Methods in Protein Analysis

Western Blot

Recombinant Proteins

Immunoprecipitation

Protein Pull Down Assay

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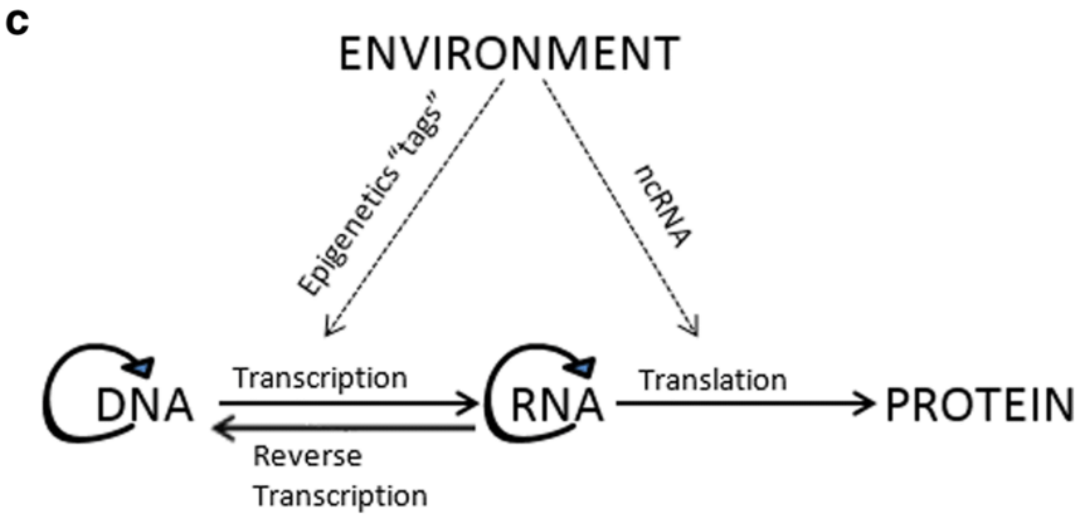
Methods in Protein Analysis

- Protein extraction
- Protein electrophoresis
  - Naïve vs denaturing conditions
- Identification of proteins
  - Mass spectrometry
  - Western Blot
    - Antibodies
- Recombinant protein
- Immunoprecipitation/Pull down

Why do we study proteins?

- Proteins are the final effector in the cells

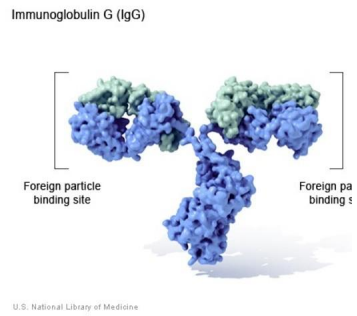
- They do most of the work in cells and are required for the structure, function, and regulation of the body's tissues and organs

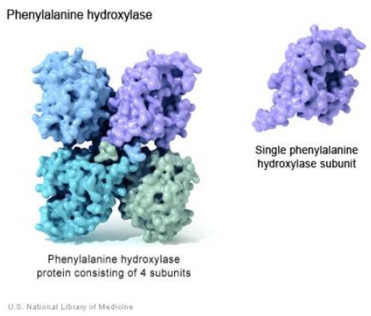


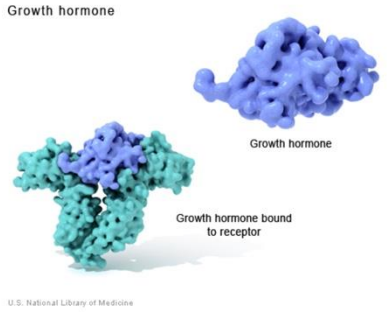
Why do we study proteins?

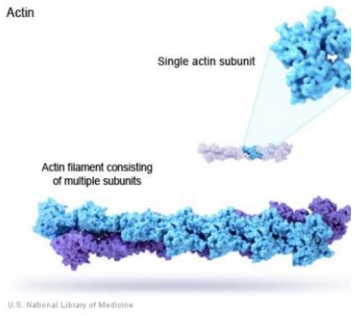
- Proteins are the final effector in the cells; as effectors they can act in many ways so the final level of the protein is the determinant of the function

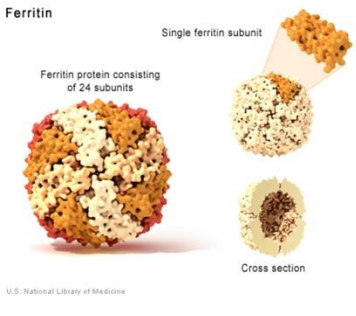
Function	Description	Example
Antibody	Antibodies bind to specific foreign particles, such as viruses and bacteria, to help protect the body.	Immunoglobulin G (IgG)
Enzyme	Enzymes carry out almost all of the thousands of chemical reactions that take place in cells. They also assist with the formation of new molecules by reading the genetic information stored in DNA.	Phenylalanine hydroxylase
Messenger	Messenger proteins, such as some types of hormones, transmit signals to coordinate biological processes between different cells, tissues, and organs.	Growth hormone
Structural component	These proteins provide structure and support for cells. On a larger scale, they also allow the body to move.	Actin
Transport/storage	These proteins bind and carry atoms and small molecules within cells and throughout the body.	Ferritin





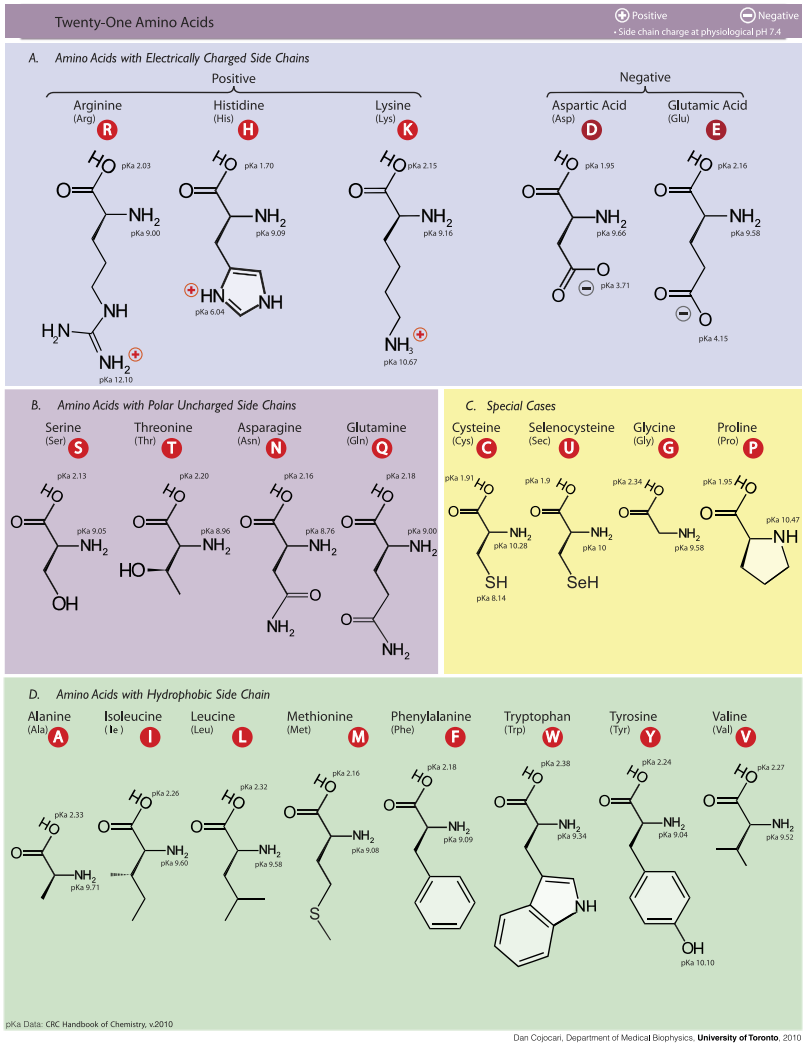


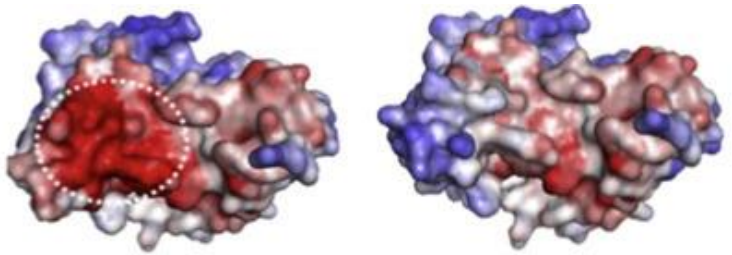




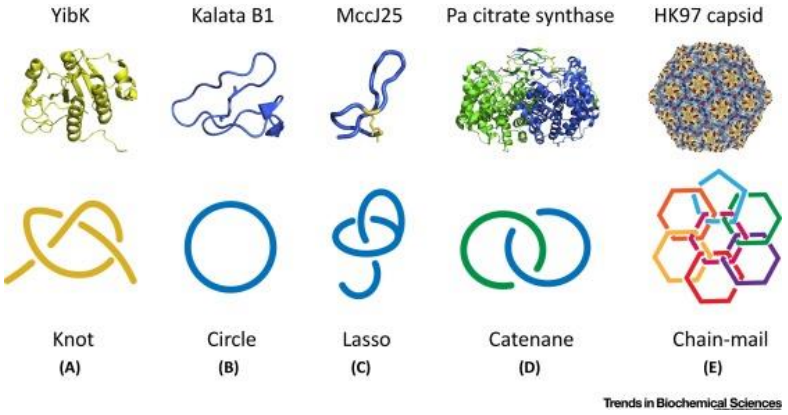
Why do we study proteins?

- Proteins are biochemically much more complicated that nucleic acids



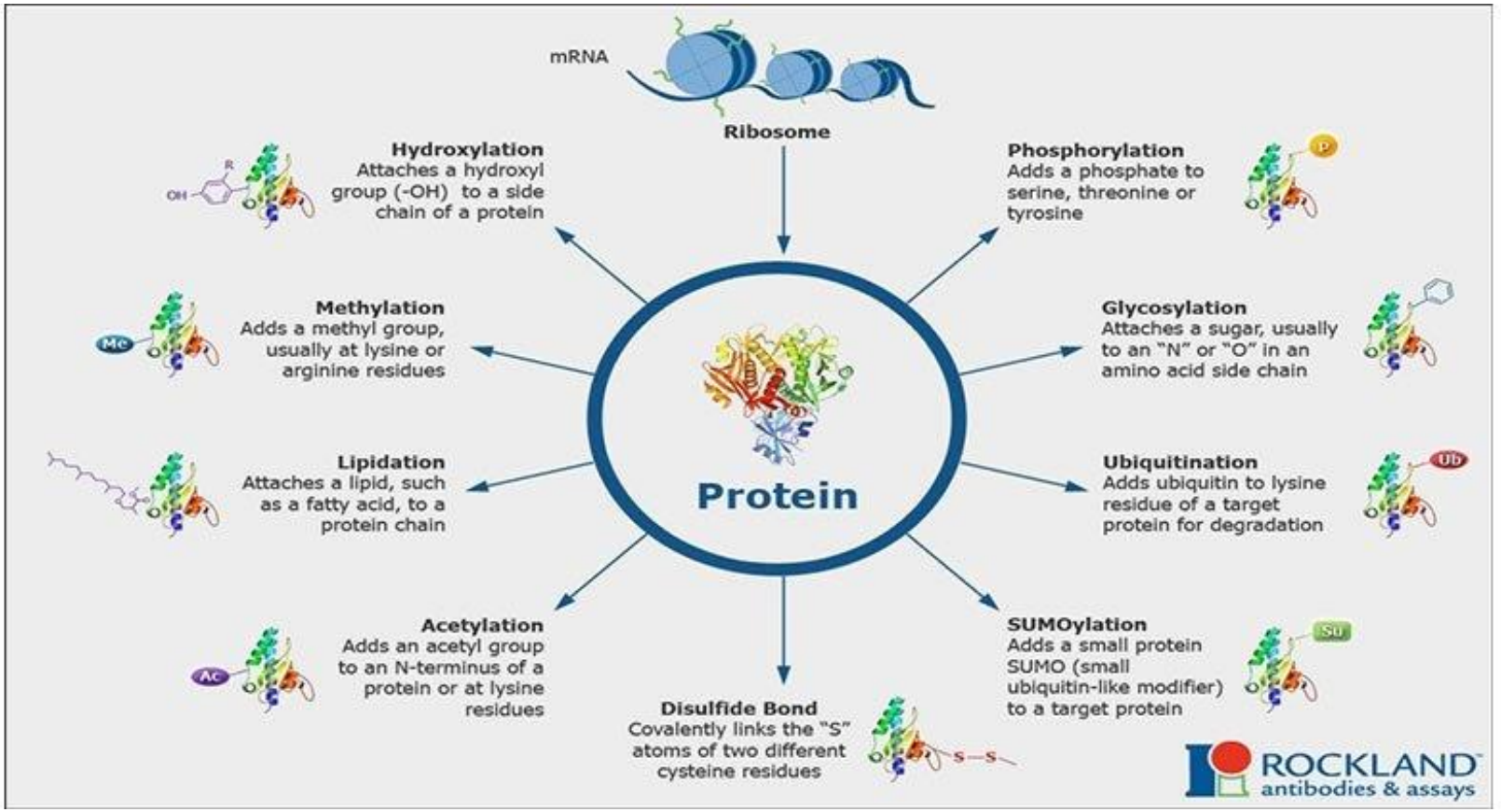


Depiction of surface polarity distribution (red is non-polar, blue is polar) on surface of scFv. A non-polar patch on the wild type is shown by the white circle on the left. On the right is the polarity distribution after introducing three salt bridges into the patch.



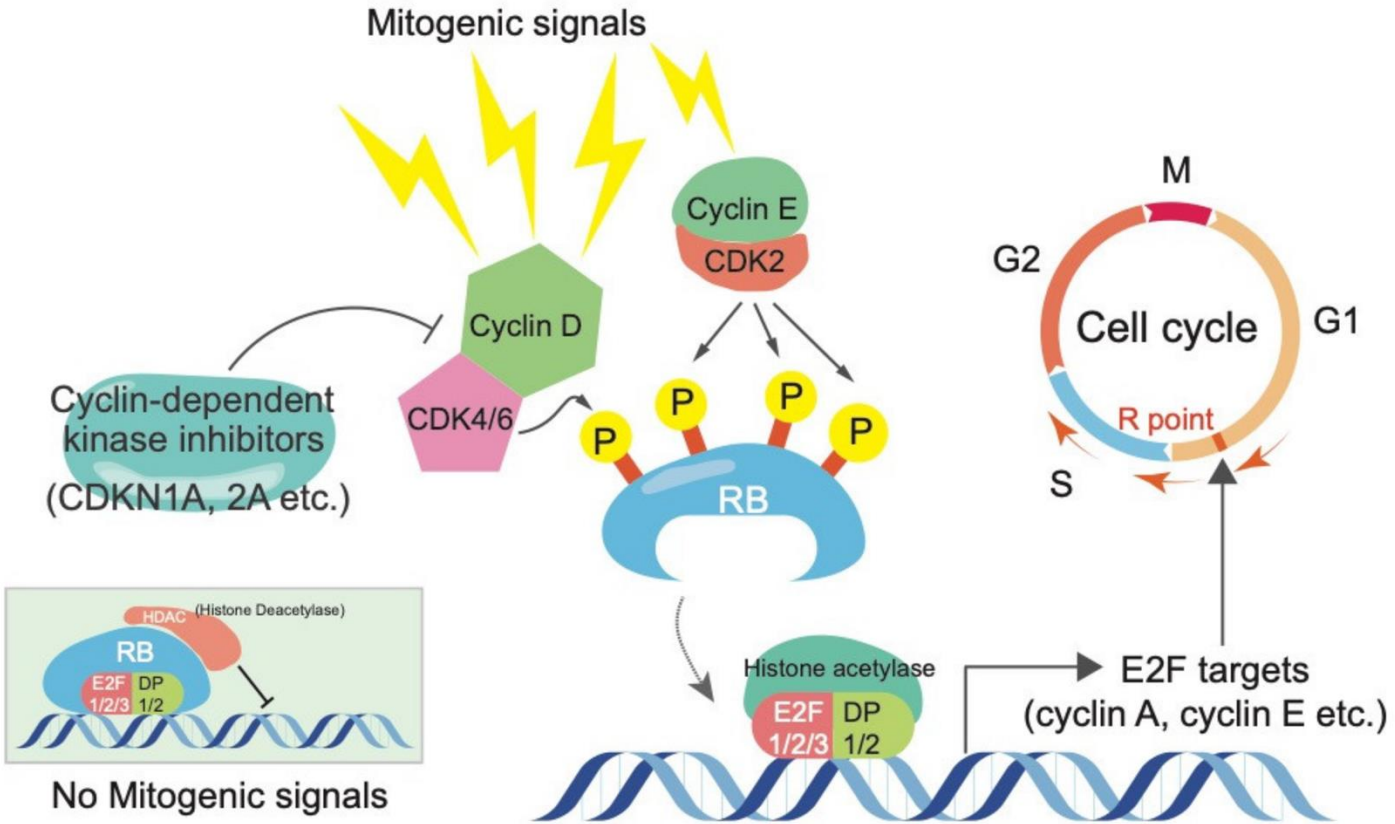
Why do we study proteins?

