SDS-PAGE e Western Blot

SDS-PAGE (PolyAcrylamide Gel Electrophoresis)

- **PAGE**: **PolyAcrylamide Gel Electrophoresis**, is a technique widely used in biochemistry,

  forensics, genetics and molecular biology:
- to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain / molecular weight and charge of the polypeptide).

SDS-PAGE (PolyAcrylamide Gel Electrophoresis)

- **SDS-PAGE**: **Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis**, is a technique widely used in biochemistry, forensics, genetics and molecular biology:

- to separate proteins according to their size, and no other physical feature.

…SDS-PAGE

- **SDS** (sodium dodecyl sulfate) is a detergent (soap) that can dissolve hydrophobic molecules but also has a negative charge (sulfATE) attached to it.

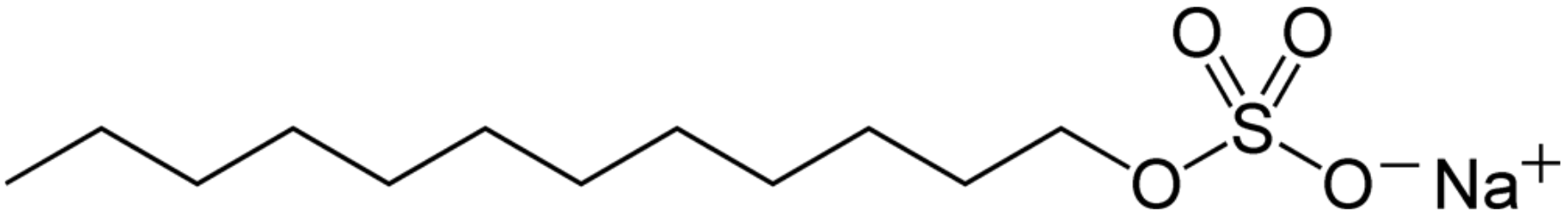
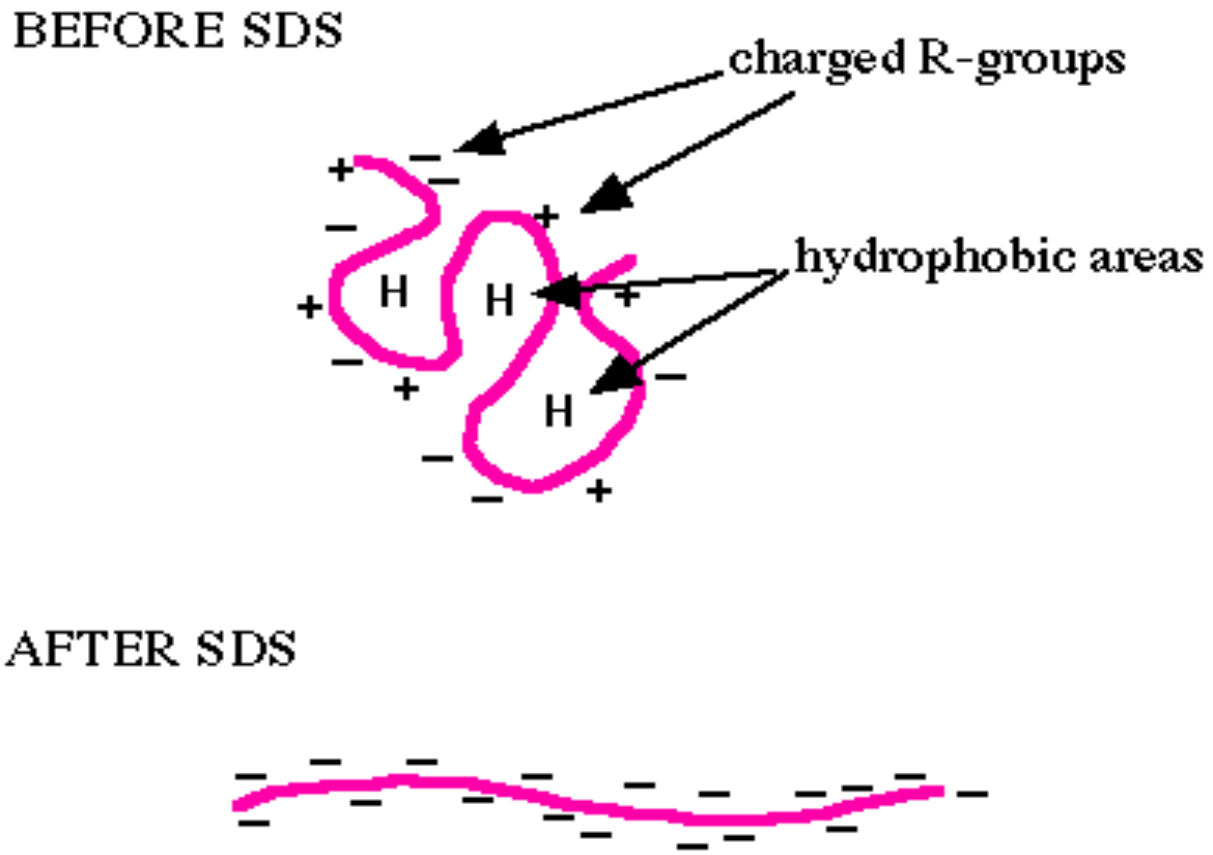


