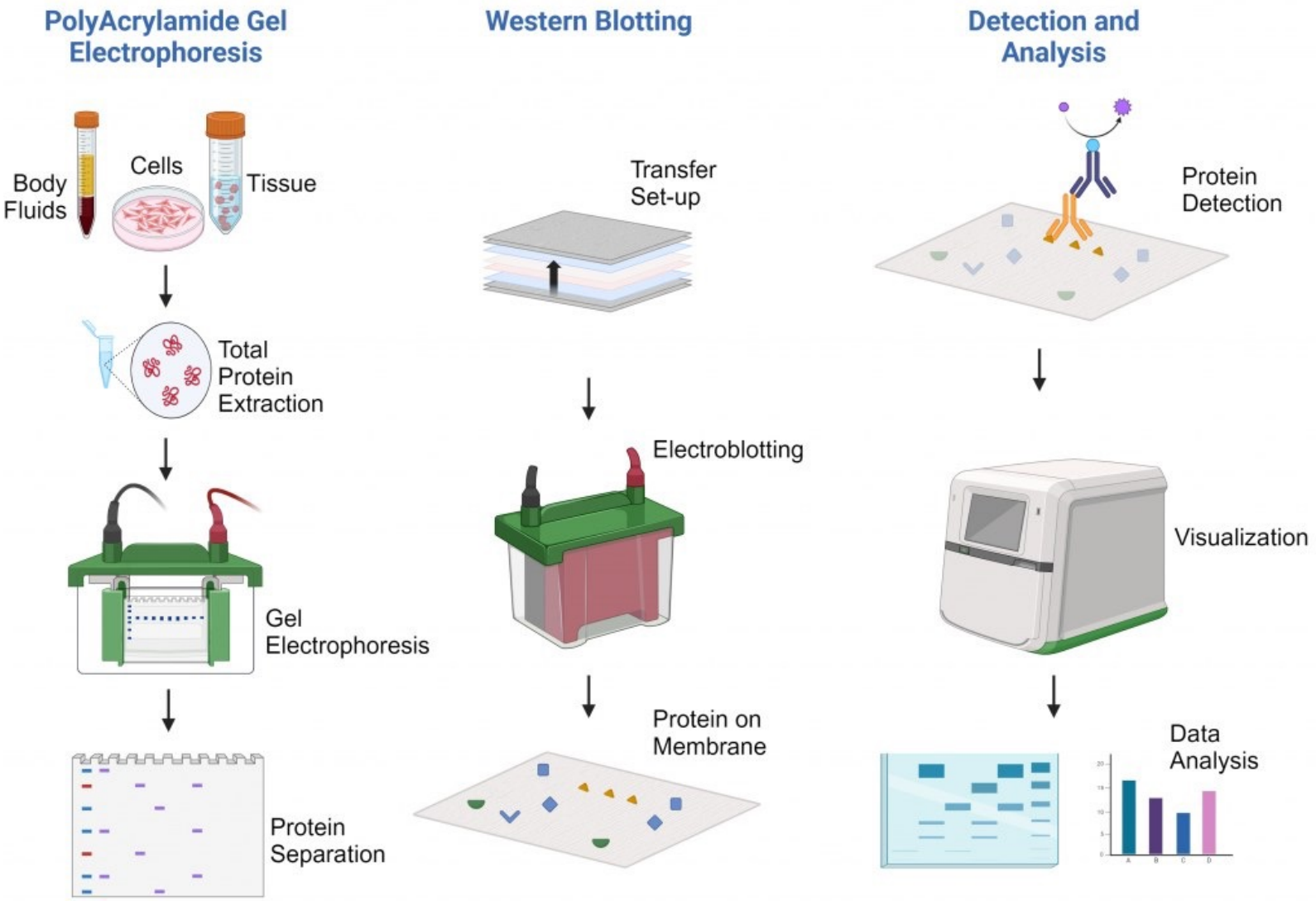
Western blot



Protein Extraction

- **Steps:**

  -Cell lysis in conditions ensuring:

  Membrane break, protein dissociation (strenght depending on the analysis), protease inhibition.

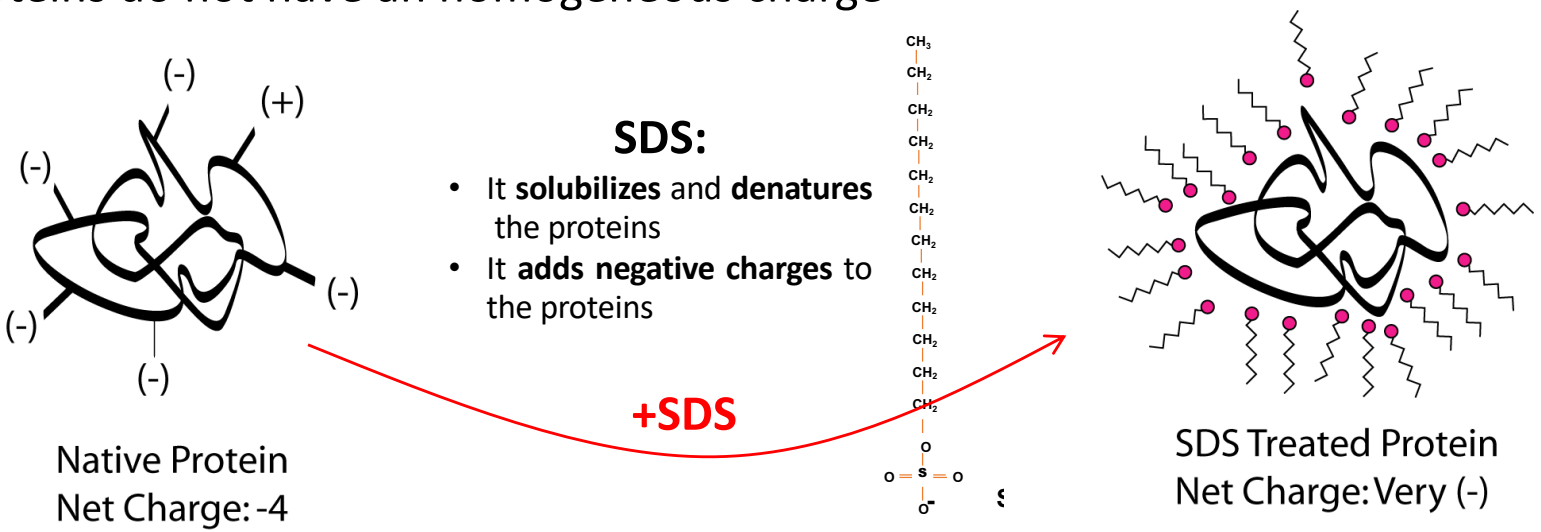
  -Spectrophotometric determination (concentration).

