Stripping for reprobing western blots

➢ **Stripping is the removal of primary and secondary antibodies from a western blot membrane. Stripping is useful when one wants to investigate more than one protein on the same blot, for instance a protein of interest and a loading control (i.e. check equal loading).**

➢ **The procedure removes some sample protein from the membrane and may affect quantification.**

➢ ***A PVDF membrane is highly recommended to minimize loss of sample protein. Chemiluminescent reagents such as ECL are recommended as they will not leave a stain and are more sensitive than colorimetric reagents.***

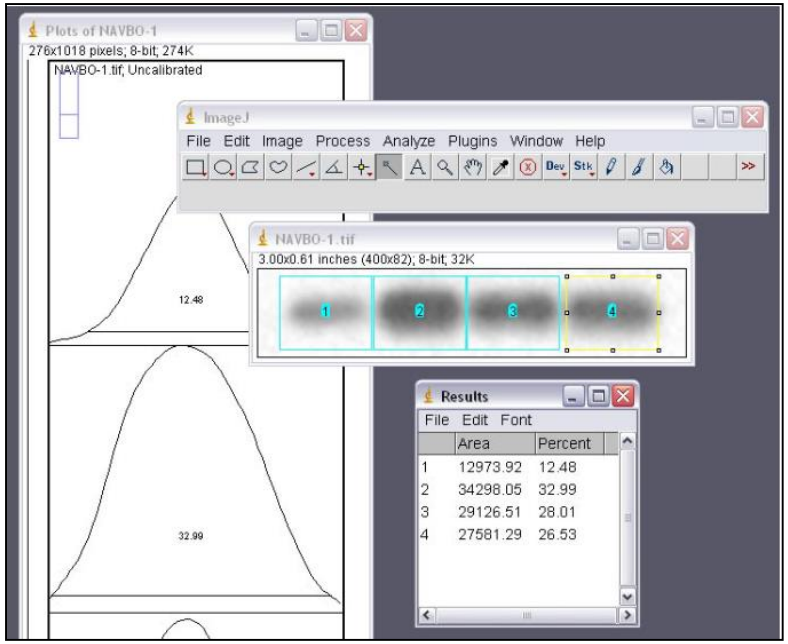
| Loading control | Sample type | Molecular weight (kDa) | Caution |
|---|---|---|---|
| Beta Actin | Whole Cell / cytoplasmic | 43 | Not suitable for skeletal muscle samples. Changes in cell-growth conditions and interactions with extracellular matrix components may alter actin protein synthesis (Farmer *et al*, 1983). |
| GAPDH | Whole Cell / cytoplasmic | 30-40 | Some physiological factors, such as hypoxia and diabetes, increase GAPDH expression in certain cell types. |
| Tubulin | Whole Cell / cytoplasmic | 55 | Tubulin expression may vary according to resistance to antimicrobial and antimitotic drugs (Sangrajrang S. *et al*, 1998, Prasad V *et al*, 2000) |
| VDCA1/Porin | Mitochondrial | 31 | |
| COXIV | Mitochondrial | 16 | Many proteins run at the same 16 kDa size as COXIV. |
| Lamin B1 | Nuclear | 66 | Not suitable for samples where the nuclear envelope is removed. |
| TATA binding protein TBP | Nuclear | 38 | Not suitable for samples where DNA is removed |

# ➢ Semi-qantification of western blots

**Using software such as:**

***1- Image-J (Image processing and analysis in Java)***

***2- Li-Cor Image Studio Lite***




# ➢ Detection of Phosphoproteins by WB

➢ Phosphorylation of proteins plays a vital role in cell signaling, oncogenesis, apoptosis, and immune disorders. Around a third of all eukaryotic gene products can be post-translationally phosphorylated.

➢The specific phosphorylation (by Kinases) of serine, threonine, or tyrosine residues is the most common mechanism for the regulation of cellular protein activity. The activity of protein kinases is regulated by various intracellular key signals, e.g., the concentration of cyclic AMP or Ca2+. Phosphatases catalyze the specific dephosphorylation of protein, allowing enzymes to switch between phosphorylated and dephosphorylated states.