Fig.1Before SDS: Protein (pink line) incubated with the denaturing detergent SDS showing negative and positive charges due to the charged R-groups in the protein.
The large H's represent hydrophobic domains where nonpolar R-groups have collected in an attempt to get away from the polar water that surrounds the protein.
After SDS: SDS disrupt hydrophobic areas (H's) and coat proteins with many negative charges which overwhelms any positive charges the protein had due to positively charged R-groups.
The resulting protein has been denatured by SDS (reduced to its primary structure-aminoacid sequence) and as a result has been linearized.



..SDS

- SDS (the detergent soap) breaks up hydrophobic areas and coats proteins with negative charges thus overwhelming positive charges in the protein.
- The detergent binds to hydrophobic regions in a constant ratio of about 1.4 g of SDS per gram of protein.

..SDS

- Therefore, if a cell is incubated with SDS, the membranes will be dissolved, all the proteins will be solubalized by the detergent and all the proteins will be covered with many negative charges.

PAGE

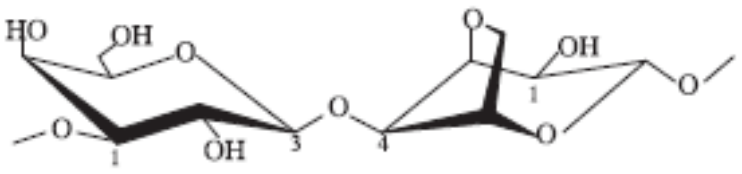
- If the proteins are denatured and put into an electric field (only), they will all move towards the positive pole at the same rate, with no separation by size.
- However, if the proteins are put into an environment that will allow different sized proteins to move at different rates.
- The environment is polyacrylamide.
- the entire process is called **polyacrylamide gel electrophoresis** (PAGE).

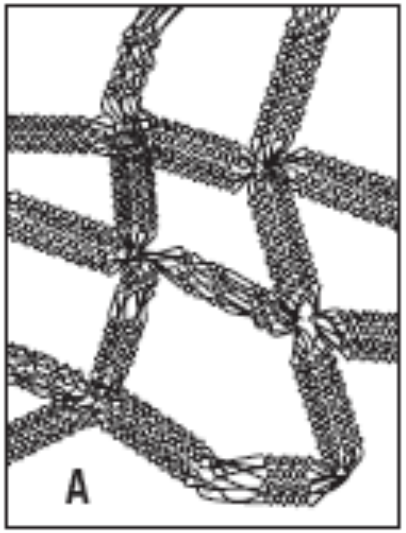
Gels

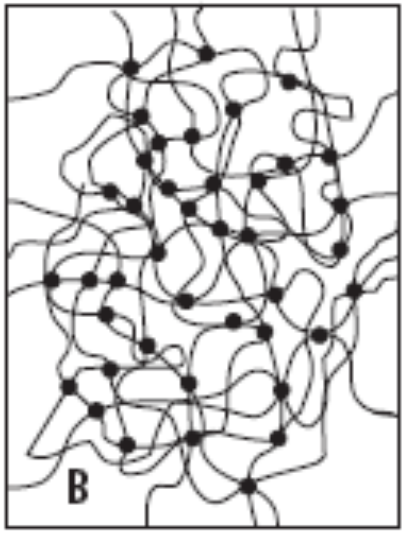
acrylamide

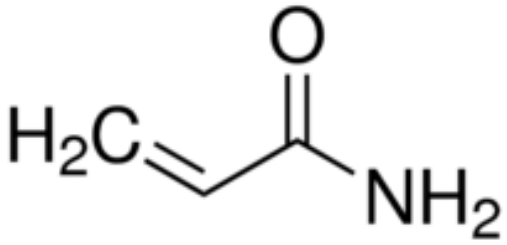
$H_2C = CH\text{-}C(=O)\text{-}NH_2$

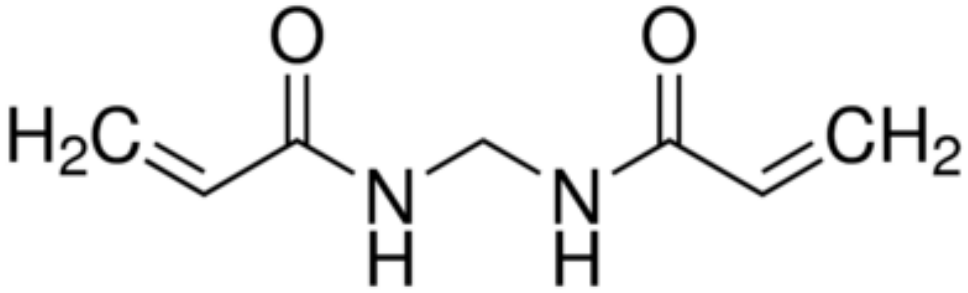
agarose











Polymerization, T%, C%

$$\%T = \frac{g(\text{acrylamide} + \text{bisacrylamide})}{100\ ml} \times 100$$

$$\%C = \frac{g(\text{bisacrylamide})}{g(\text{acrylamide} + \text{bisacrylamide})} \times 100$$

..PAGE

- Small molecules move through the polyacrylamide forest faster than big molecules.
- Big molecules stays near the well.