- Moreover modification in the proteins can alter the function; studying protein modification will tell us information about the protein function.



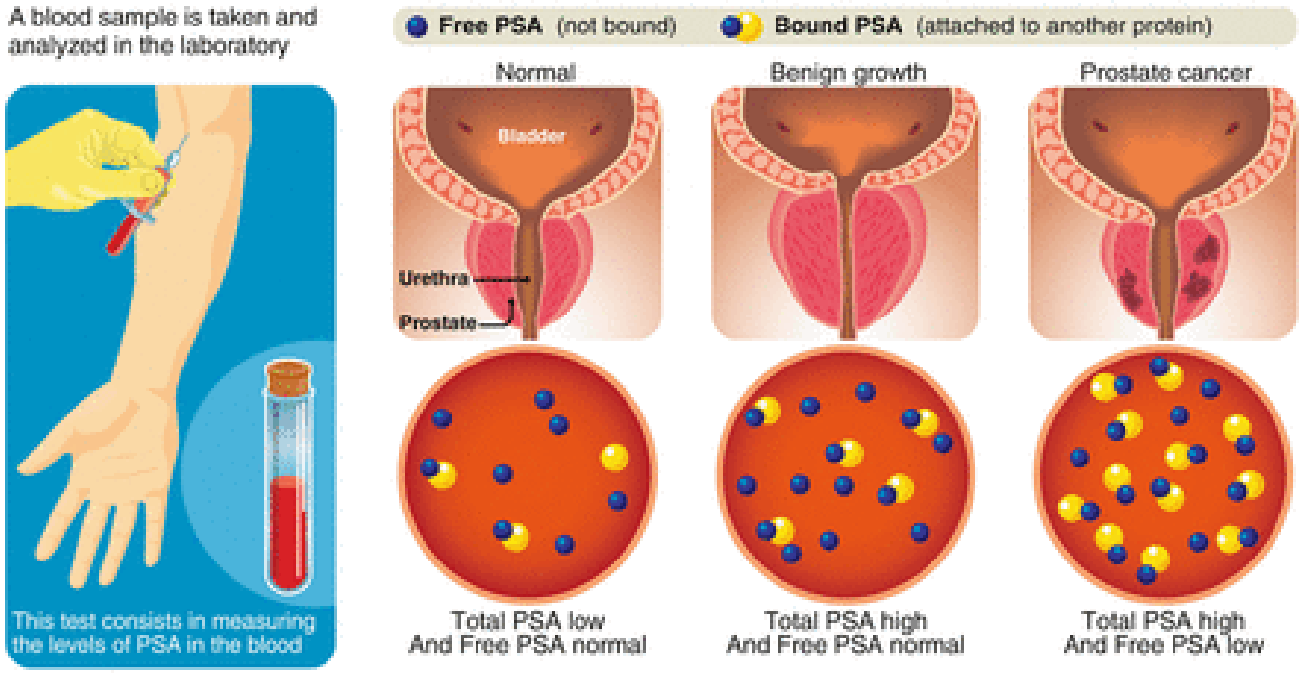
Why do we study proteins?

- Example Phosphorylation state of Retinoblastoma protein can tell us the proliferative state of the cell.



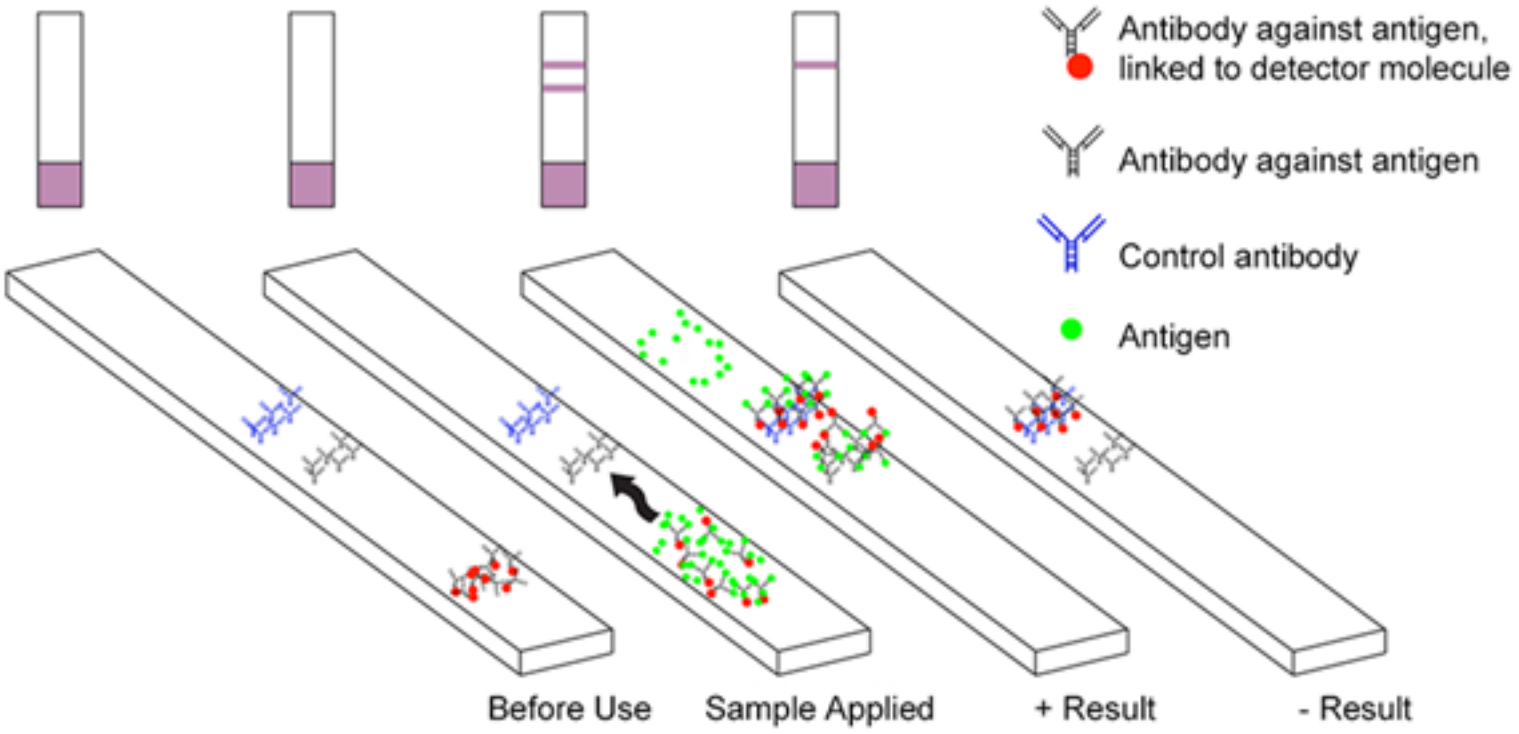
Why do we study proteins?

- Example 2 PSA protein. Levels of Prostate specific Antigen (PSA) in blood indicates the healthy state of prostate.



Why do we study proteins?

- Example Lateral Flow covid test. Cheap and fast detection of virus in samples.



Adapted from: Ian M. Campell, https://commons.wikimedia.org/wiki/File:Diagnostic_Medical_Dipstick.png

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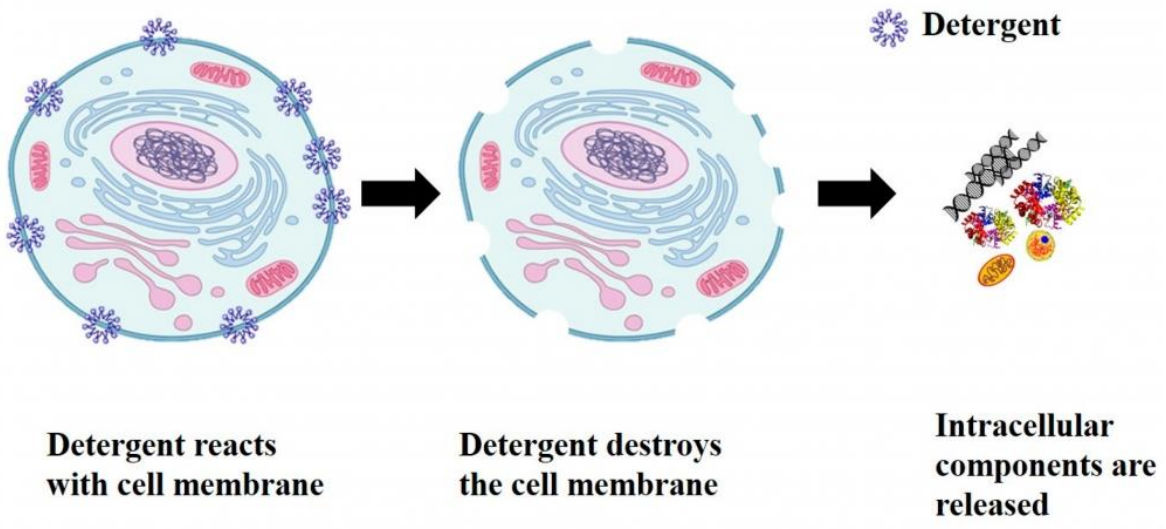
Protein Extraction: Total

- More complicated than nucleic acid extraction:
  - Proteins are in different cellular compartments, might be in the membrane.
  - Proteins can be polar/non-polar, hydrophobic, non soluble, etc…
  - Enzymes and catalytic activities.

- -Cell lysis in conditions ensuring:

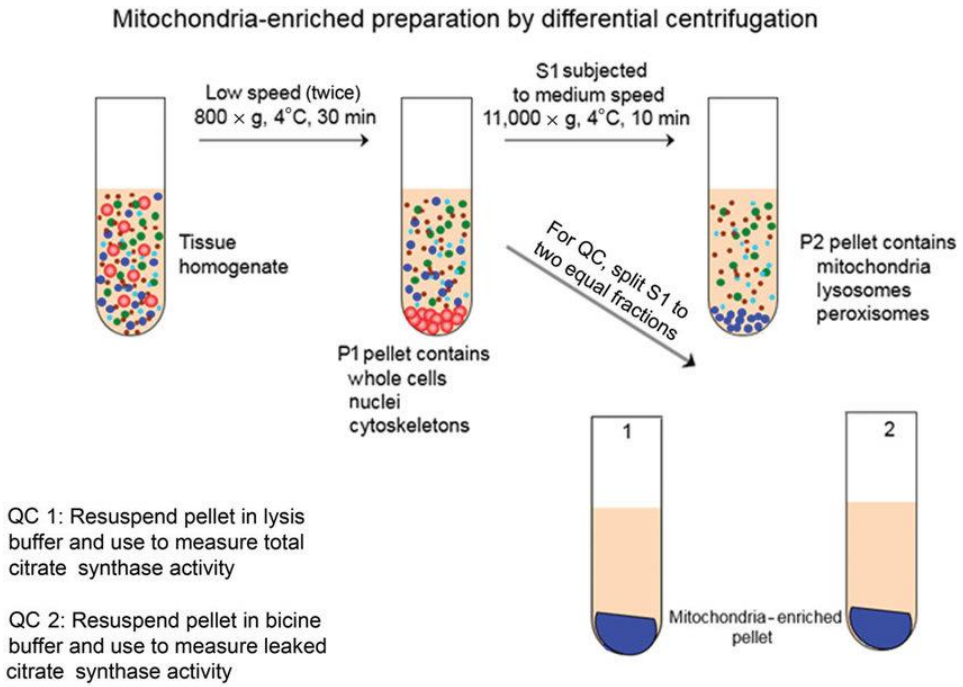
  Membrane break, protein dissociation → <u>Detergents</u> : SDS, Triton, Tween

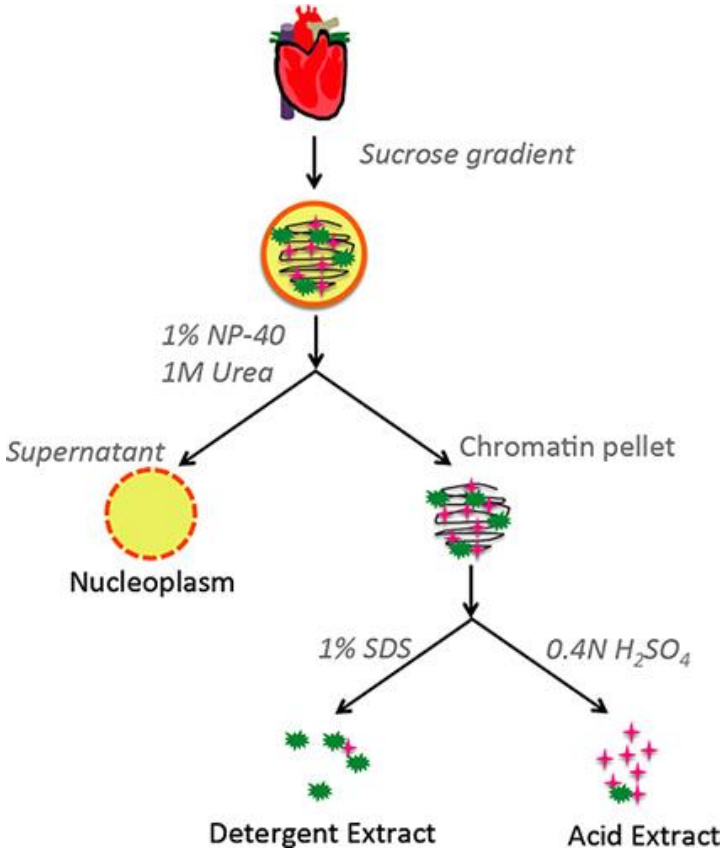
  Protein Inhibitors; Leupeptin, Pepstatin, PMSF, EDTA, 4 C



Protein Extraction:partial

- We can separate also specific compartments in the cell





- Mitochondria
- Chromatin

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Protein Electrophoresis

Harder than nucleic acid electrophoresis:

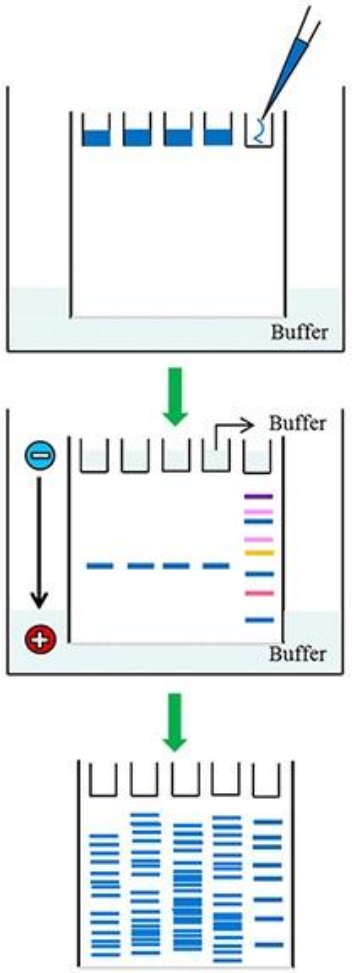
Proteins differ in charge

Proteins differ in conformation

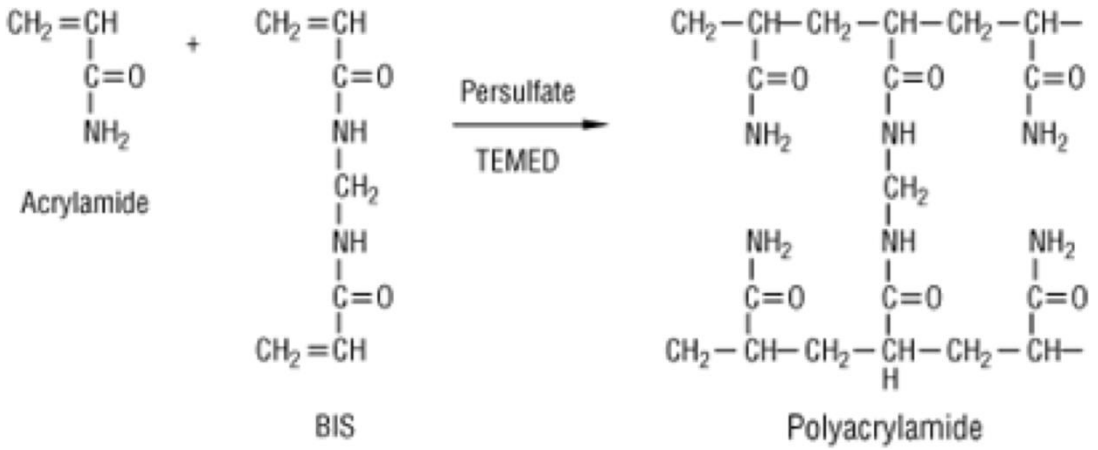
A polyacrylamide gel can be:

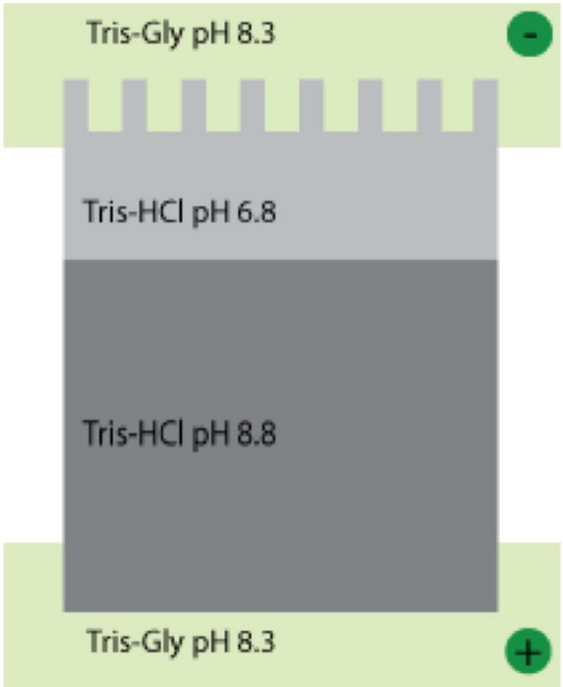
Native
(non denaturing conditions)

SDS-gel
(denaturing conditions)

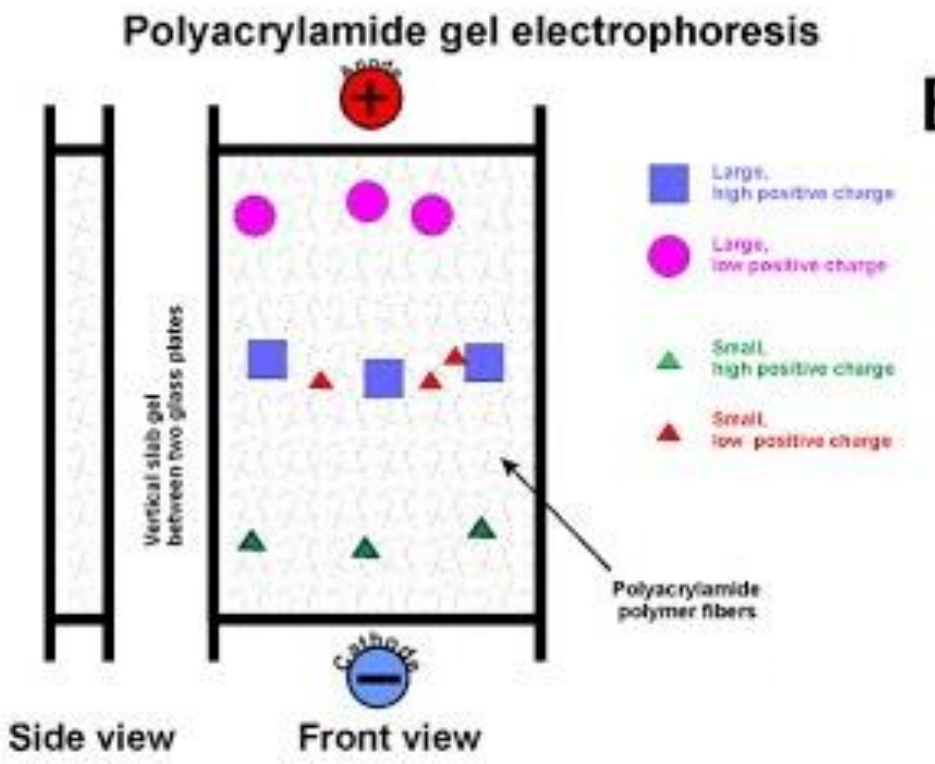


Polyacrylamide Gel





Polyacrylamide Gel Electrophoresis (PAGE)



Native gel Electrophoresis

Proteins migrate depending **m** and **q**

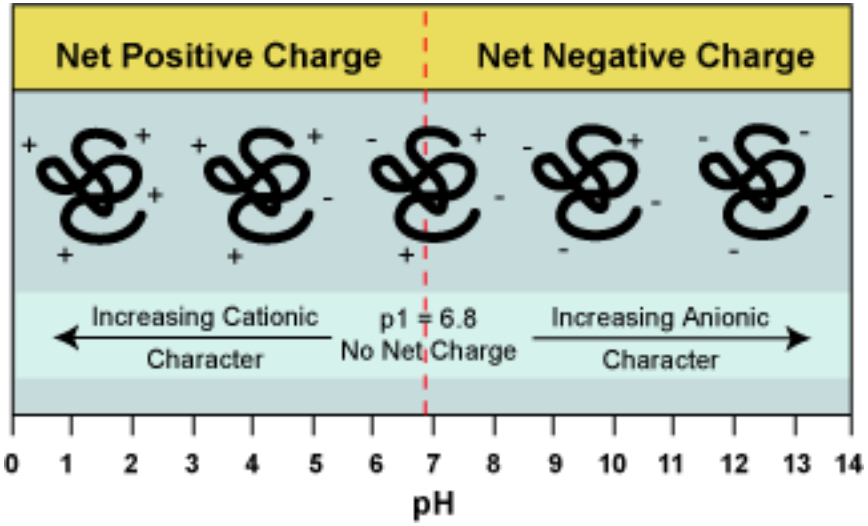
Proteins keep conformation

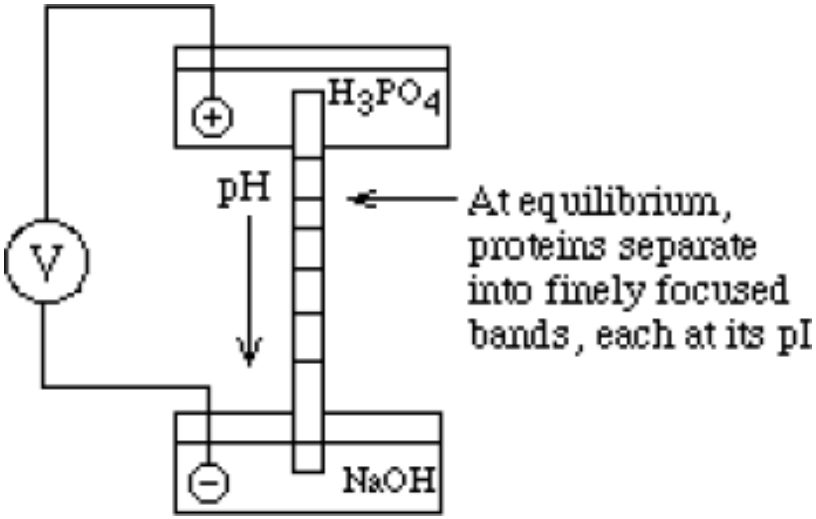
Isoelectric Focusing Electrophoresis (IFE)

-Fractionation based on Isoelectric Point (pI)

-The buffer generates a pH gradient

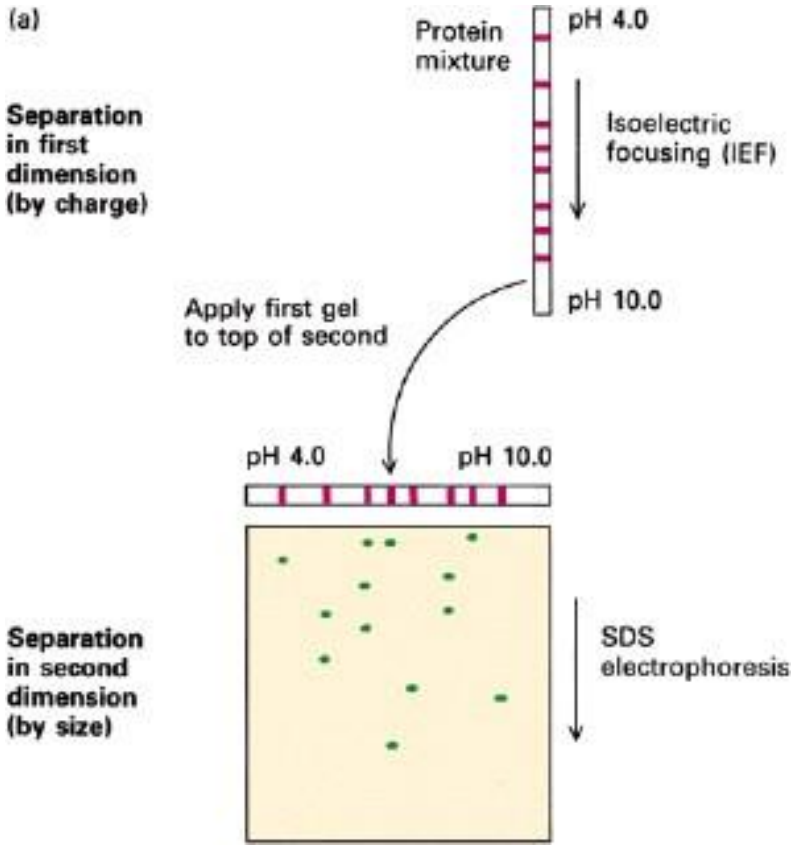
-When reaches the pI, the protein loses its charge (q=0) and stops in the gel





2D-PAGE

- First separation based on Isofocused Electrophoresis (pI)
- Second separation by SDS-PAGE (size)
- High resolution
- Proteomics study

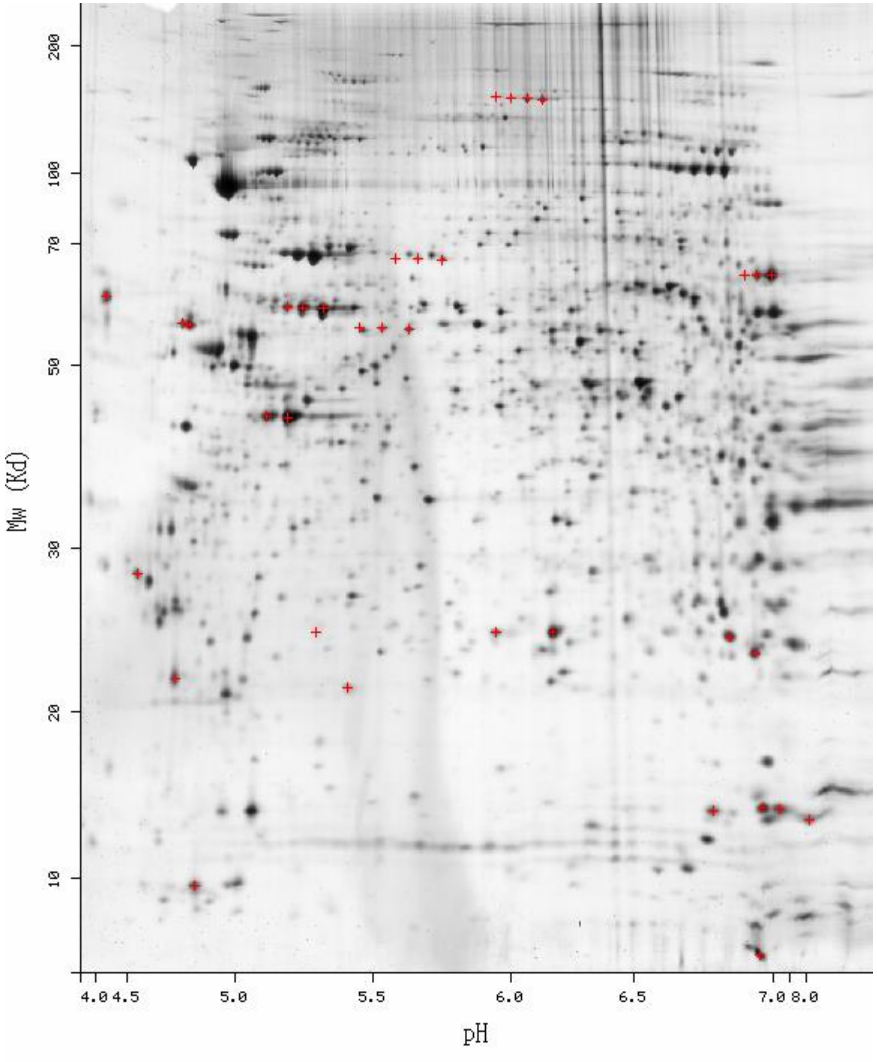


2-dimensional Gel Electrophoresis

A second electrophoretic run, orthogonal to the previous one and governed by protein size, allows proteins to be highly resolved as single spots