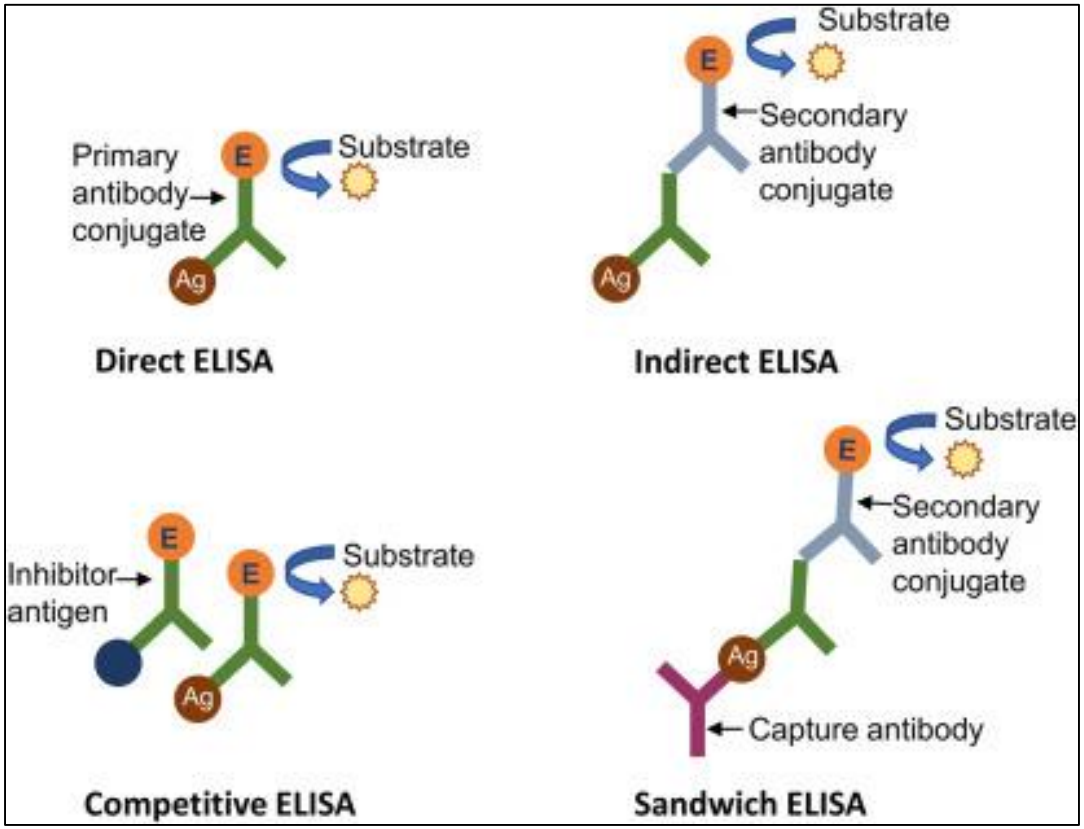
➢ Phosphoproteins can be detected by using SDS-PAGE followed by specific antibody to the phosphoprotein ( some proteins are phosphorylated at different sites)

➢ OR by using PhosphoProtein Purification Kits (affinity chromatography) to separate phospho and non-phospho prorteins followed by WB using non-phosphorylated antibody to detect both protein forms.

# ➢ Enzyme-linked immunosorbent assay (ELISA)

➢ **ELISA is a plate-based technique enabling the detection of antigens in biological samples.**

➢**In an ELISA, the antigen is immobilized to a solid surface either directly or more commonly via a capture antibody, itself immobilized to the surface. The surface is washed, then incubated with detection antibodies conjugated to molecules such as enzymes or fluorophores.**