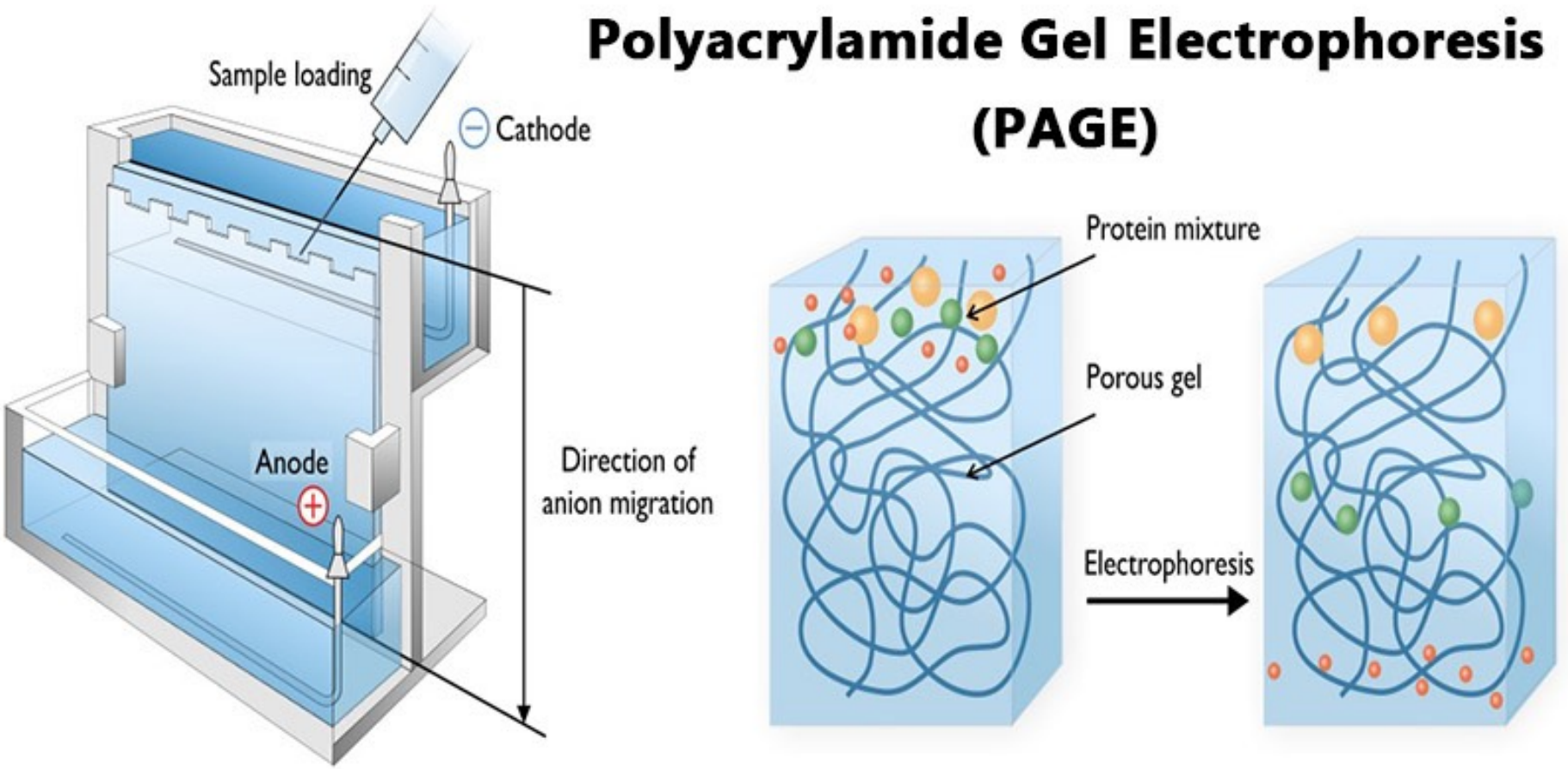
…SDS-PAGE

- The end result of SDS- PAGE has two important features:

1) all proteins contain only primary structure &

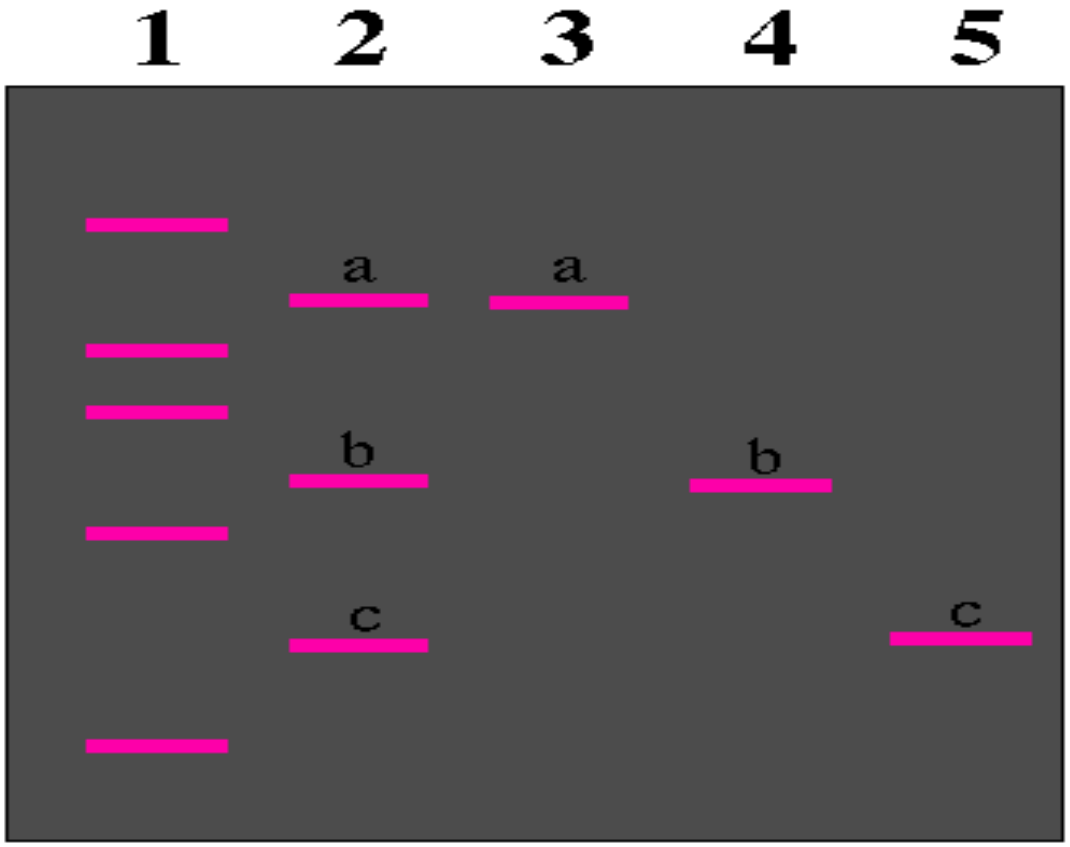
2) all proteins have a large negative charge which means they will all migrate towards the positive pole when placed in an electric field.