Why Polyacrylamide Gel?

<u>Polyacrylamide gels have a denser texture</u>
The pores have smaller dimensions than in agarose gels
Proteins are much smaller than DNA
Average amino acid = 110 Da
Average nucleotide = 300 Da
1 kilobase of DNA = 650 kDa
1 kilobase of DNA codes for 333 amino acids = 36 kDa

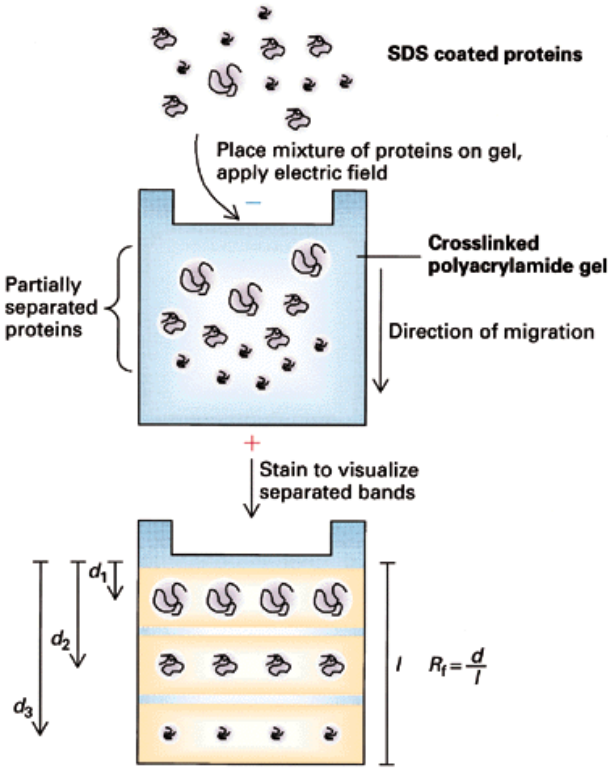
How can we make proteins separable **only** on the basis of **molecular weight?**

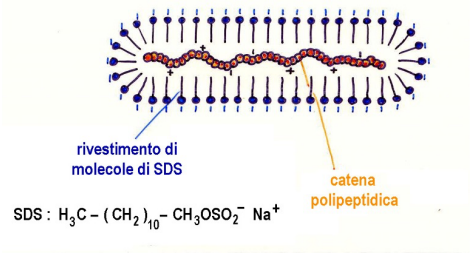
Proteins do not have an homogeneous charge



SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

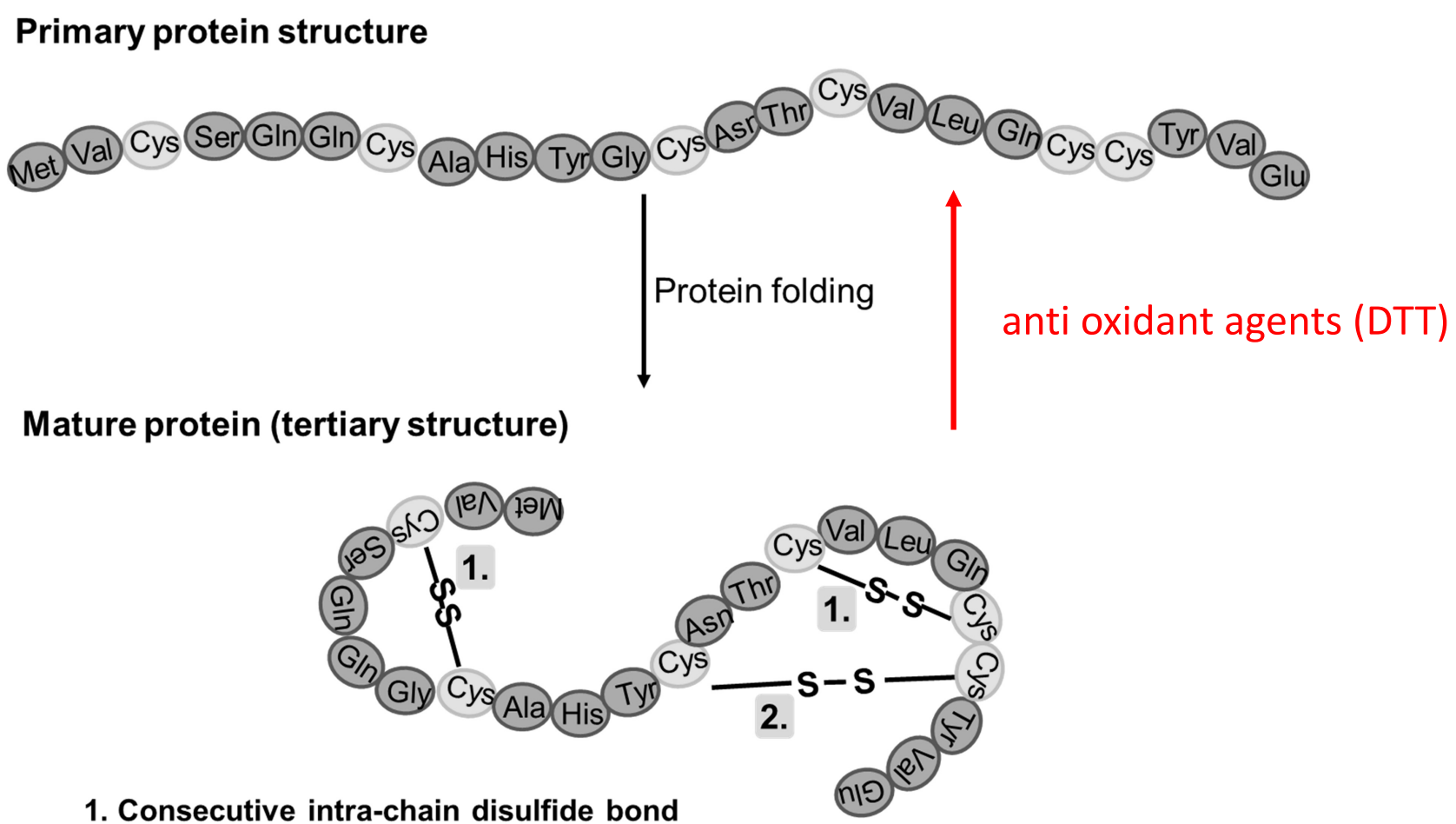
- Once negatively charged, all proteins run toward +
- The smaller are the proteins, the faster they run
- Denaturing conditions allow proteins to move according to their size only, no influence from conformation