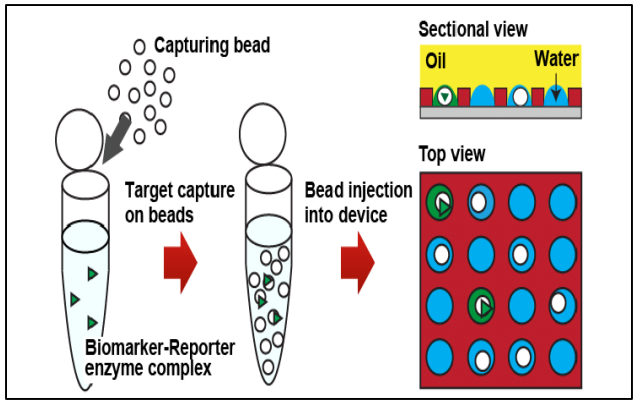
# ➢ ELISA vs. WB

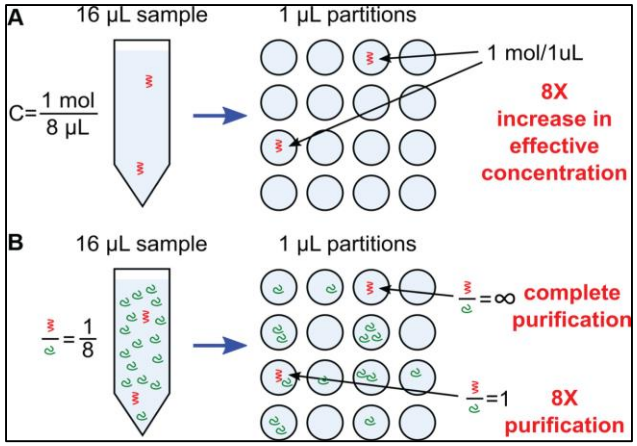
| ASPECT | ELISA | WESTERN BLOTTING |
|---|---|---|
| Principle | Immunoassay utilizing specific antibodies | Separation of proteins by gel electrophoresis |
| Target Detection | Soluble proteins, antigens, or antibodies | Soluble and membrane proteins |
| Detection Range | Low to high protein concentrations | Low to medium protein concentrations |
| Sensitivity | Highly sensitive, capable of detecting low levels | Moderate to high sensitivity |
| Specificity | High specificity with proper antibody selection | High specificity with specific antibody probes |
| Protein Size | No information about protein size | Information about protein molecular weight |
| Multiplexing | Multiple targets can be analyzed simultaneously | Limited multiplexing capability |
| Quantification | Quantitative measurement of protein concentration | Semi-quantitative estimation of protein levels |
| Sample Requirement | Small sample volumes required | Relatively larger sample volumes required |
| Time and Labor | Relatively faster and less labor-intensive | Time-consuming and labor-intensive |
| Cost | Cost effective | Costly |
| Equipment Requirement | Standard laboratory equipment | Specialized equipment (e.g., gel electrophoresis |