Effetto "setaccio" in un gel uniforme



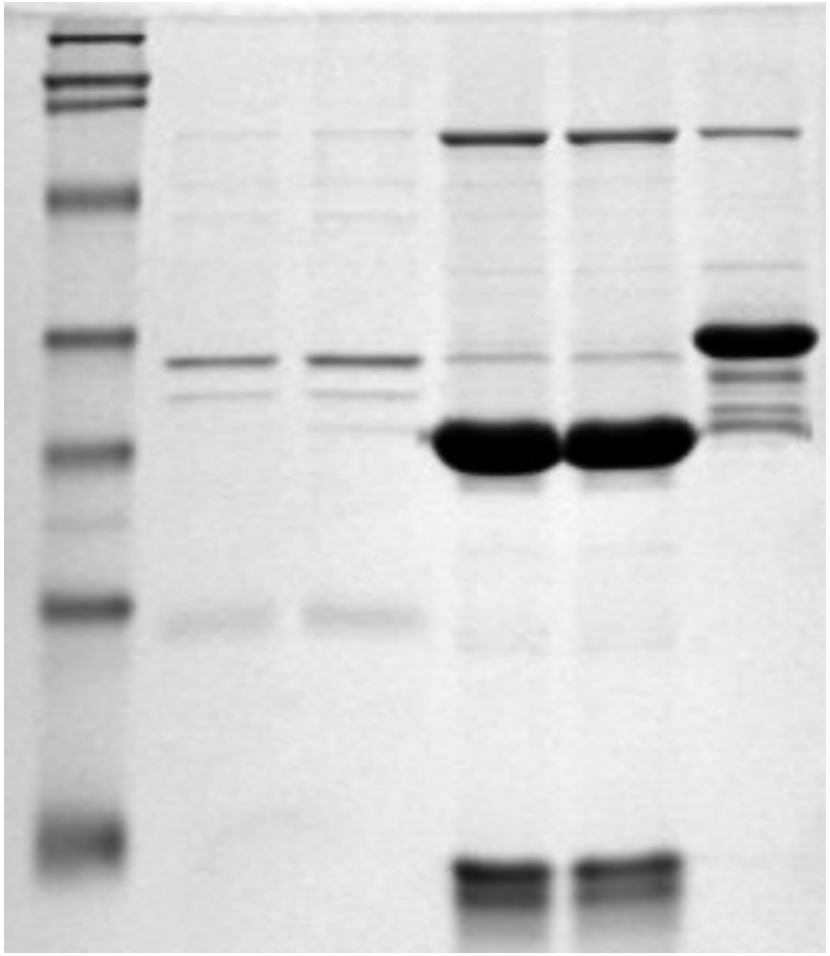
SDS-PAGE

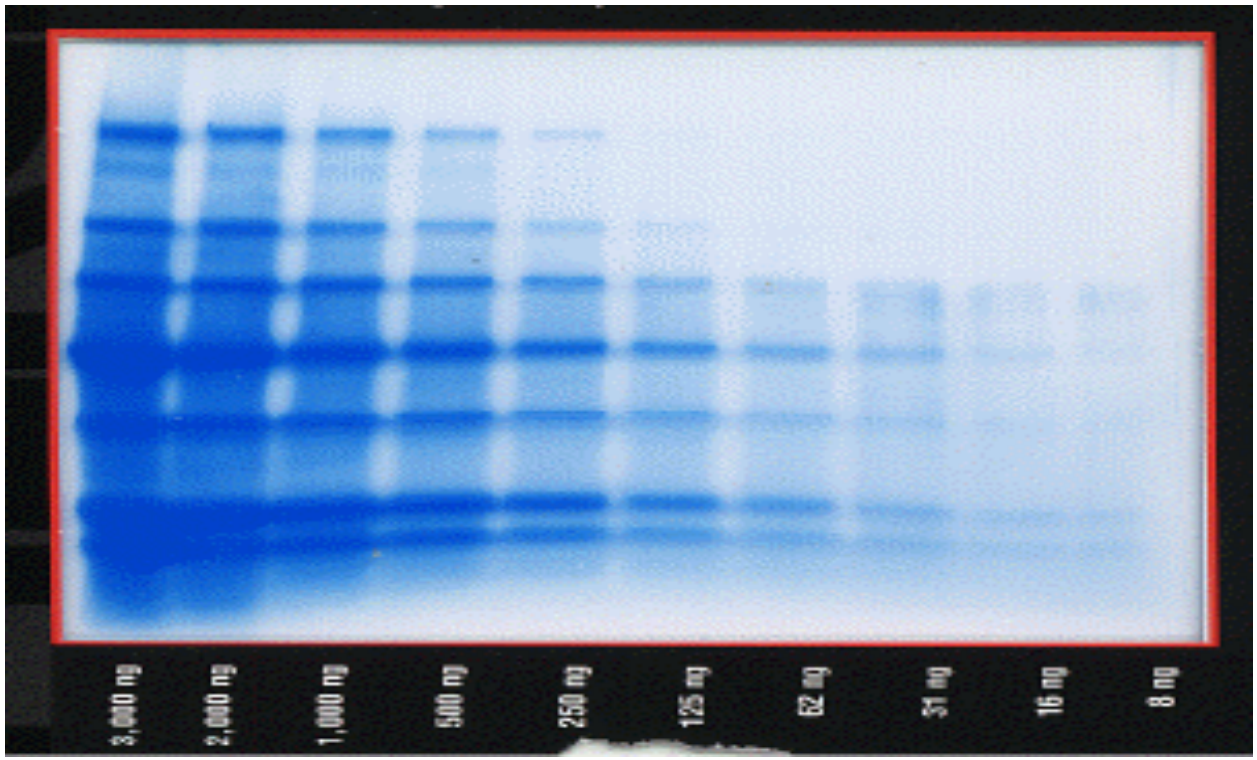
(SDS-PolyAcrilamide Gel Electrophoresis)



- The actual bands are equal in size, but the proteins within each band are of different sizes.

Sample of SDS- PAGE





Protein gel (SDS-PAGE) that has been stained with Coomassie Blue.

What happens after electrophoresis?

- 1. Fix the proteins in the gel and them stain them.
- 2. Electrophorectic transfer to a membrane and then probe with antibodies- (Western blotting) (Refer Western Blot first few slides)

..Western blotting

- Western blot analysis can detect **one** protein in a mixture of any number of proteins while giving you information about the size of the protein.
- This method is, however, dependent on the use of a high-quality antibody directed against a desired protein.
- This antibody is used as a probe to detect the protein of interest.