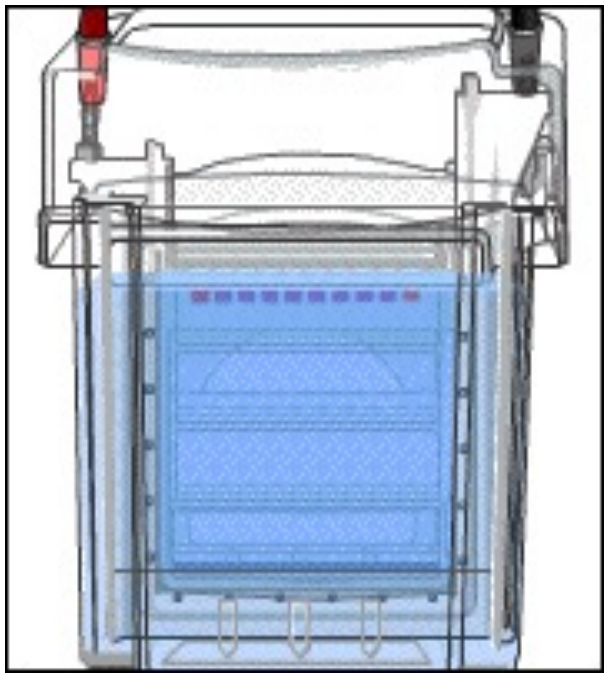
Anionic detergent tightly binding proteins in a fixed ratio (1 mol SDS/2 mol aminoacids)