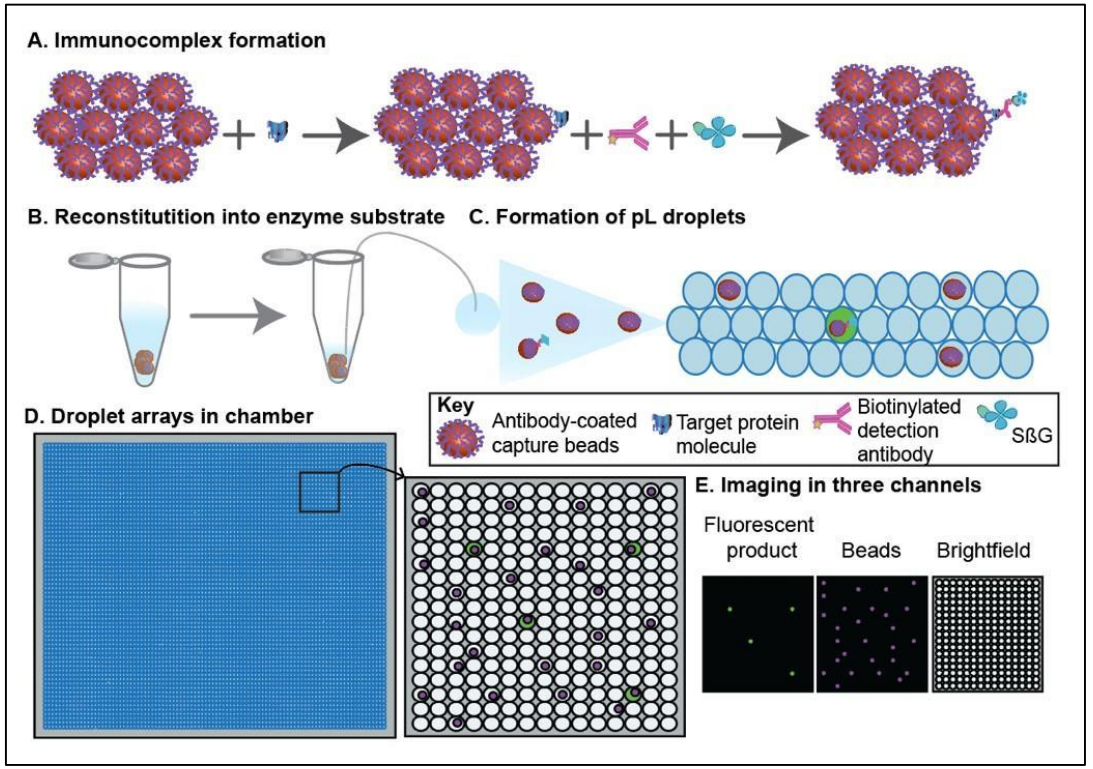
# ➢Digital ELISA

➢ **Digital ELISA was designed to be highly efficient in the capturing of target proteins, labeling of these proteins, and their detection in single molecule arrays (SiMoA).**

➢ **Compared to conventional ELISA, digital ELISA lowers the detection limits by almost 3 log to $10^{-16}$ M.**



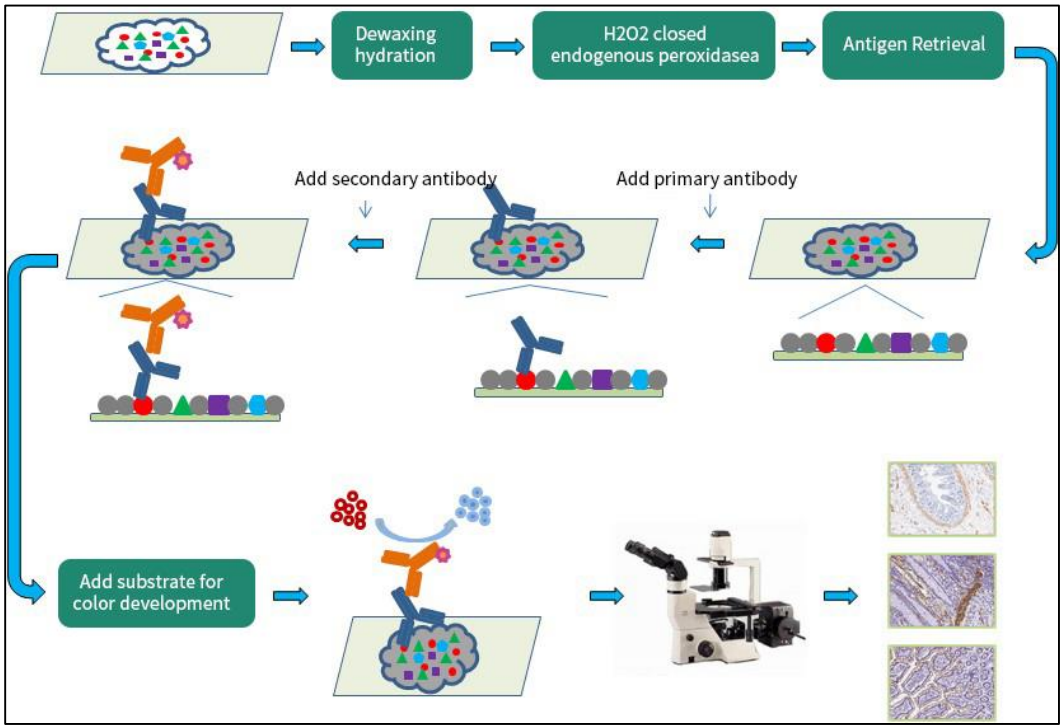



# ➢ Immunohistochemistry (IHC)

➢ **IHC combines histological, immunological and biochemical techniques for the identification of specific tissue components by means of a specific antigen/antibody reaction tagged with a visible label.**