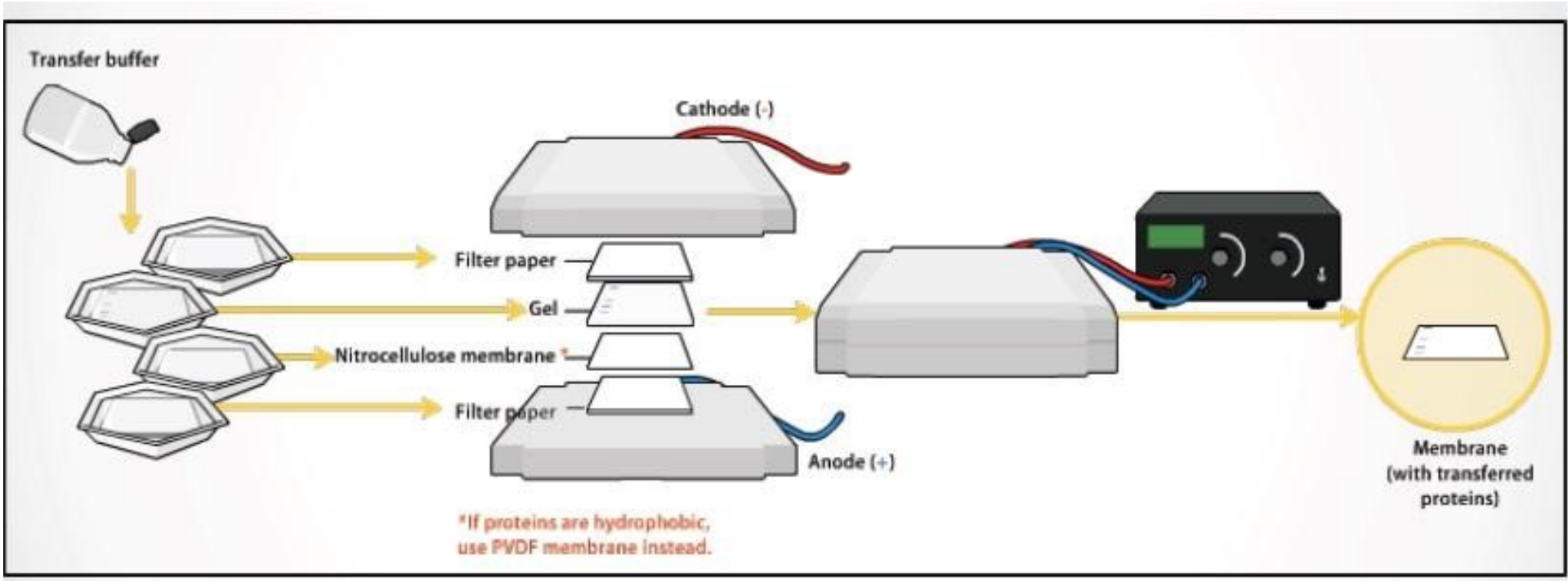
Western Blot followed by SDS

- Proteins are separated using SDS-polyacrylamide gel electrophoresis which separates proteins by size.
- Nitrocellulose membrane is placed on the gel. The actual blotting process may be active (electroblotting) or passive (capillary).
- Electroblotter is used for faster and more efficient transfer of protein from gel to membrane
- Sandwich of filter paper, gel, membrane and more filter paper is prepared in a cassette, which is placed between platinum electrodes.
- An electric current is passed through the gel causing the proteins to electrophorese out of the gel and onto the nitorcellulose membrane.

Terminologies..