Spot coordination

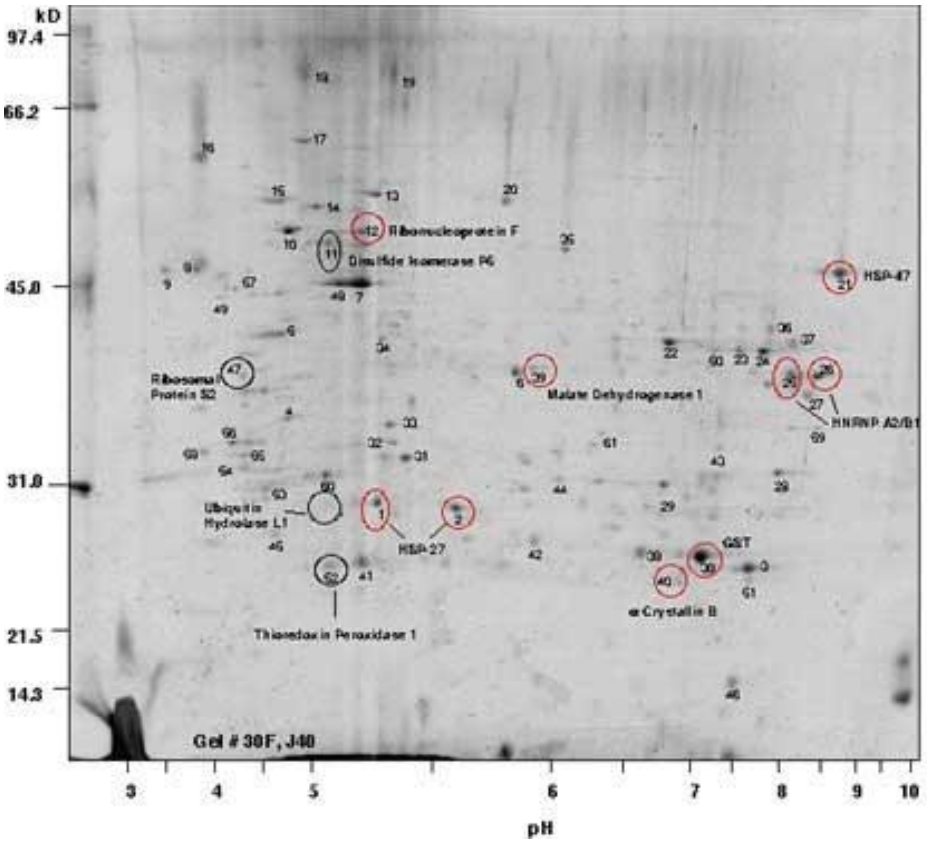
- pI
- MW



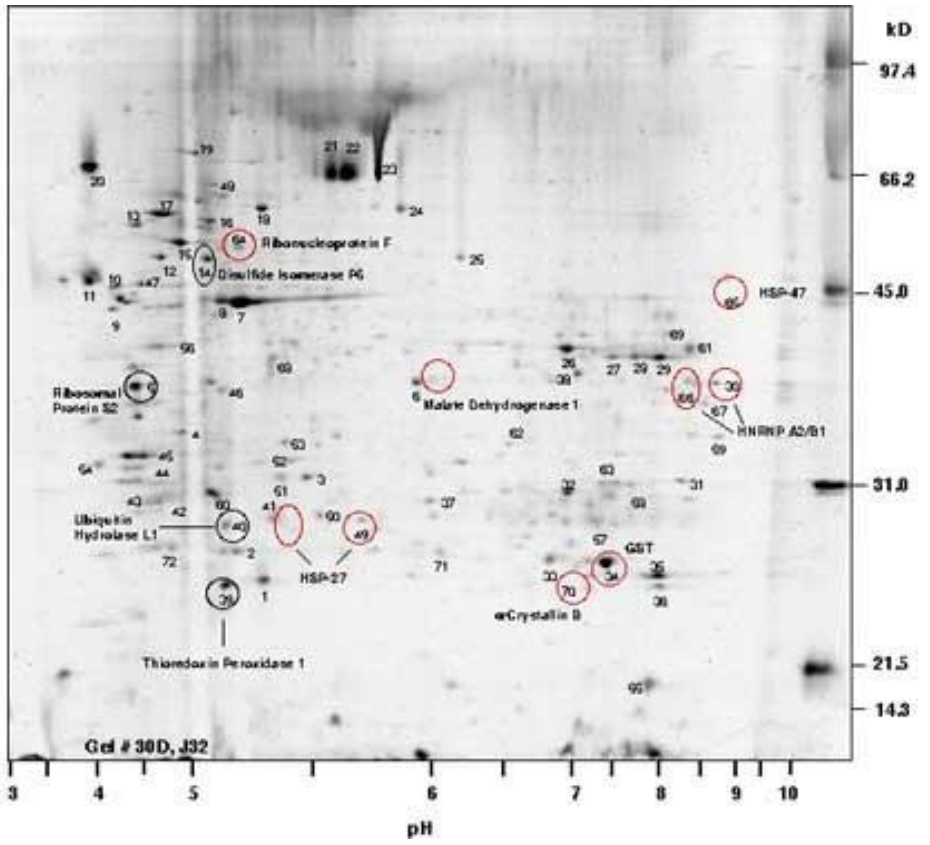
2-dimensional Gel Electrophoresis

Application: Proteomics

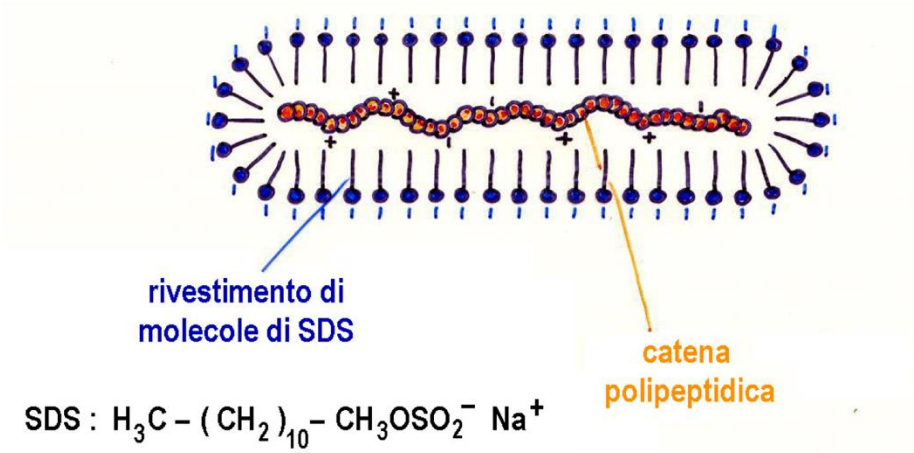
Condition A



Condition B

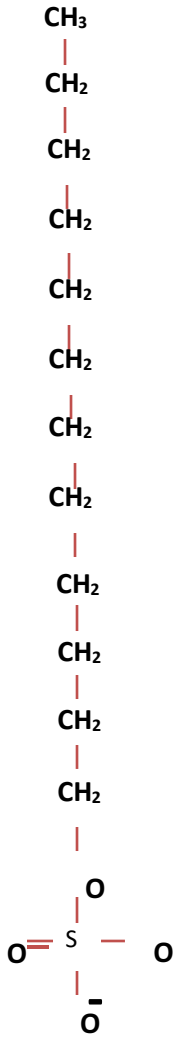


SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Denaturing conditions



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

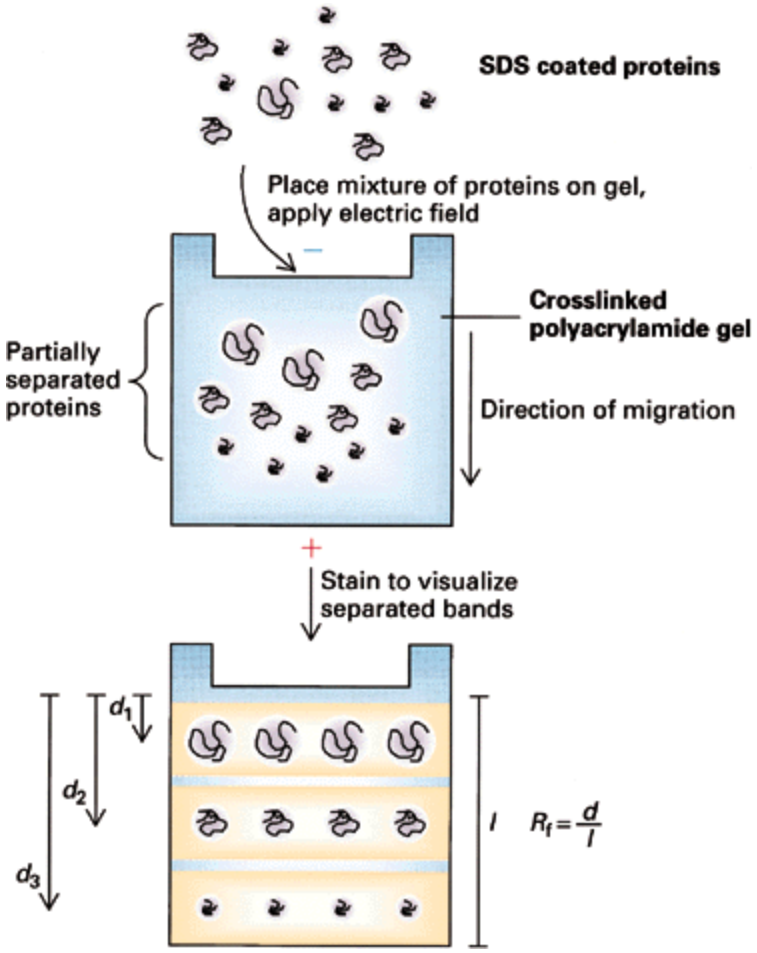
For each SDS denatured protein, the ratio between **m and q is constant**.



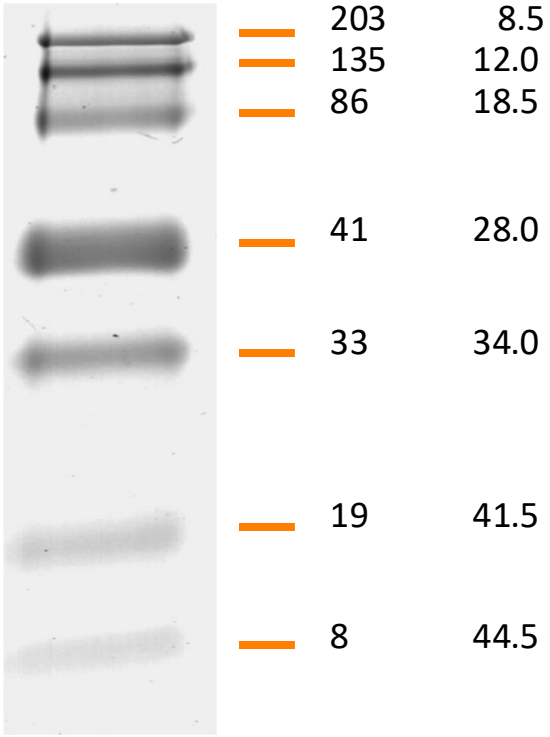
SDS

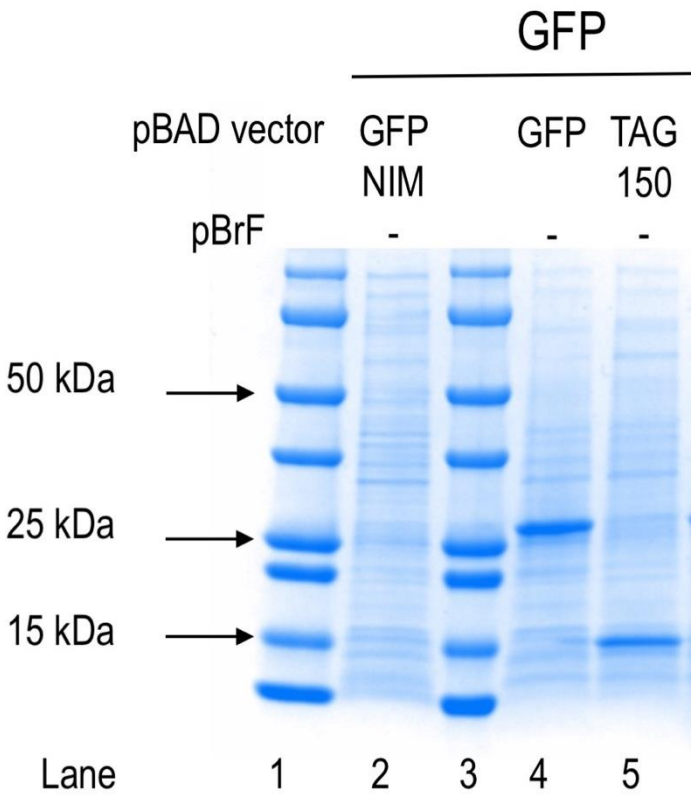
Considerations

- 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



Molecular Weights





- We can separate proteins by mass.

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    - Antibodies
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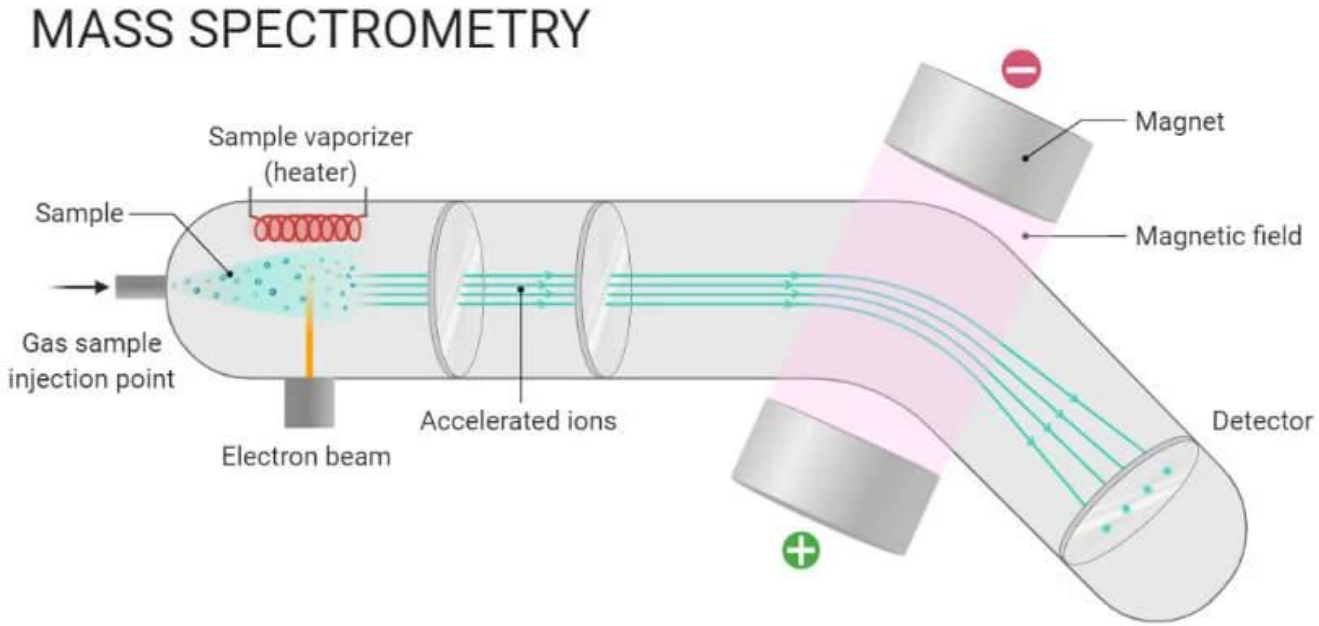
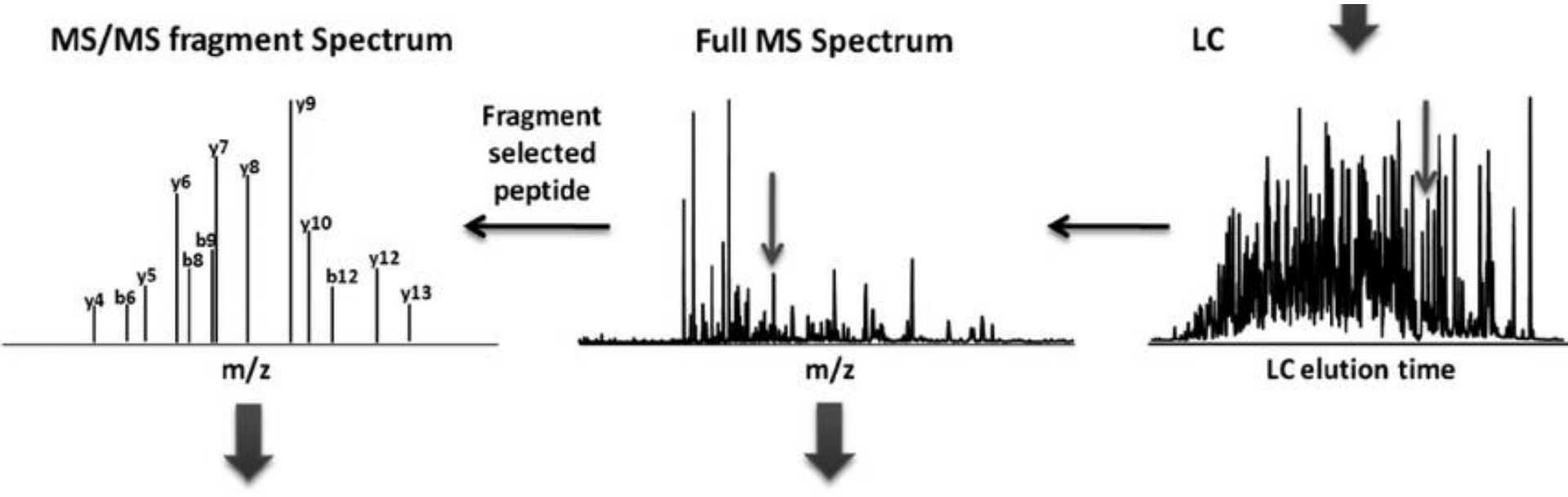
Mass spectometry (Brief, do not panic)