Eliminate the S-S Disulfide bonds



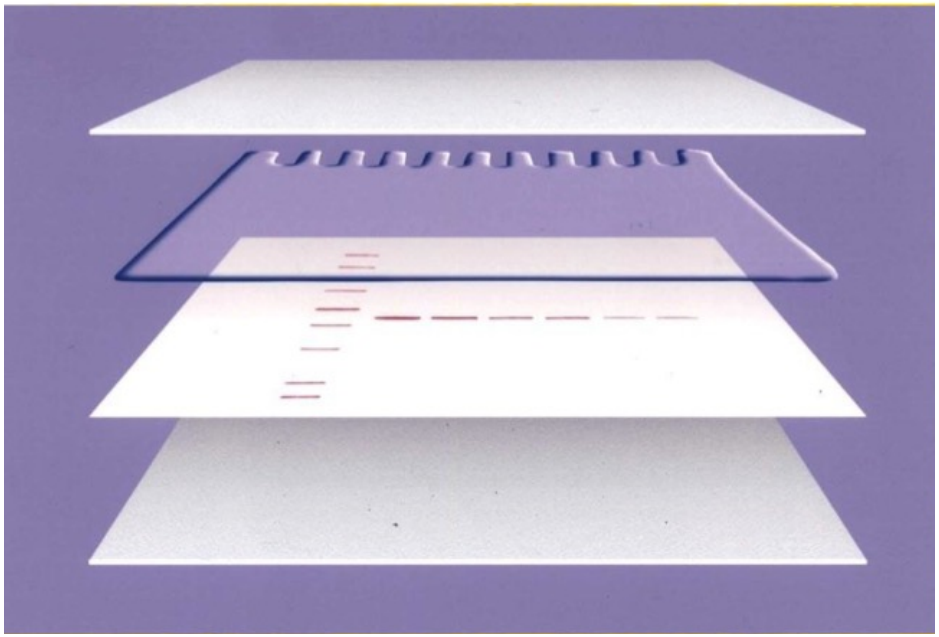
SDS-PAGE

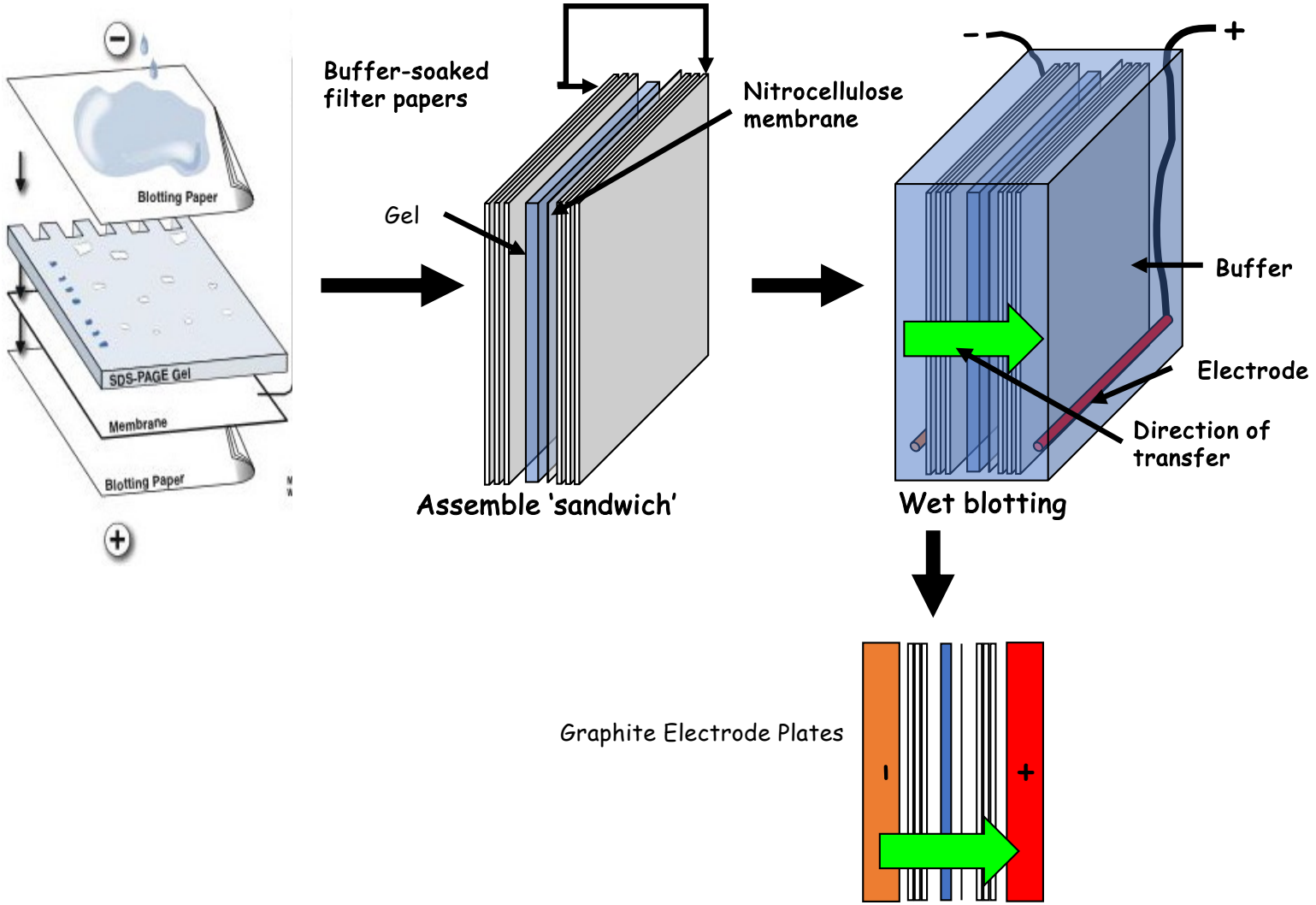
acrilammide/bis-acrilammide 29:1 (6-20%)+ SDS 0.1%



Colorazione con Blu di Coomassie

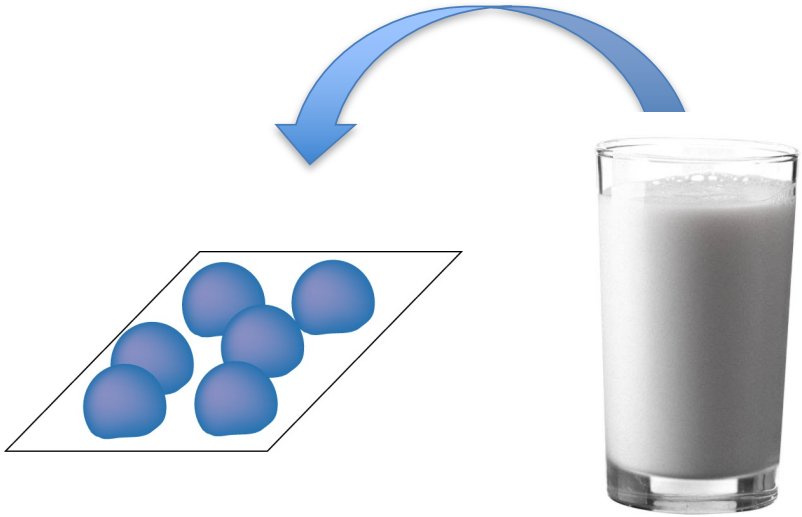
Blot




Blotting Paper
SDS-PAGE Gel
Membrane
Blotting Paper
Buffer-soaked filter papers