➢**IHC makes it possible to visualize the distribution and localization of specific cellular components within a cell or tissue. The most popular type of IHC are chromogenic and fluorescence detection mediated by an enzyme or a fluorophore, respectively.**

➢ **Although less quantitative than western blot or ELISA, IHC offers the advantage of characterizing protein expression in the context of intact tissue.**

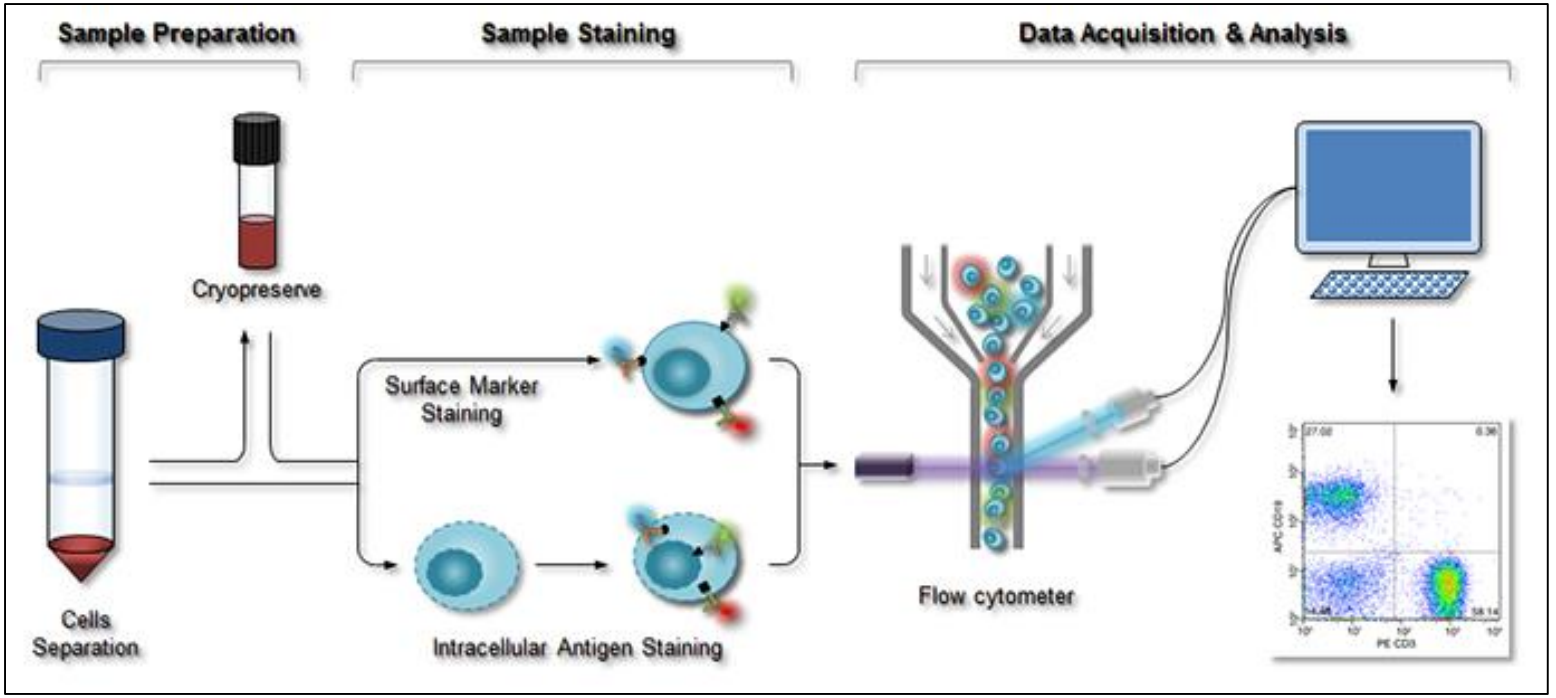


# ➢ Flow cytometry and FACS

➢ **Flow cytometry is a popular laser-based technology used to analyze the characteristics of cells or particles (not for tissues). The technique measures fluorescence emitted by labeled antibodies bound to individual cells in a mixed population. Also, the scattering of light by different cells is used to determine their size and properties.**