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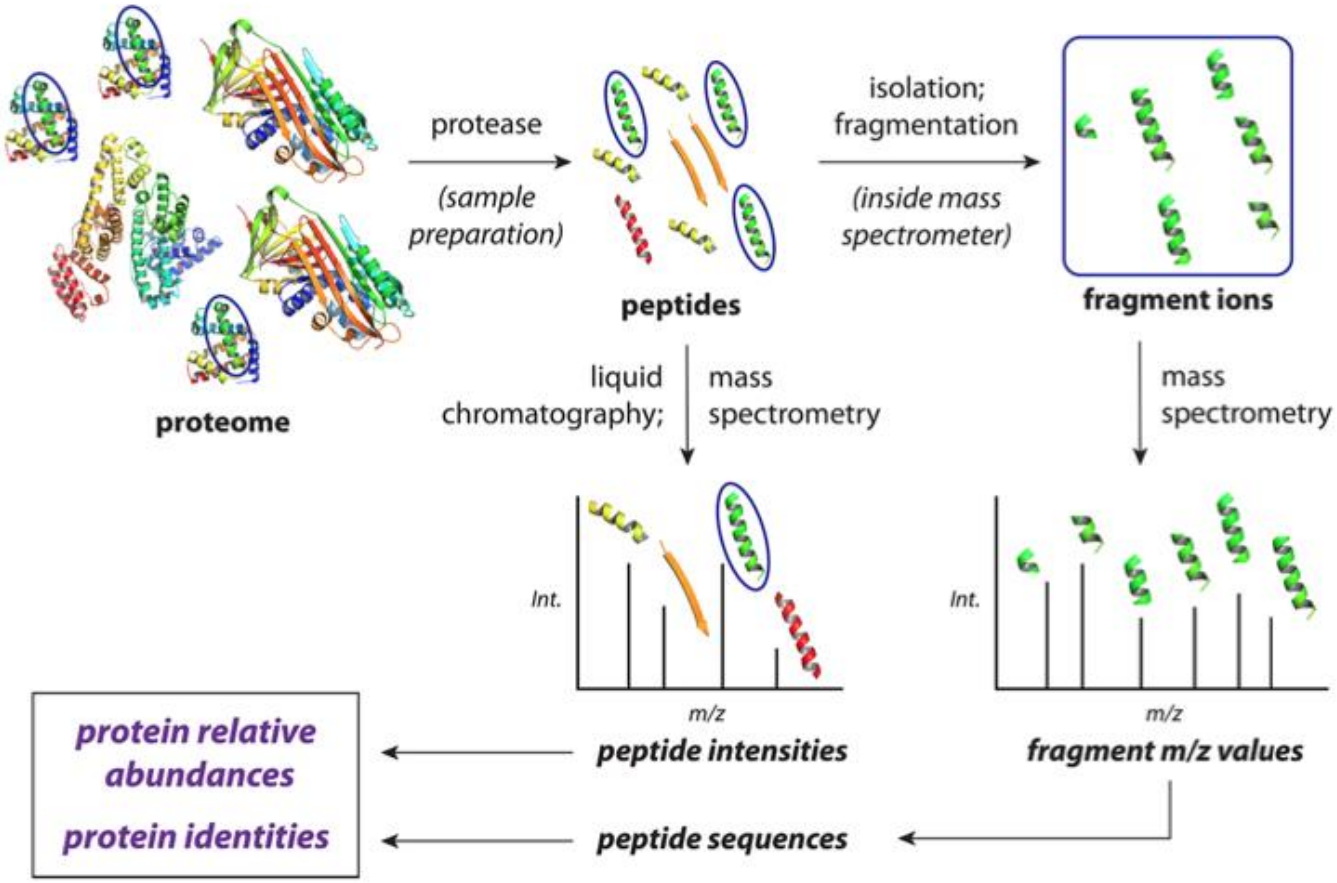
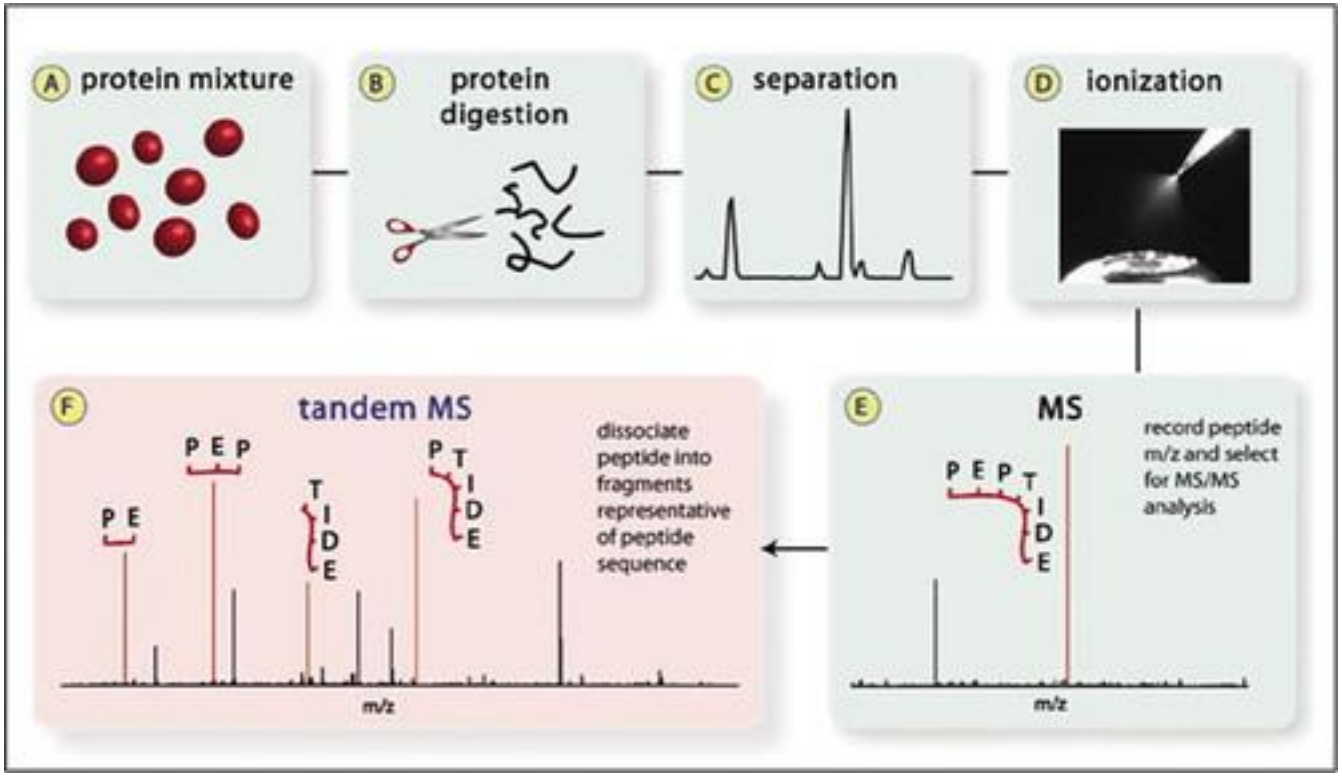
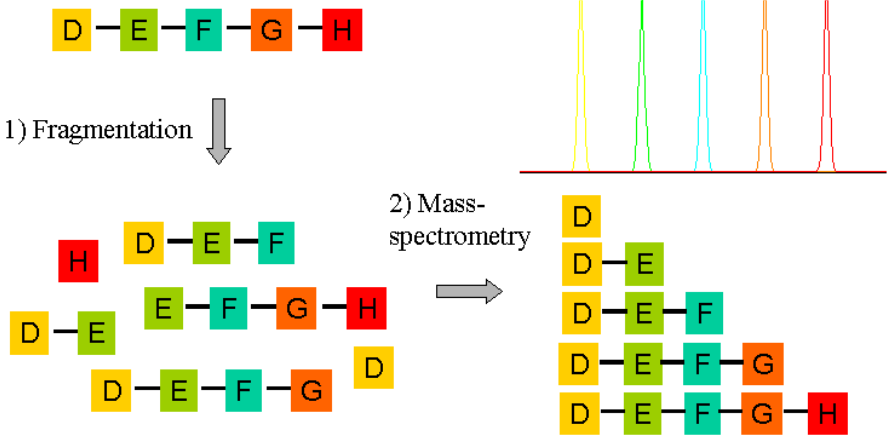


Mass spectometry





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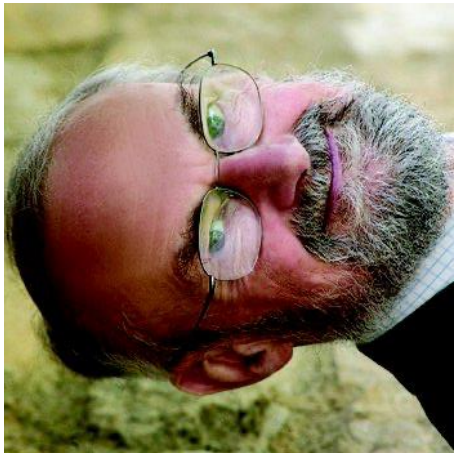
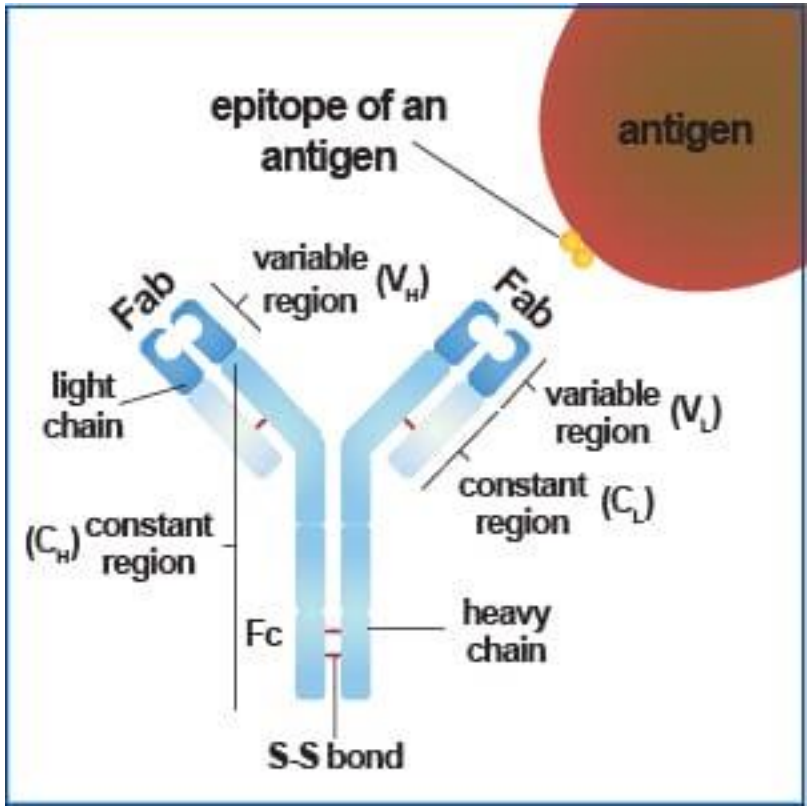
How we identify proteins: Western Blot assay

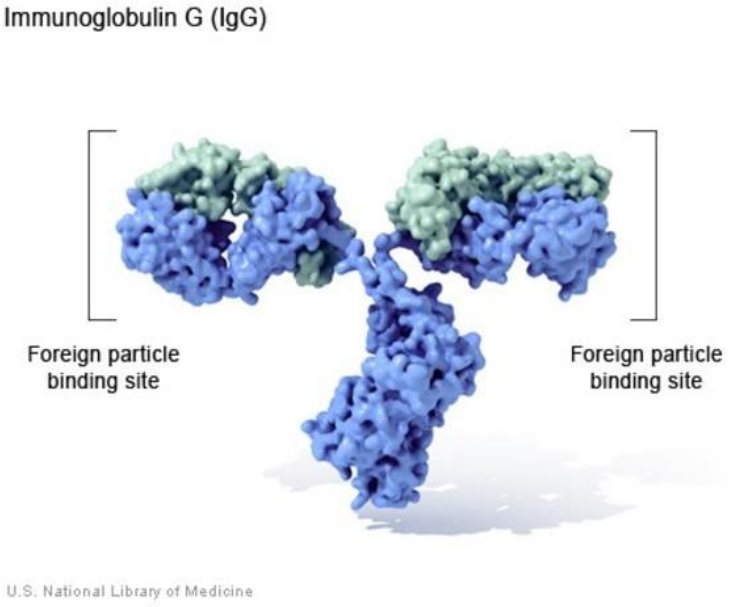
- **Specific protein detection (presence/absence)**
- **Gene expression analysis**
- **Comparing different conditions**



How we identify proteins: Antibodies

An antibody (Ab), also known as an immunoglobulin (Ig), is a large, Y-shaped protein used by the immune system to identify and neutralize foreign objects such as pathogenic bacteria and viruses. The antibody recognizes a unique molecule of the pathogen, called an antigen.





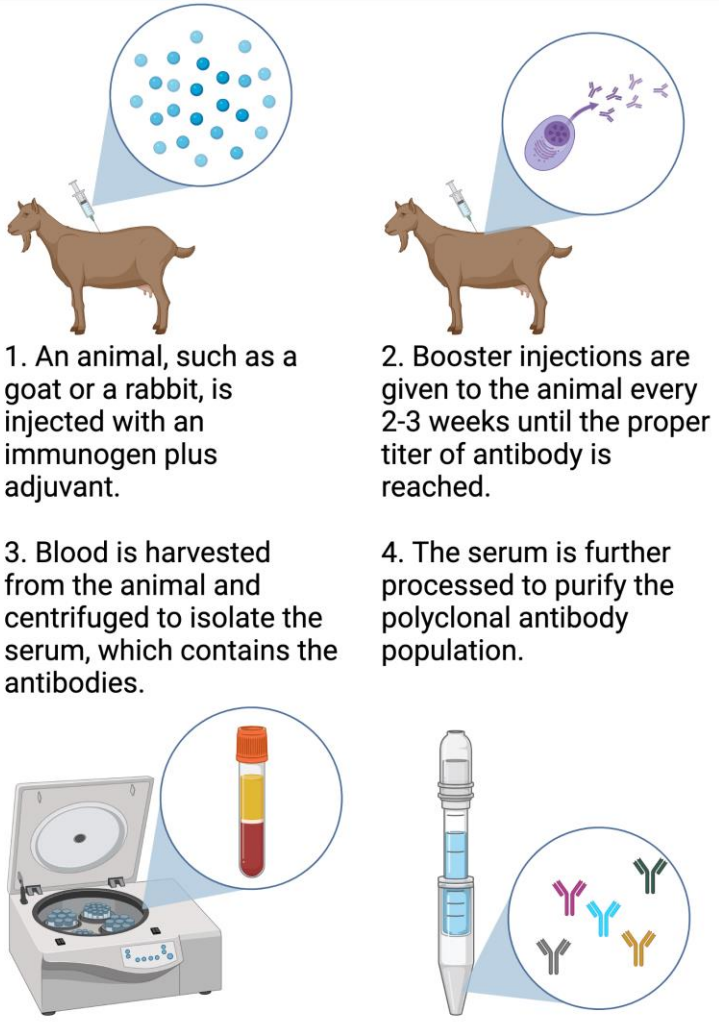
Antibody production
We can produce antibodies to recognise different proteins

POLICLONAL

Multiple immunization rounds of the animal through injection of the antigen (peptide, purified protein, recombinant protein)