Gel
Nitrocellulose membrane
Assemble 'sandwich'
Buffer
Electrode
Direction of transfer
Wet blotting
Graphite Electrode Plates

Blocking

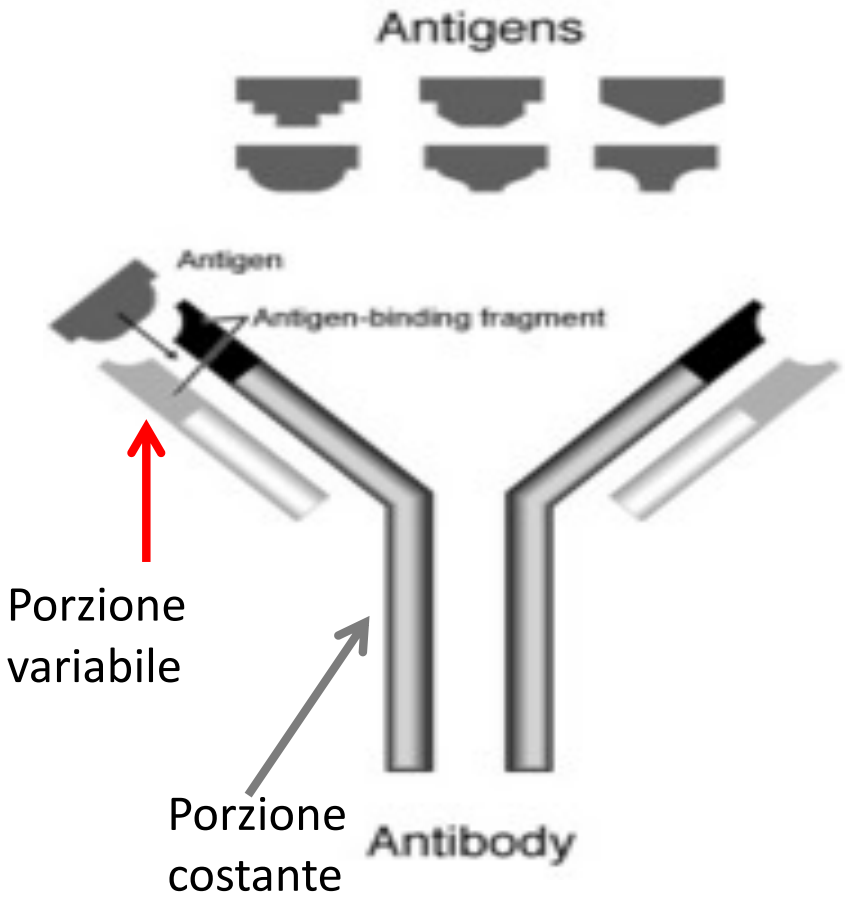
- Saturation of free hydrophobic spots on the membrane
- Avoids unspecific binding of primary Ab to the membrane
- Skimmed milk or Bovin Serum Albumin used