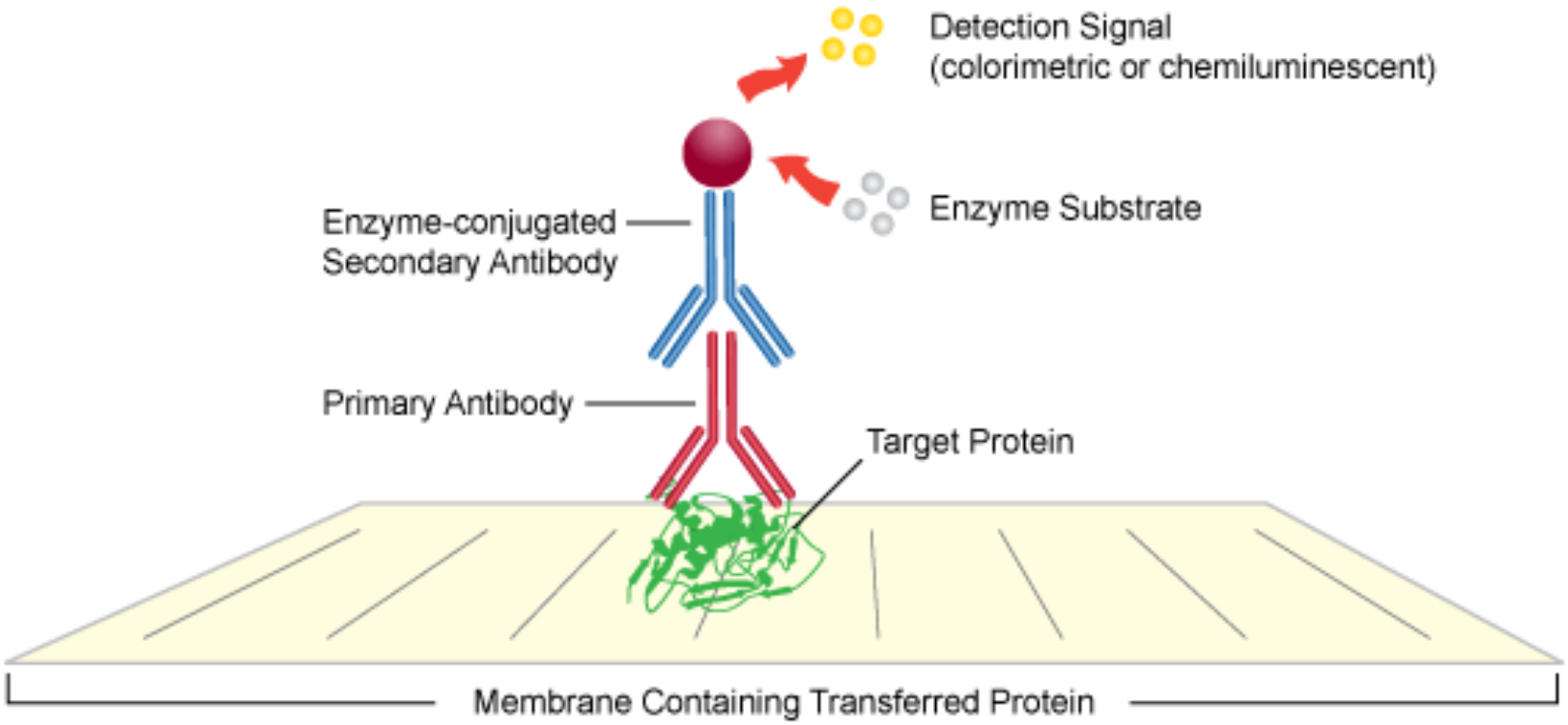
- The **Western blot** (alternatively, **protein immunoblot**) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract.
- A **Southern blot** is a method routinely used in molecular biology for detection of a specific DNA sequence in DNA samples.
- The **northern blot** is a technique used in molecular biology research to study gene expression by detection of RNA.
- **Southwestern blotting**, based along the lines of Southern blotting (which was created by Edwin Southern) and first described by B. Bowen and colleagues in 1980, is a lab technique which involves identifying and characterizing DNA-binding proteins (proteins that bind to DNA).

Western Blot



Sviluppo di un Western Blot

Anticorpi secondari e/o marcati



Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine (Fatto)
- Legame dell'anticorpo primario

Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine
- Legame dell'anticorpo primario (30 - 60 minuti / 4° - 20° - 37°C)

??? In cosa diluisco l'anticorpo????

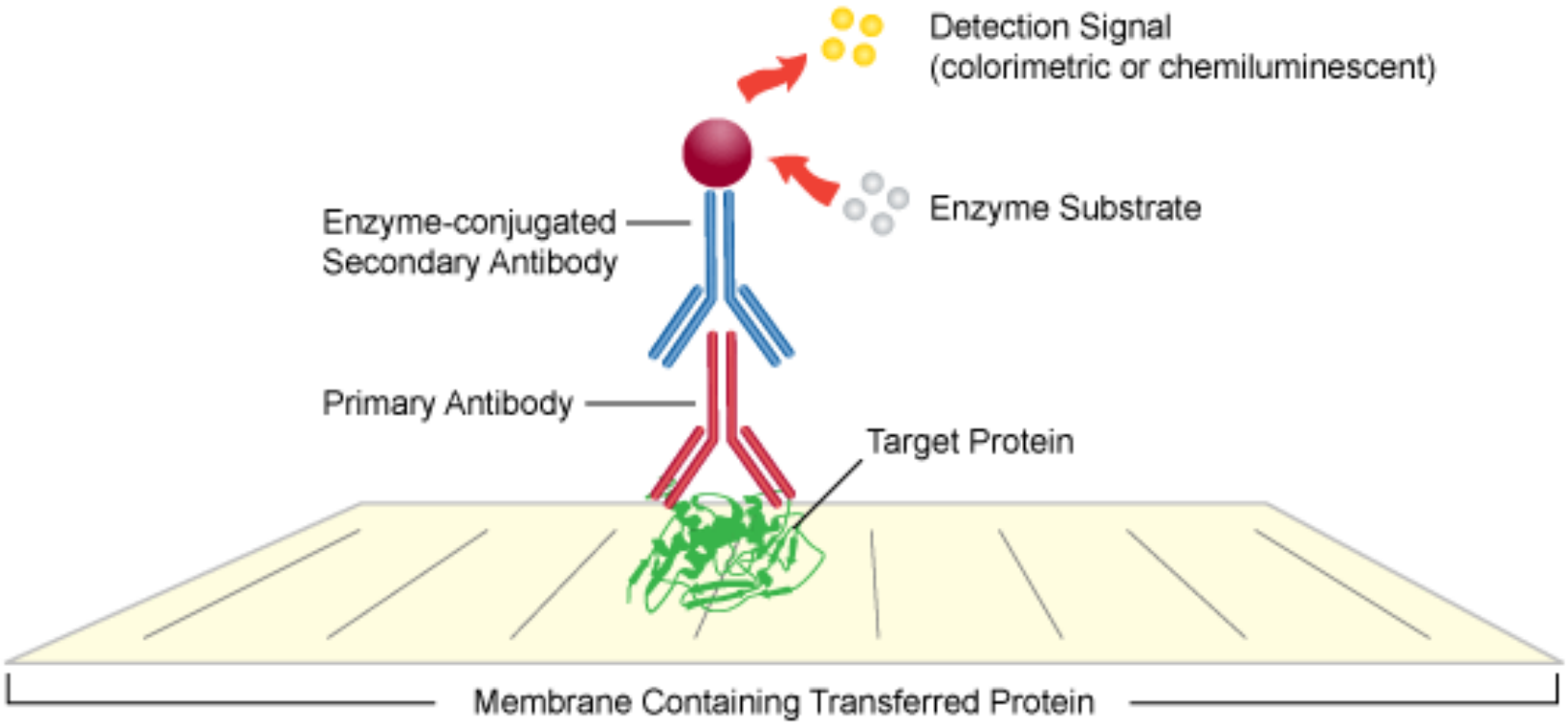
Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine (Fatto)
- Legame dell'anticorpo primario
- Lavaggio per eliminare gli anticorpi non legati (3 x 3 minuti)