Blood collection and serum purification

Heterogeneous pool of antibodies against different immunogenic epitopes



Antibody production
We can produce antibodies to recognise different proteins

MONOCLONAL

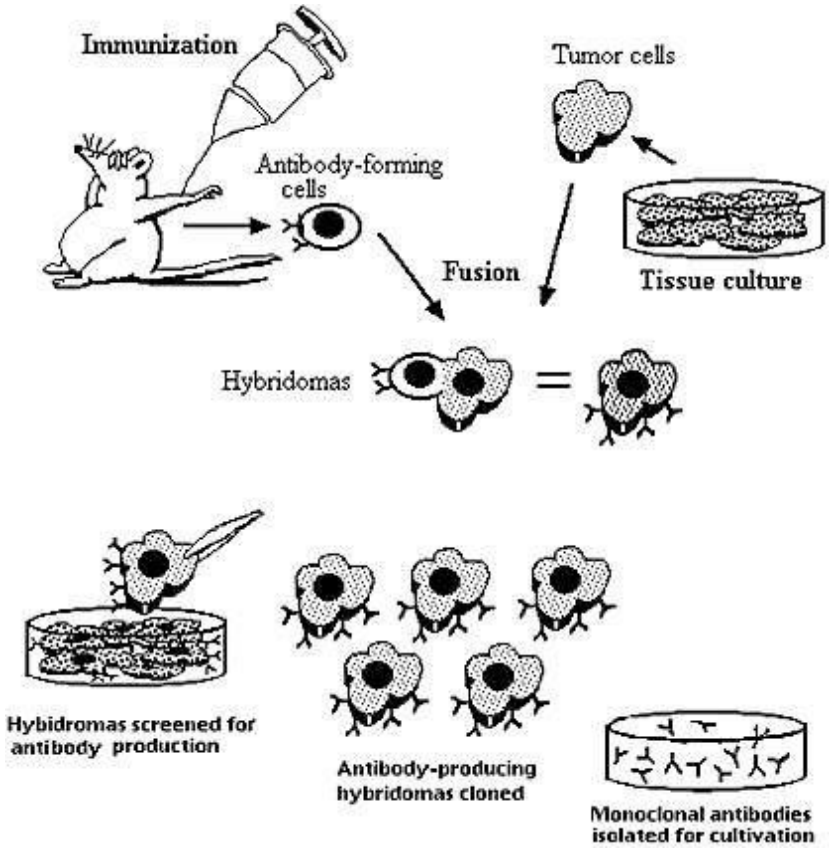
Multiple immunization rounds of the animal through injection of the antigen (peptide, purified protein, recombinant protein)

Selection of antibody production cells

Hybridization with tumour cell to immortalise the cell line.

Selection and amplification of best cell/antibody

Single clone antibody against one immunogenic epitope.



Western Blot Assay: steps

–SDS-PAGE

–Blot

–Blocking

–Binding of primary Ab

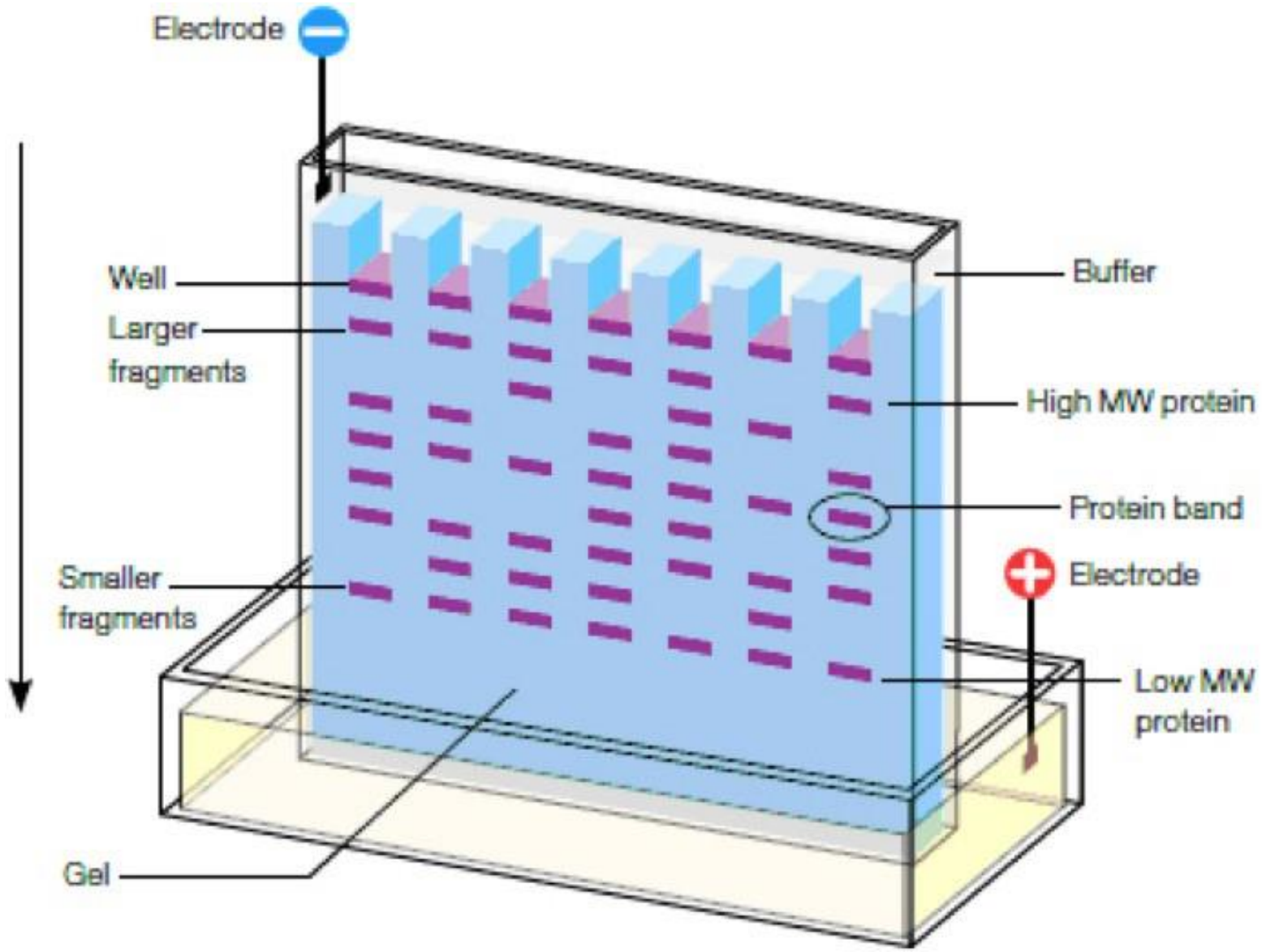
–Wash by buffer

–Binding of secondary Ab

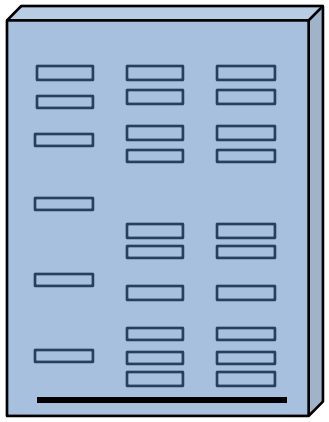
–Wash by buffer

–Signal Detection

SDS-PAGE


Electrode
Well
Larger fragments
Smaller fragments
Gel
Buffer
High MW protein
Protein band
Electrode
Low MW protein

Blot

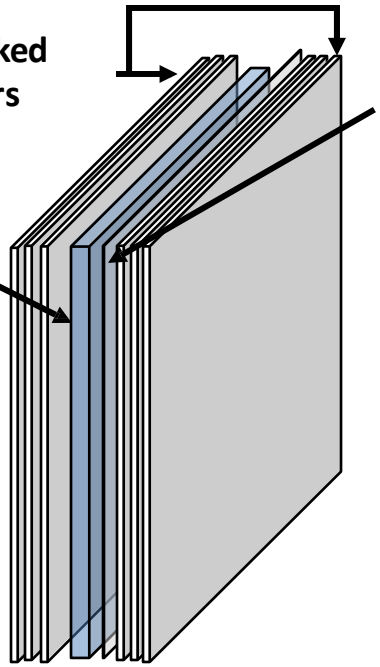


SDS-PAGE

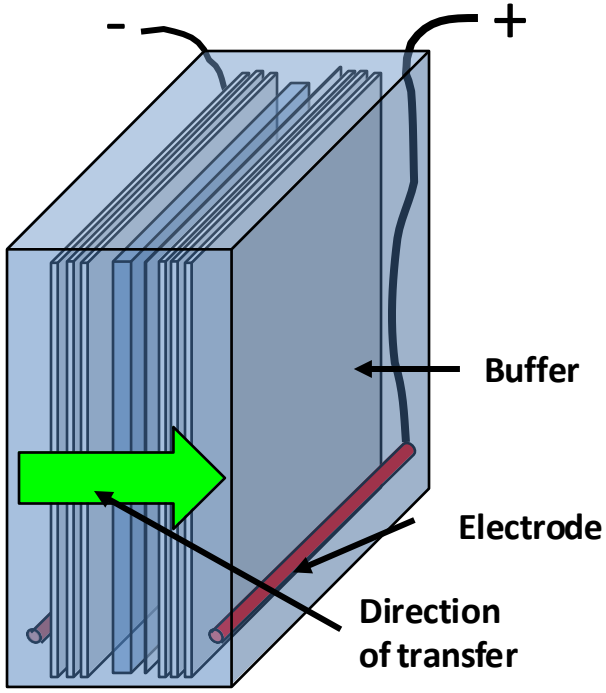
Buffer-soaked filter papers

Nitrocellulose membrane

Gel

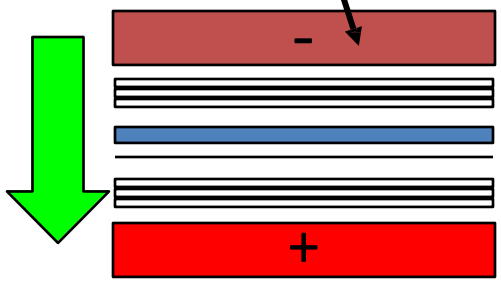


Assemble ‘sandwich’

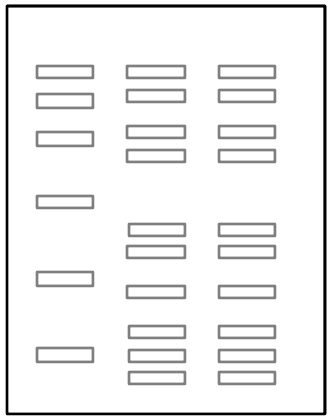


Wet blotting

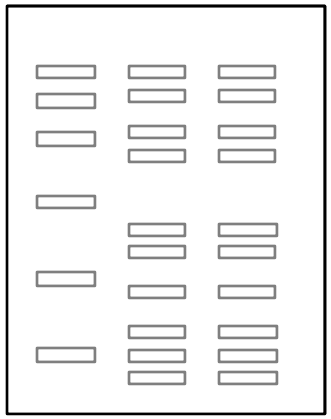
Graphite Electrode Plates



Semi-dry blotting

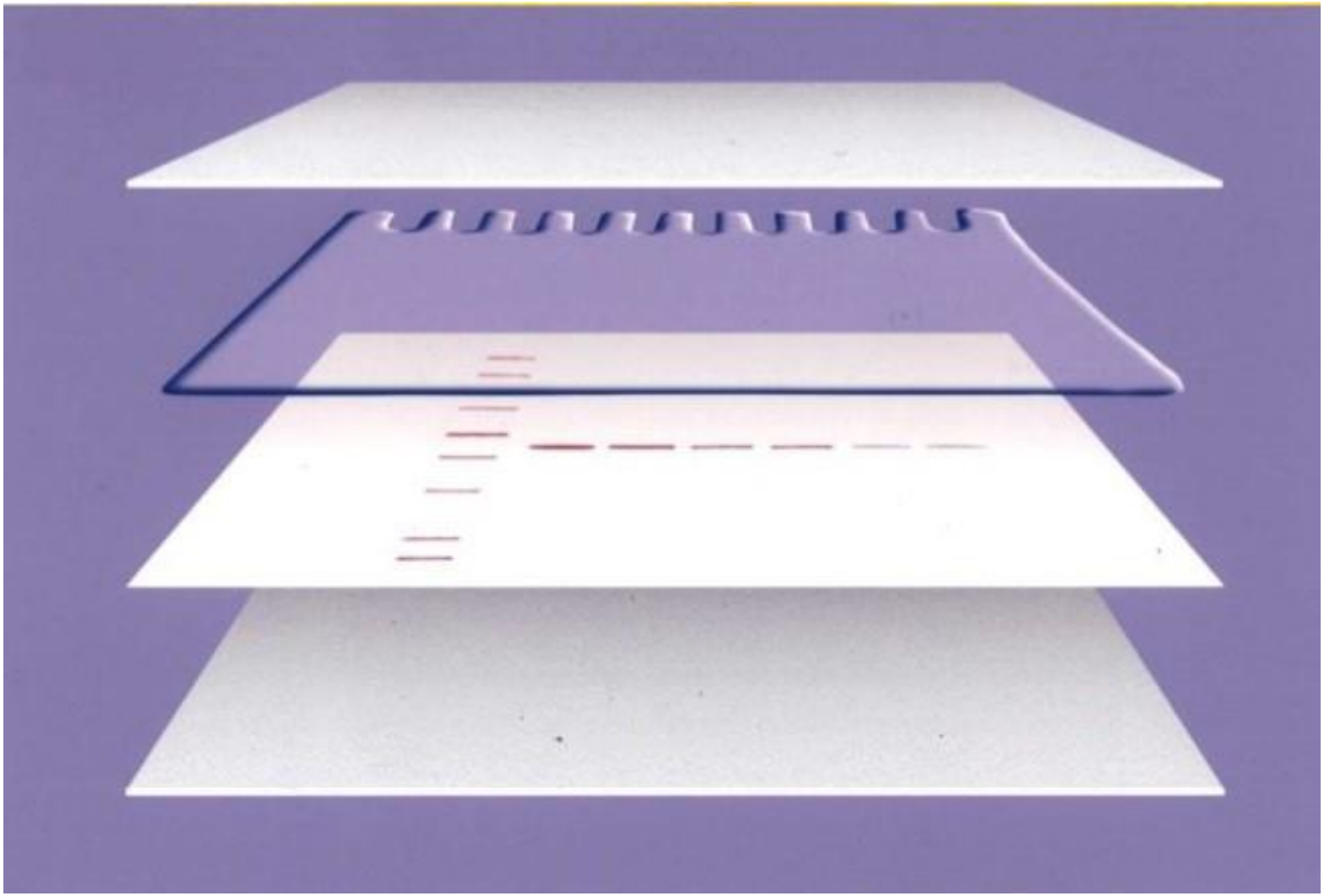


Nitrocellulose with bound proteins



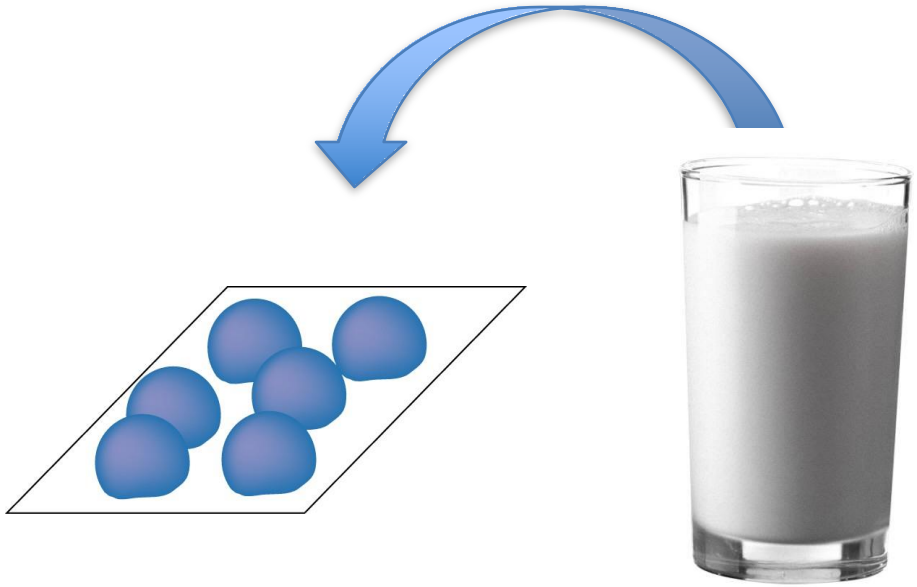
Stained

Blot



Blocking

Saturation of free hydrophobic spots on the membrane Avoids aspecific 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