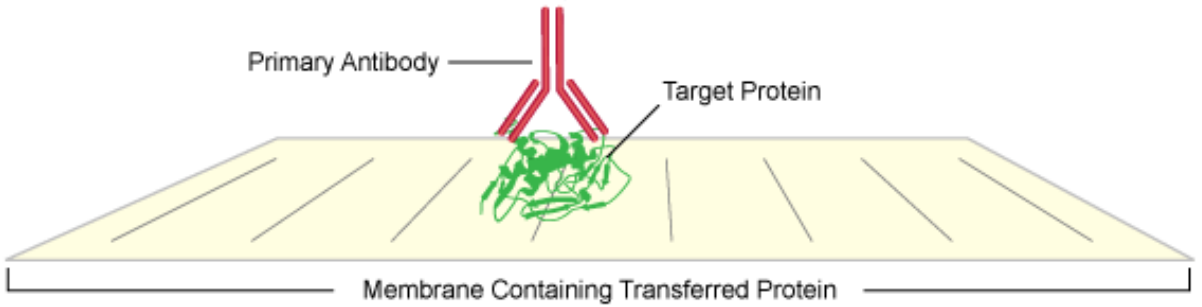
This reduces **background leading to clearer results**, and eliminates false positives

Antibodies

- **Antibodies (Immunoglobulins, Ig)**
- A Y-shaped protein secreted into the blood in response to a specific antigen, such as a bacterium or a virus, which neutralizes the antigen by specifically binding to it and eliciting an immune response.



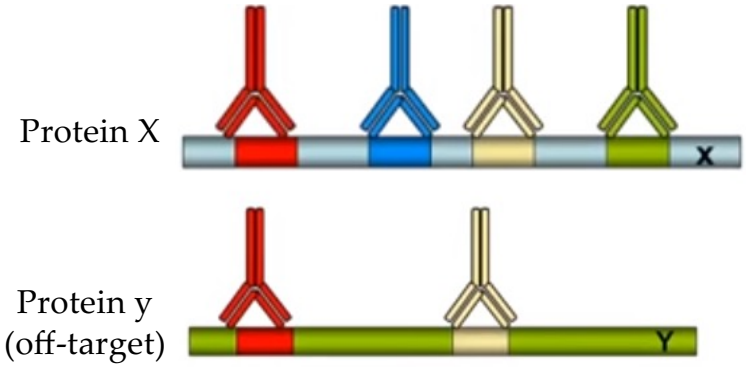
Antibody binding



Antibodies

polyclonal

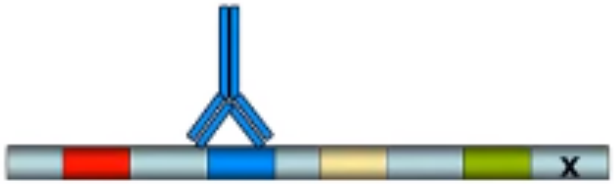
Specific to multiple epitopes on a single antigen