??? Con cosa lavo le membrane ????

Sviluppo di un Western Blot

Anticorpi secondari e/o marcati



Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine (Fatto)
- Legame dell'anticorpo primario
- Lavaggio per eliminare gli anticorpi non legati
- Legame dell'anticorpo secondario (30-60 minuti)

Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine (Fatto)
- Legame dell'anticorpo primario
- Lavaggio per eliminare gli anticorpi non legati
- Legame dell'anticorpo secondario
- Lavaggio per eliminare gli anticorpi secondari non legati (3 x 3 minuti)

Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine (Fatto)
- Legame dell'anticorpo primario
- Lavaggio per eliminare gli anticorpi non legati
- Legame dell'anticorpo secondario
- Lavaggio per eliminare gli anticorpi secondari non legati
- Sviluppo con substrato precipitante

Sviluppo di un Western Blot

- Blocco dei siti della membrana non impegnati nel trasferimento delle proteine (Fatto)

- Legame dell'anticorpo primario (40 minuti)

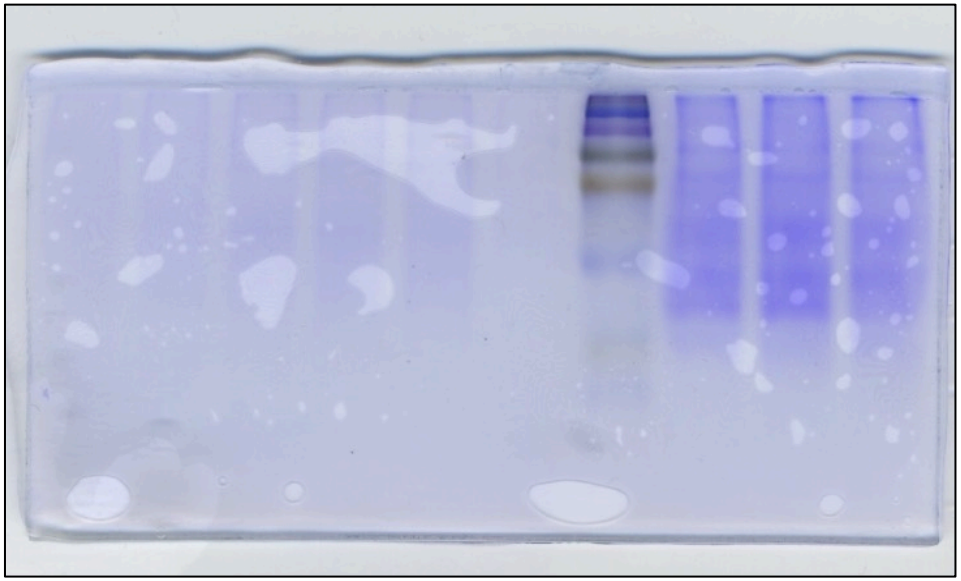
- Lavaggio per eliminare gli anticorpi non legati (10 minuti)

- Legame dell'anticorpo secondario (40 minuti)

- Lavaggio per eliminare gli anticorpi secondari non legati (10 minuti)

- Sviluppo con substrato precipitante (10 minuti)

SDS-PAGE



1 2 3 4 5 6 7 8 9 10

Western Blot