Antibody Bound

SECONDARY ANTIBODIES

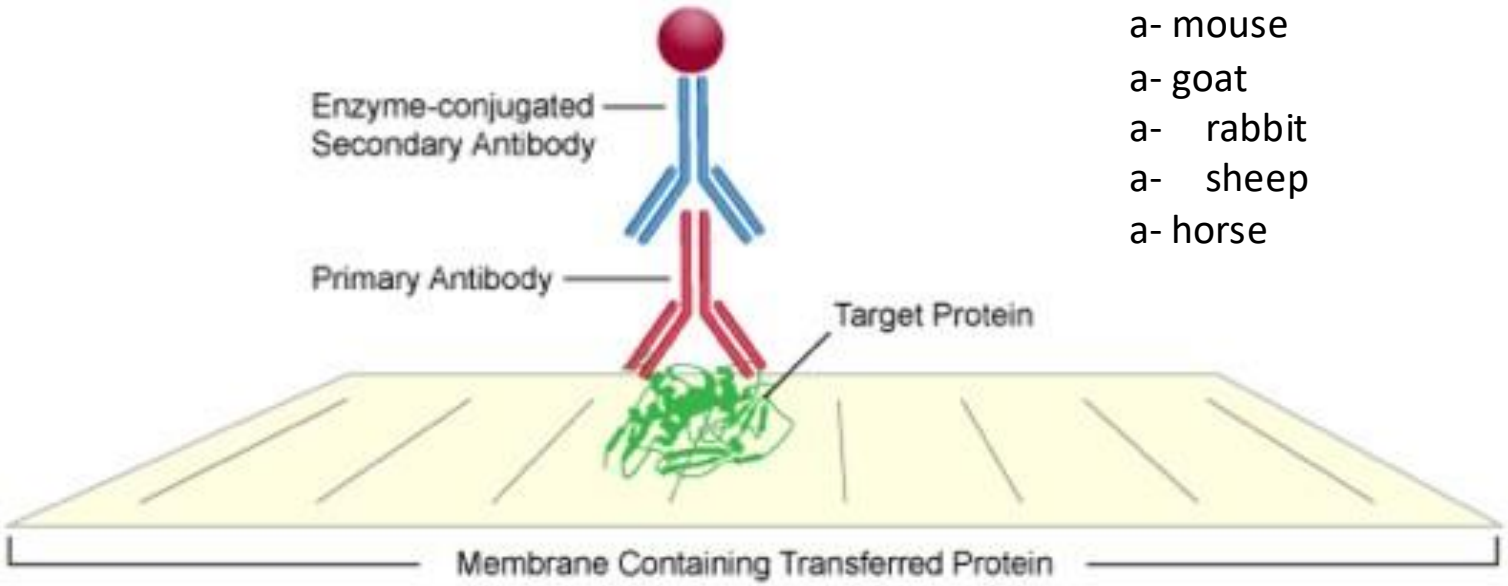
a- mouse

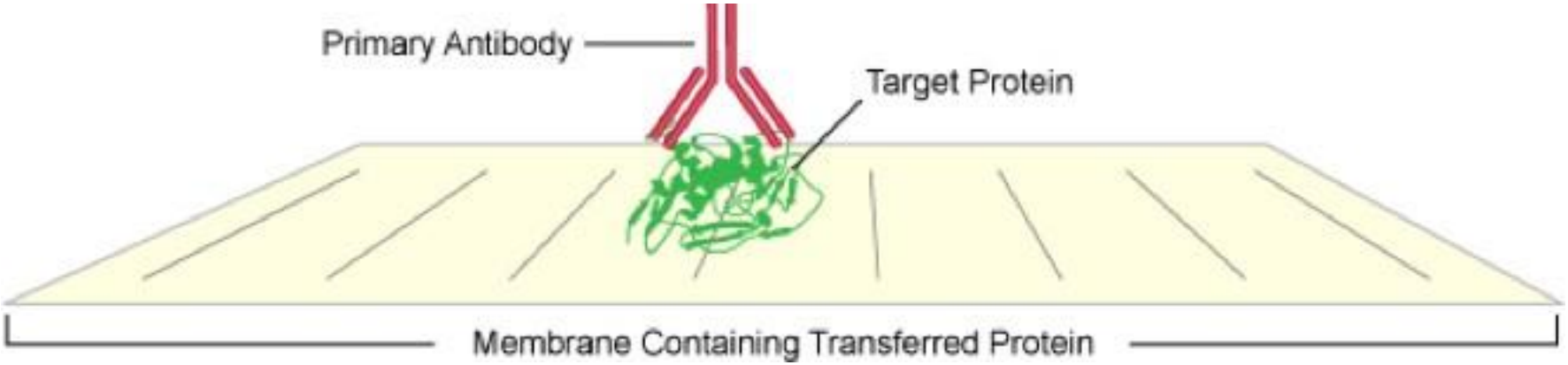
a- goat

a- rabbit

a- sheep

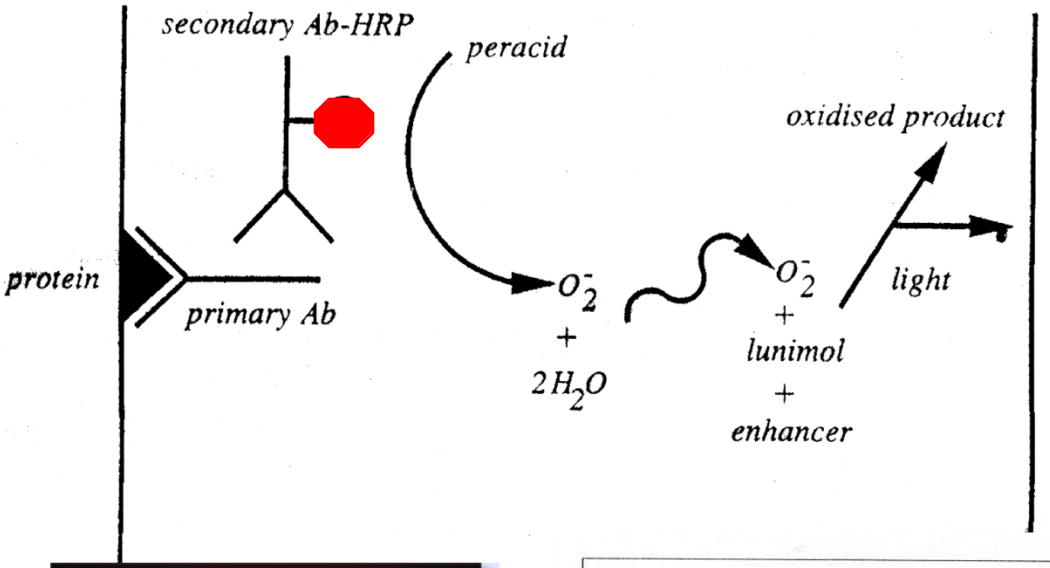
a- horse





ECL (Enhanced Chemio-Luminescence) method

membrane **Autoradiographic film**



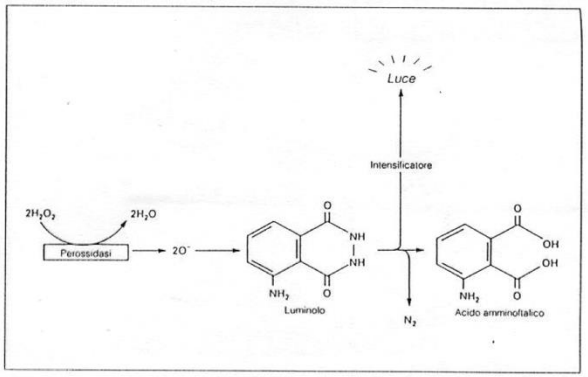
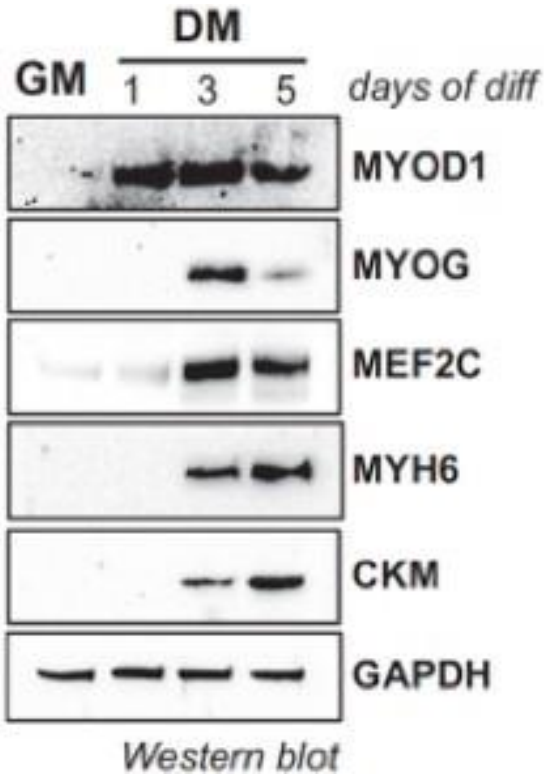


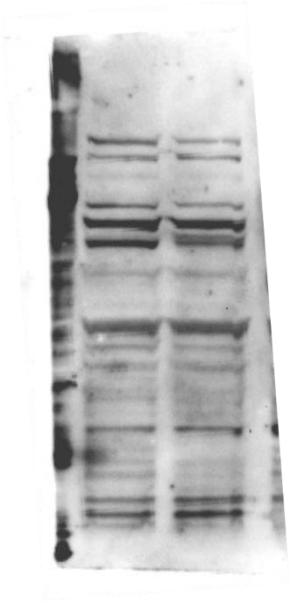
Figura 9.14 Uso della chemiluminescenza intensificata per la rivelazione della perossidasi di rafano.

The substrate is metabolised by HRP (peroxidase) emitting light

Protein Detection



Adapted from Ballarino et al, 2015



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

Methods in Protein Analysis

- Protein extraction
- Protein electrophoresis
  - Naïve vs denaturing conditions
- Identification of proteins
  - Mass spectrometry
  - Western Blot
    - Antibodies
- Recombinant protein
- Immunoprecipitation/Pull down

Protein Tagging

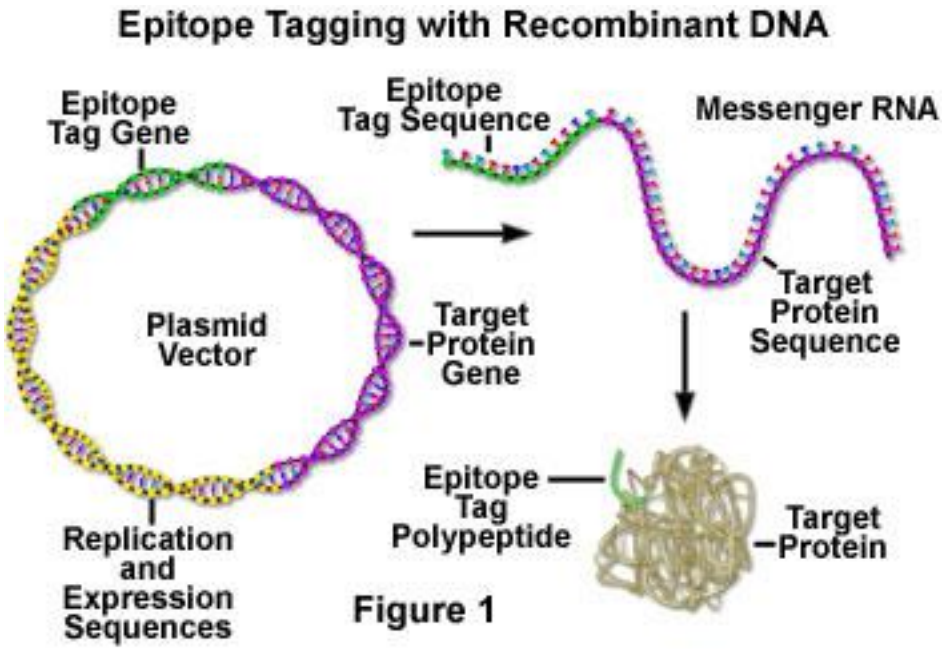
•Protein Tagging is a strategy for fusing a protein to a well- characterized peptide. The peptide (TAG) confers the protein with the possibility to go through easy/detection purification, allowing to isolate it in big amounts or to identify multiprotein or RNA/DNA/protein complexes.

•Tagged proteins can be obtained by cloning into expression vectors:

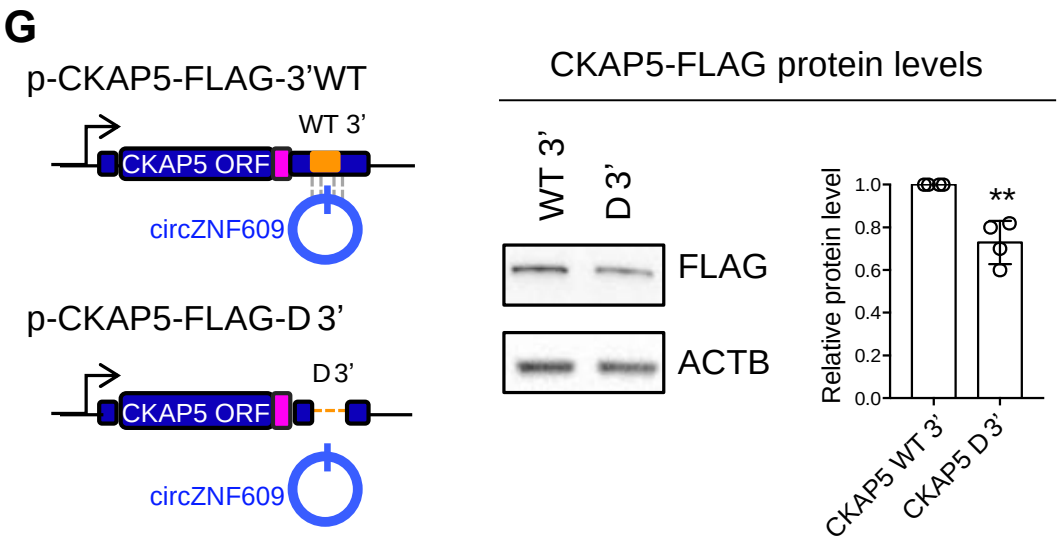
DNA encoding for the protein + DNA encoding for the Tag

The fusion protein is a **recombinant protein**

Protein Tagging



- Protein TAGs
  - FLAG
    DYKDDDDK
  - MYC
    EQKLISEEDL
  - V5
    GKPIPNPLLGLDST
  - HA
    YPYDVPDYA



Recombinant proteins

- Biomedical research
- Commercially relevant factors
- Therapeutic molecules

Recombinant vaccines
Cytomegalovirus
Diphtheria
Hepatitis B
Hepatitis C
Influenza
HIV
Malaria
Poliomyelitis

Hormones
ACTH
TSH (Tiretropin)
HGH (Growth hormone)
EPO
Somatotropin
Calcitonin
Glucagon Insulin

Peptide bioactive
Interferon
Interleuchin

Recombinant proteins in biomedical research

Factor VIII
Hemoglobin

Inibitori di proteasi

Growth factors
HNG (human nerve growth factor)
BGNF (brain derived neurotropic factor)
NT-3 (Neurotrophin-3) NT-4 (Neurotrophin-4)
GDNF (gliale-derived neurotrophin)
CNTF (Rat ciliary neurotrophin)

Leptin
Protein secreted by adipose cells in order to regulate the fat mass.