Pro: Signal amplification
Cons: non-specific interactions

monoclonal

Specific to one epitope on a particular antigen

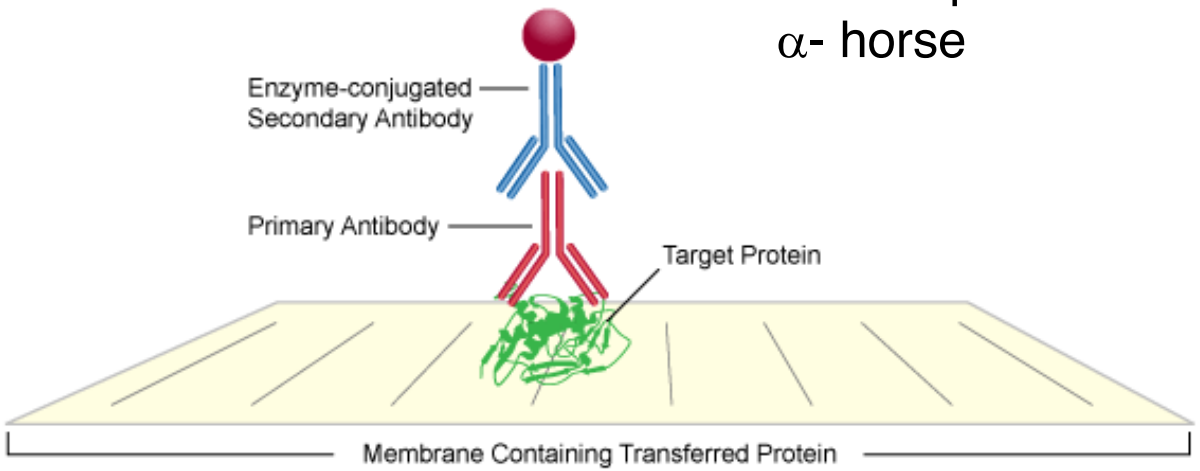


Pro: High specificity
Cons: High sensibility to damage of epitopes

Antibody binding

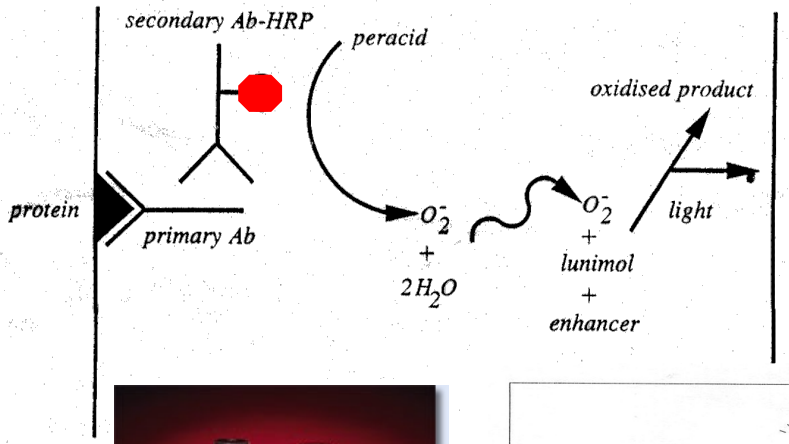
SECONDARY ANTIBODIES

α- mouse
α- goat
α- rabbit
α- sheep
α- horse



ECL (Enhanced Chemio-Luminescence) method

membrane



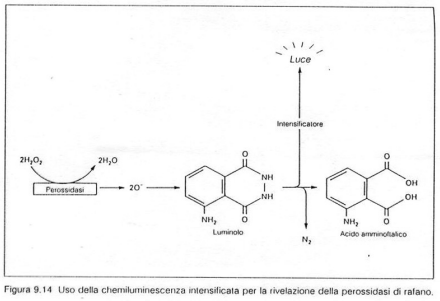
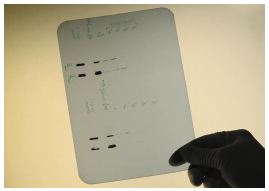
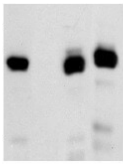


Figura 9.14 Uso della chemiluminescenza intensificata per la rivelazione della perossidasi di rafano.

Autoradiographic film or WB Imaging system

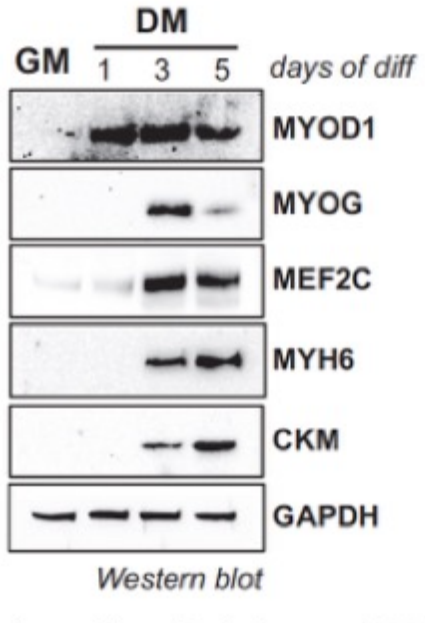




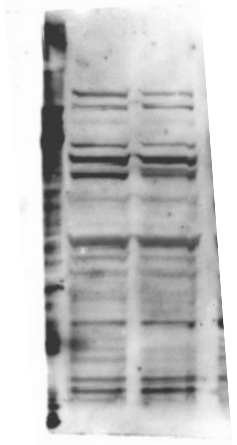
The substrate metabolized by horseradish peroxidase (HRP) emits light

Western Blot applications

- Specific protein detection (presence/absence)
- Gene expression analysis
- Comparing different conditions



Adapted from Ballarino et al, 2015



Aspecific Primary Ab?
Excess of Secondary Ab?
Unsufficient blocking?
Week wash conditions?

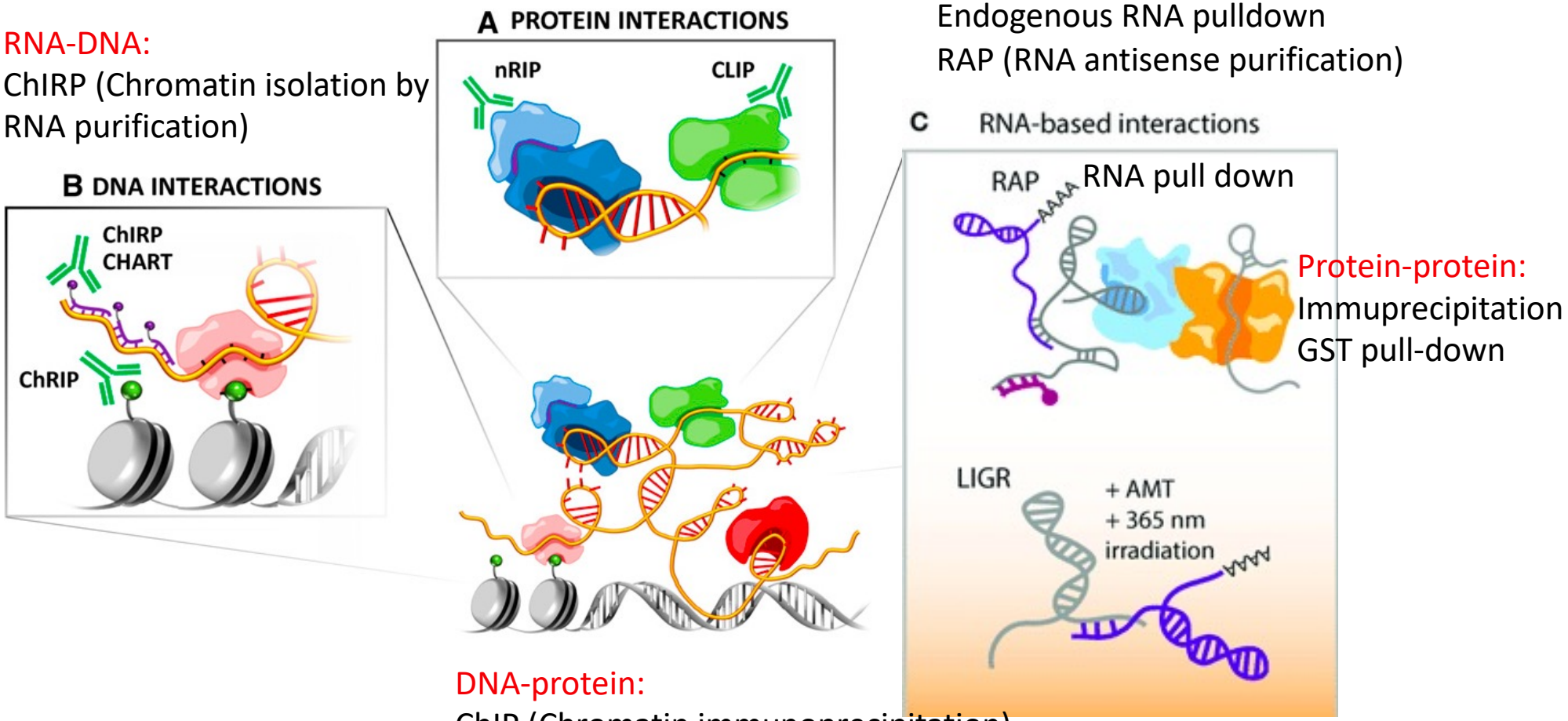
Molecular techniques for the study of the interaction between macromolecules