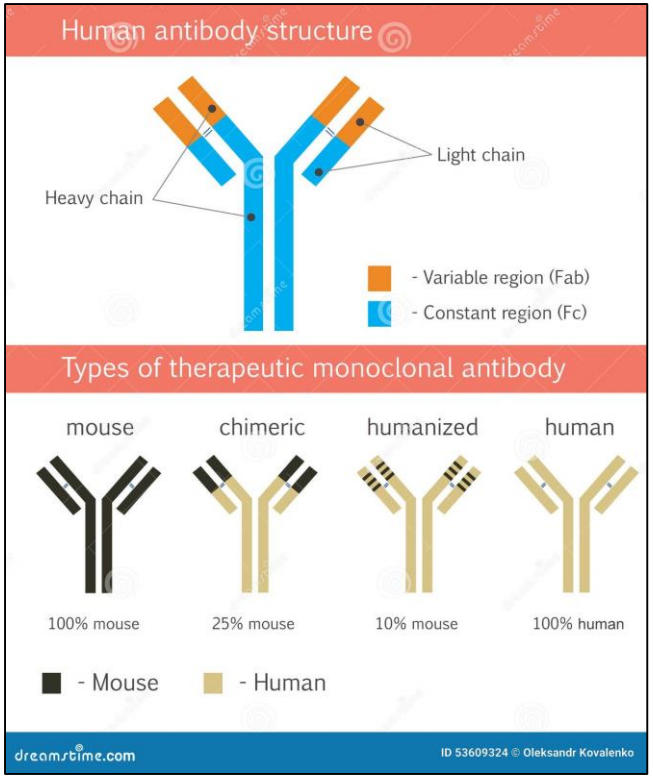
➢ **Flow cytometry enables you to analyze the expression of cell surface and intracellular molecules, characterize and define different cell types in a heterogeneous cell population, assess the purity of isolated subpopulations, and analyze cell size and volume. It allows simultaneous multi-parameter analysis of single cells.**

➢ **Fluorescence-activated cell sorting (FACS) is a derivative of flow cytometry that physically separates a population of cells into subpopulations based on fluorescent labeling.**

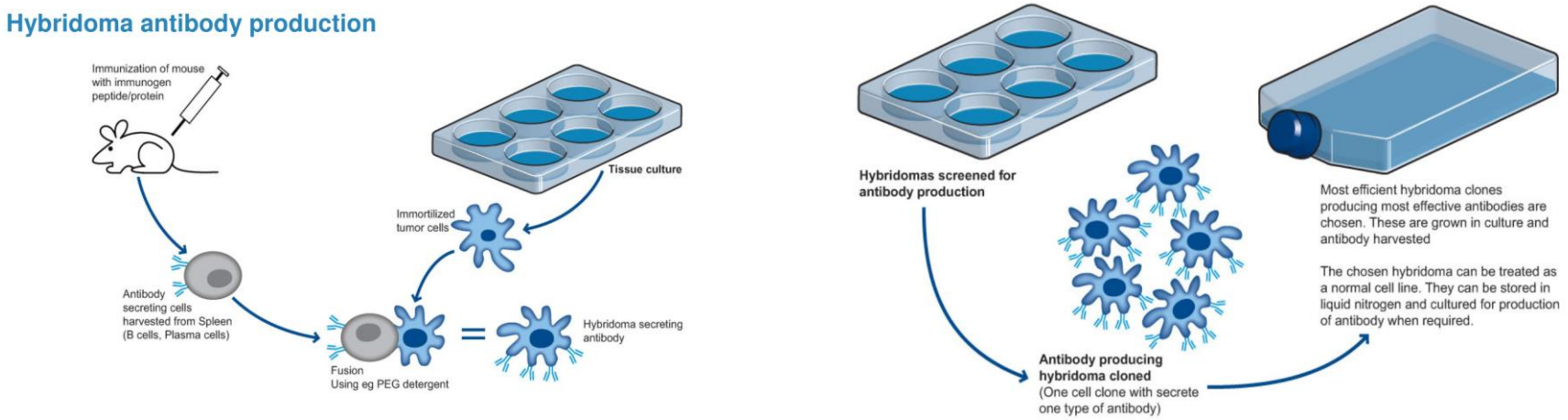


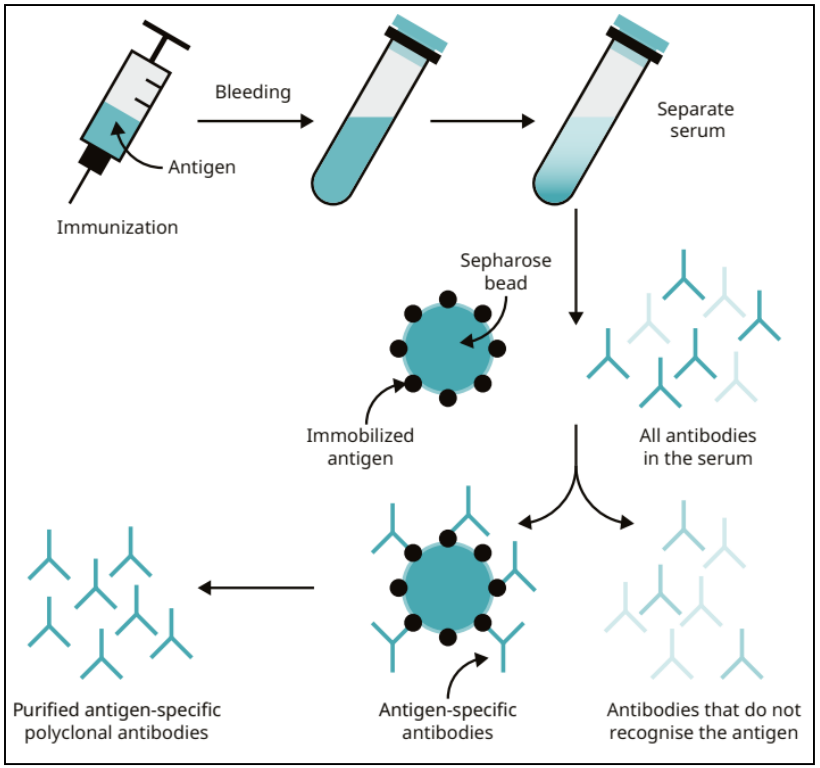
# ➢ Antibodies

| | Monoclonal | Polyclonal |
|---|---|---|
| **Production** | *in vitro* (hydridoma cells) | mostly *in vivo* |
| **Target** | one epitope | multiple epitopes |
| **Cost** | expensive | relatively cheap |
| **Technology** | highly complex | relatively easy |
| **Production time** | long (> 6 months) | short (2-3 months) |
| **Batch to batch** | identical | large variability |
| **Scale** | very large | limited |
| **Cross-reactivity** | very low | possible, depending on level of purification |



# ➢ Antibodies


Hybridoma antibody production
Immunization of mouse with immunogen peptide/protein
Tissue culture
Immortilized tumor cells
Antibody secreting cells harvested from Spleen (B cells, Plasma cells)
Fusion
Using eg PEG detergent
Hybridoma secreting antibody
Hybridomas screened for antibody production
Most efficient hybridoma clones producing most effective antibodies are chosen. These are grown in culture and antibody harvested
The chosen hybridoma can be treated as a normal cell line. They can be stored in liquid nitrogen and cultured for production of antibody when required.
Antibody producing hybridoma cloned
(One cell clone with secrete one type of antibody)


Bleeding
Antigen
Immunization
Separate serum
Sepharose bead
Immobilized antigen
All antibodies in the serum
Purified antigen-specific polyclonal antibodies
Antigen-specific antibodies
Antibodies that do not recognise the antigen

# ➢ Antibodies

**Checking the antibody's immunogen sequence alignment with your protein of interest.**

- **You can find antibody immunogen sequences using the UniProt/SwissProt protein database**

- **Take this immunogen sequence and compare it with the protein you're interested in using an online tool like CLUSTALW.**

- **An alignment score of over 85% indicates that the antibody may bind to your protein. However, this doesn't guarantee the antibody will perform well; you'll need to run several controls to ensure it works as intended.**

***Thank you***