Molecular techniques for the study of the interaction between:

Protein-RNA (Protein centric):
RIP (RNA immunoprecipitation)
CLIP (Cross-linked immunoprecipitation)

RNA-Protein (RNA centric):
Exogenous RNA pulldown,
Endogenous RNA pulldown
RAP (RNA antisense purification)

RNA-DNA:
ChIRP (Chromatin isolation by RNA purification)

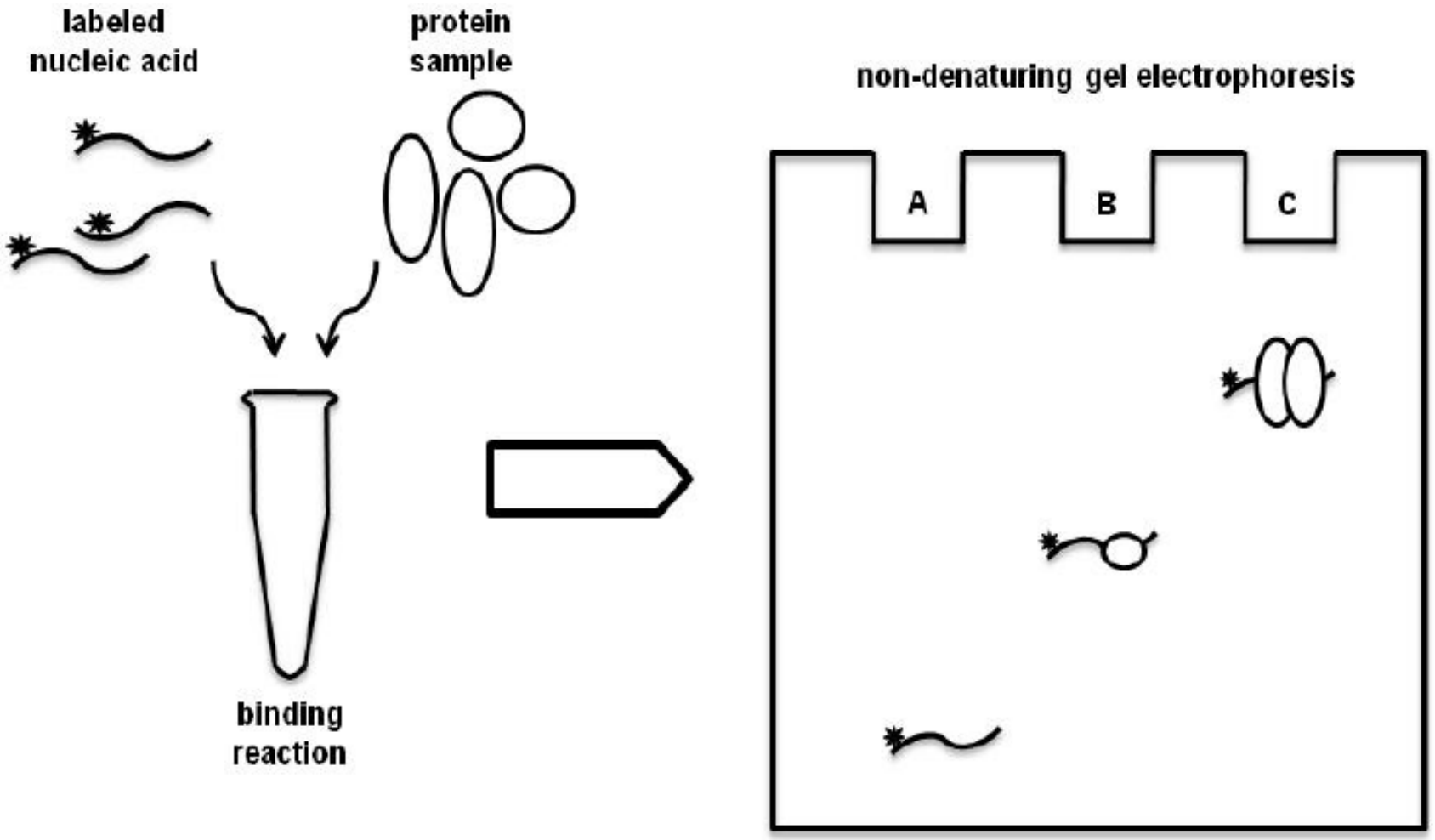


Protein-protein:
Immuprecipitation
GST pull-down

DNA-protein:
ChIP (Chromatin immunoprecipitation)
Cut&Run/Cut&Tag
ATAC-seq

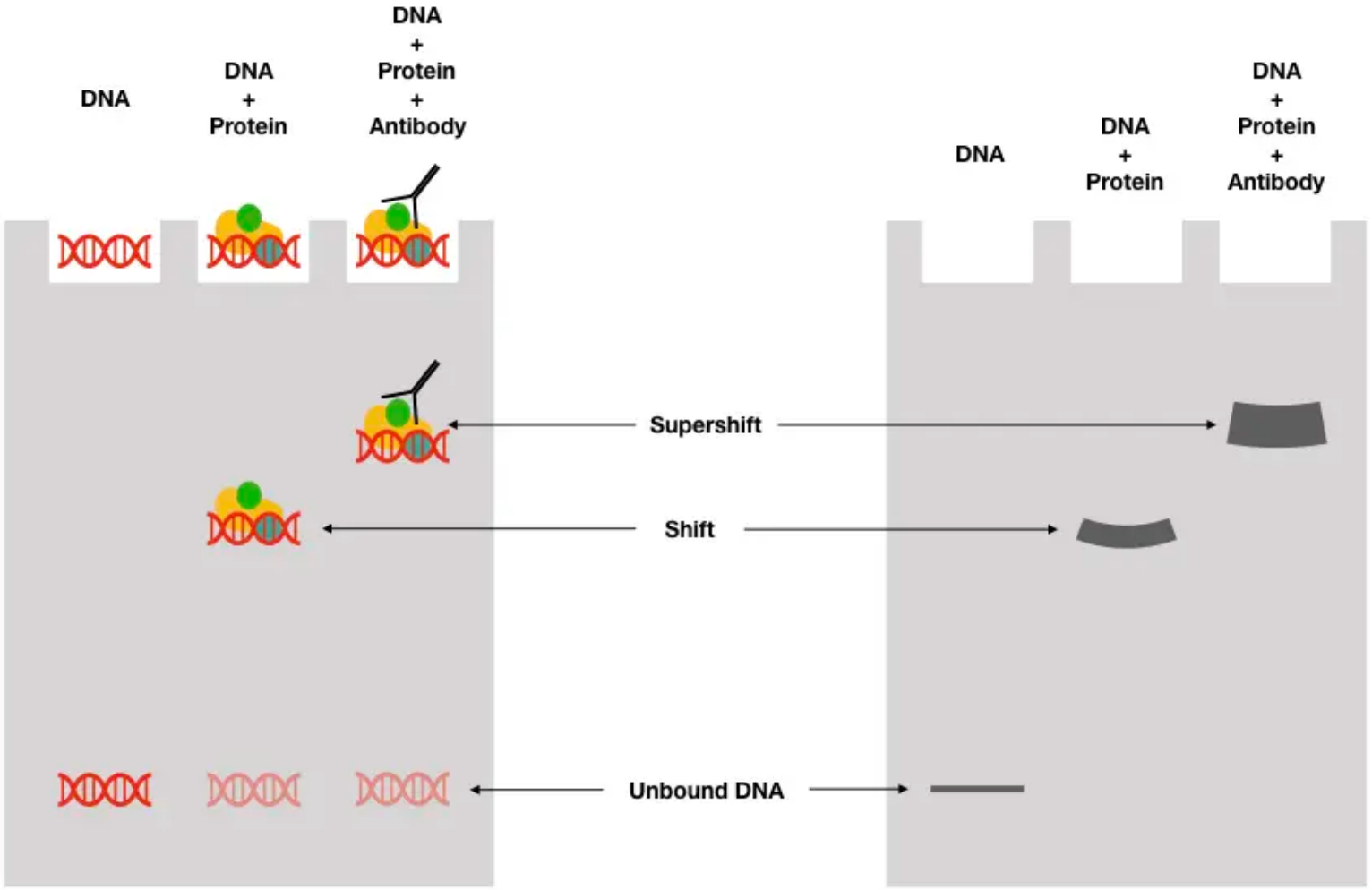
Electrophoretic mobility shift assay - Band shift assay

Does this protein bind this RNA/DNA?

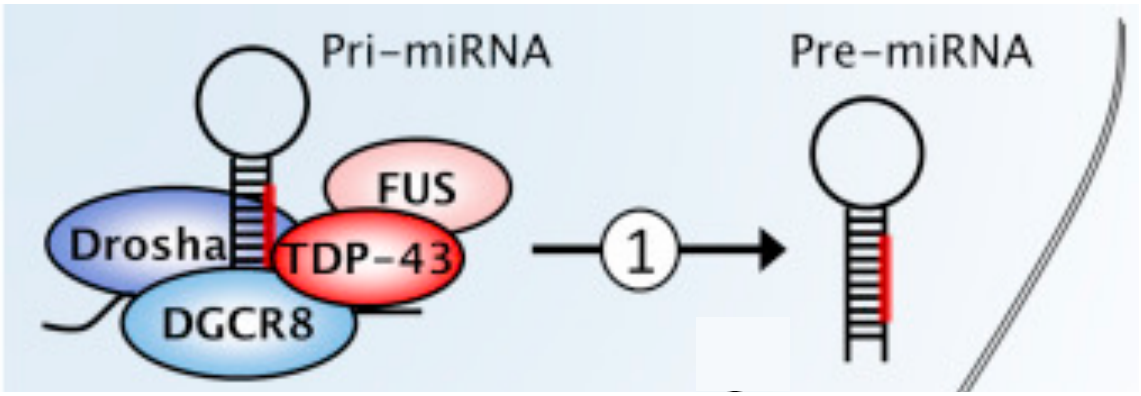


Electrophoretic mobility shift assay - Band shift assay

Is the shift specific?



FUS binds pri-miRNA and stimulate their processing



B