In order to express a protein in an heterologous system we need:

Expression vector + Expression host

Expression Host

Genes can be theoretically expressed in any system

The choice depends on the aim and on the protein features

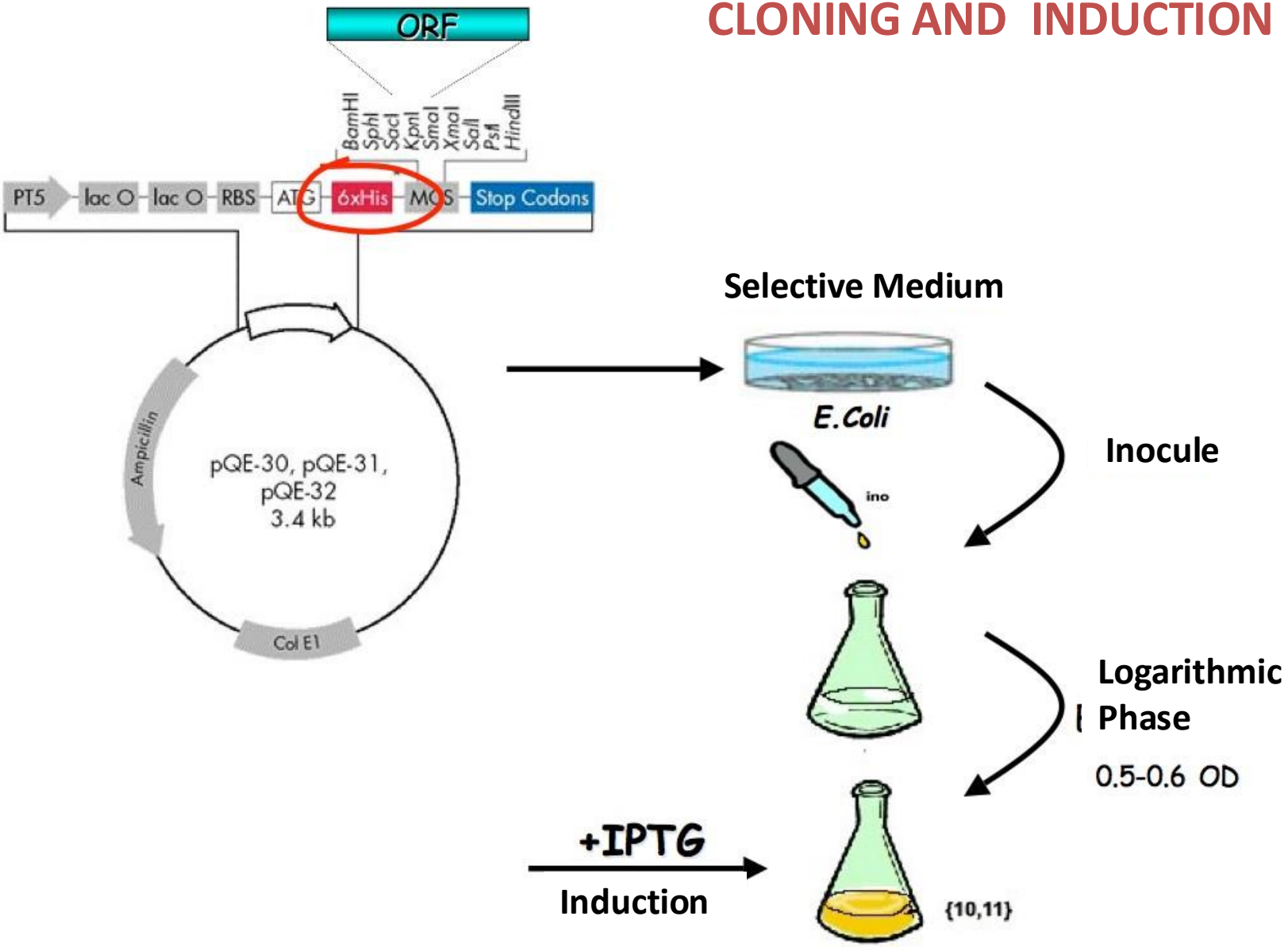
Bacteria	*Escherichia coli* *Bacillus subtilis*	
Fungi	*Sacarocmices cerevisiae* *Aspergillus nidulans*	
Plants	*Arabidopsis thaliana,* *Nicotiana tabacco*	cellule in coltura protoplasti piante transgeniche
Insects	*Dorifera californica* *Drosophila melanogaster*	cellule in coltura *org*anismi interi
Animals		oociti cellule in coltura organismi interi

	Pros	Cons
Bacteria	• Simple • Short generation time • High yeald • Low costs	• Misfolding • Inclusion bodies • Possible toxicity of exogenous proteins • Few post-translational modifications
Yeast	• Simple • Short generation time • High yeald • Low costs • Post-translational modifications	• Active proteases • Possible toxicity
Insects Plants Animals	• Post-translational modifications	• More expensive systems

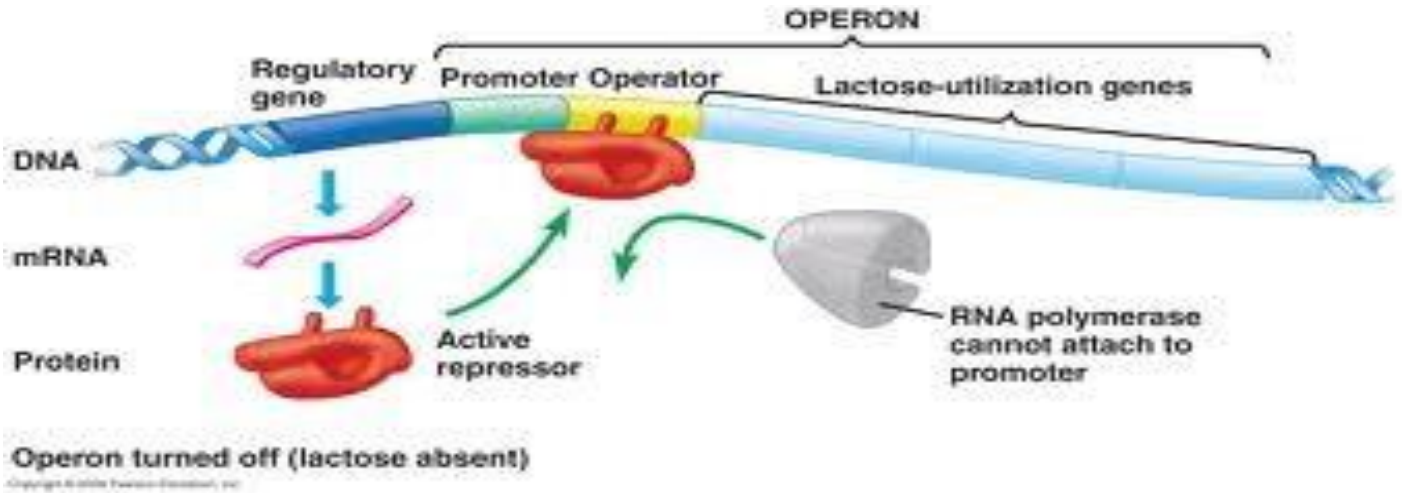
Why do we express proteins in heterologous systems?

- Big amounts of products
- Expression in higher organisms can be difficult due to gene regulation
- Simple model systems are easy to be obtained and manipulated

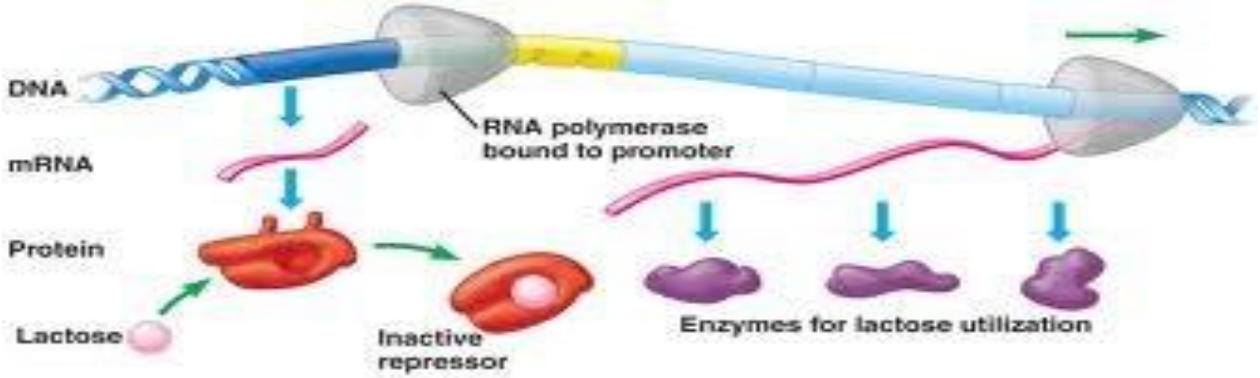
CLONING AND INDUCTION



OPERONE LAC

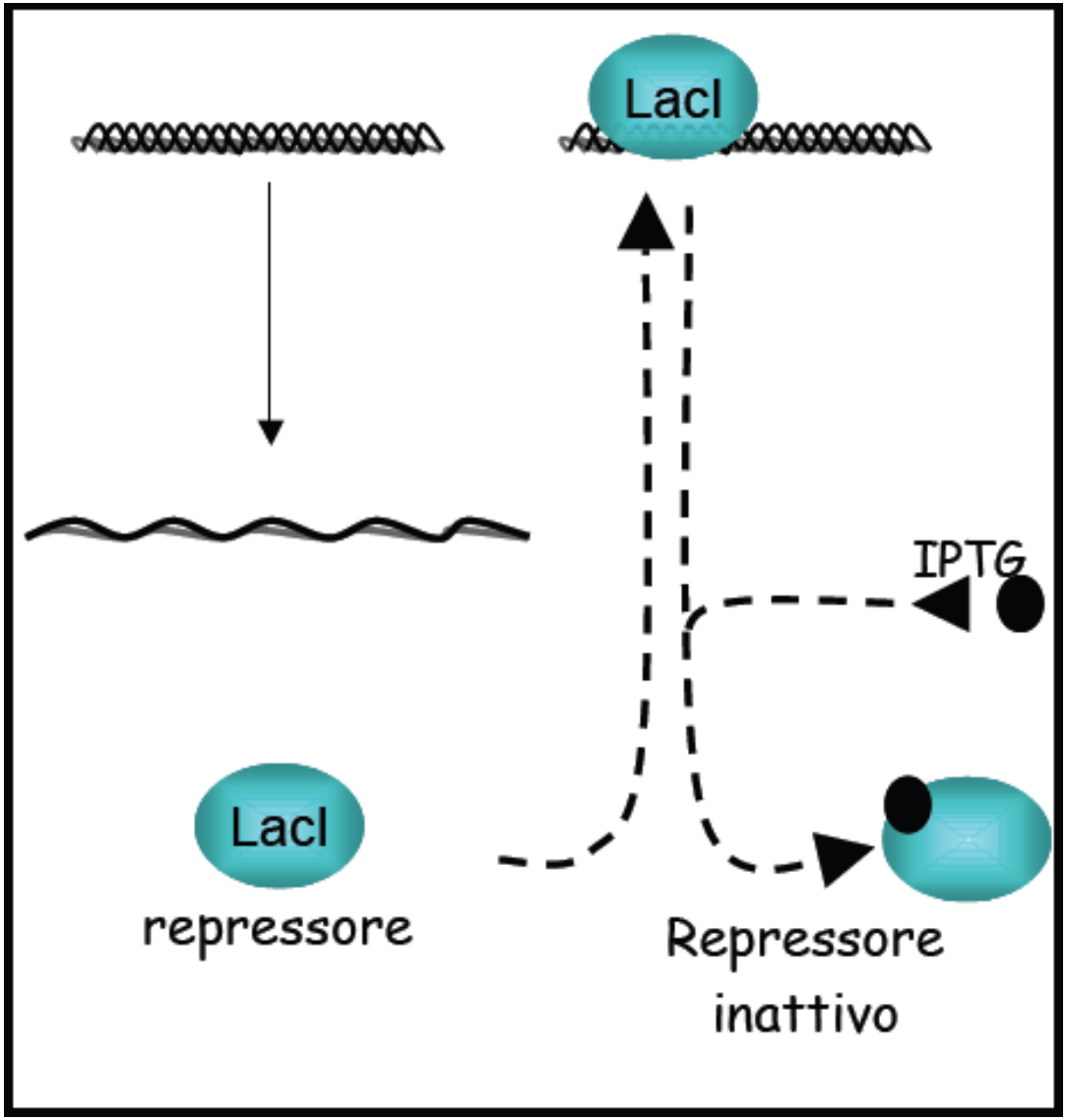


Operon turned off (lactose absent)

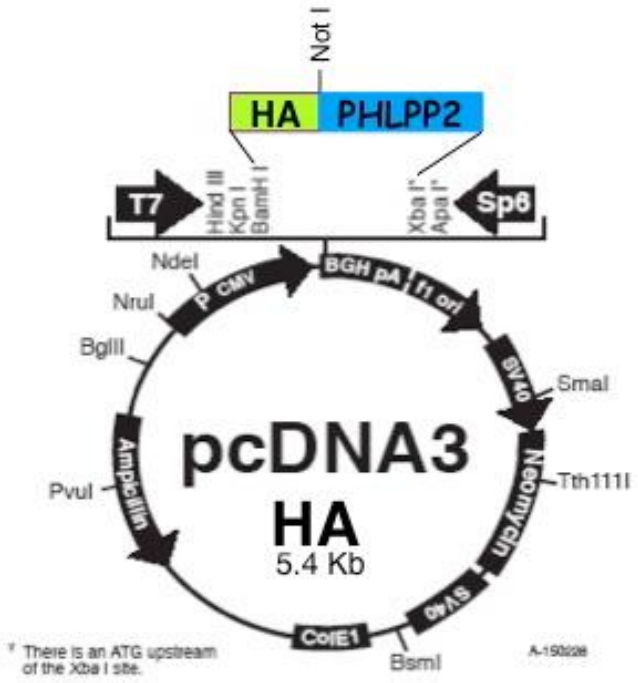


Operon turned on (lactose inactivates repressor)

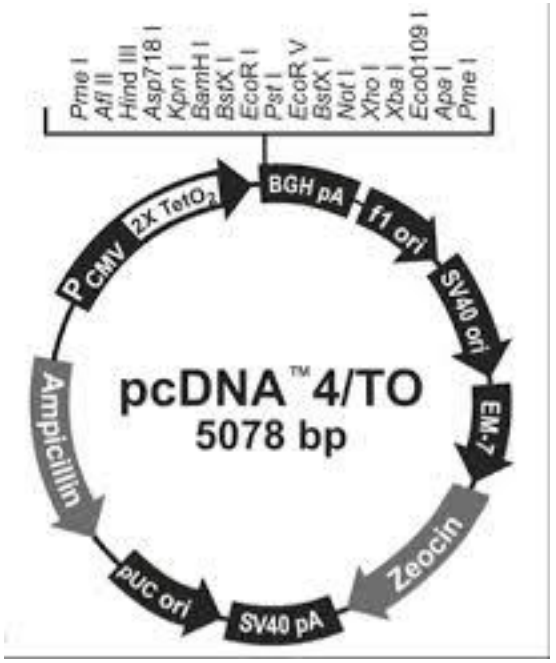
Procariotic gene expression is regulated



Eucariotic Expression Vector



Constitutive expression



Inducible expression

Expression and Purification of a recombinant protein

1. **TRANSFORMATION (Expression vector in the expression host)**.

2. **AMPLIFICATION of the positive bacterial/yeast strain**.

1. **INDUCTION of the recombinant protein.**

2. **PURIFICATION of the recombinant protein**.

Methods in Protein Analysis

- Protein extraction
- Protein electrophoresis
  - Naïve vs denaturing conditions
- Identification of proteins
  - Mass spectrometry
  - Western Blot
    - Antibodies
- Recombinant protein
- Immunoprecipitation/Pull down

Purification of a (recombinant) proteins

1. Purifications of proteins
   - Antibodies
   - TAG

2. Purifications of proteins

→**Protein production**

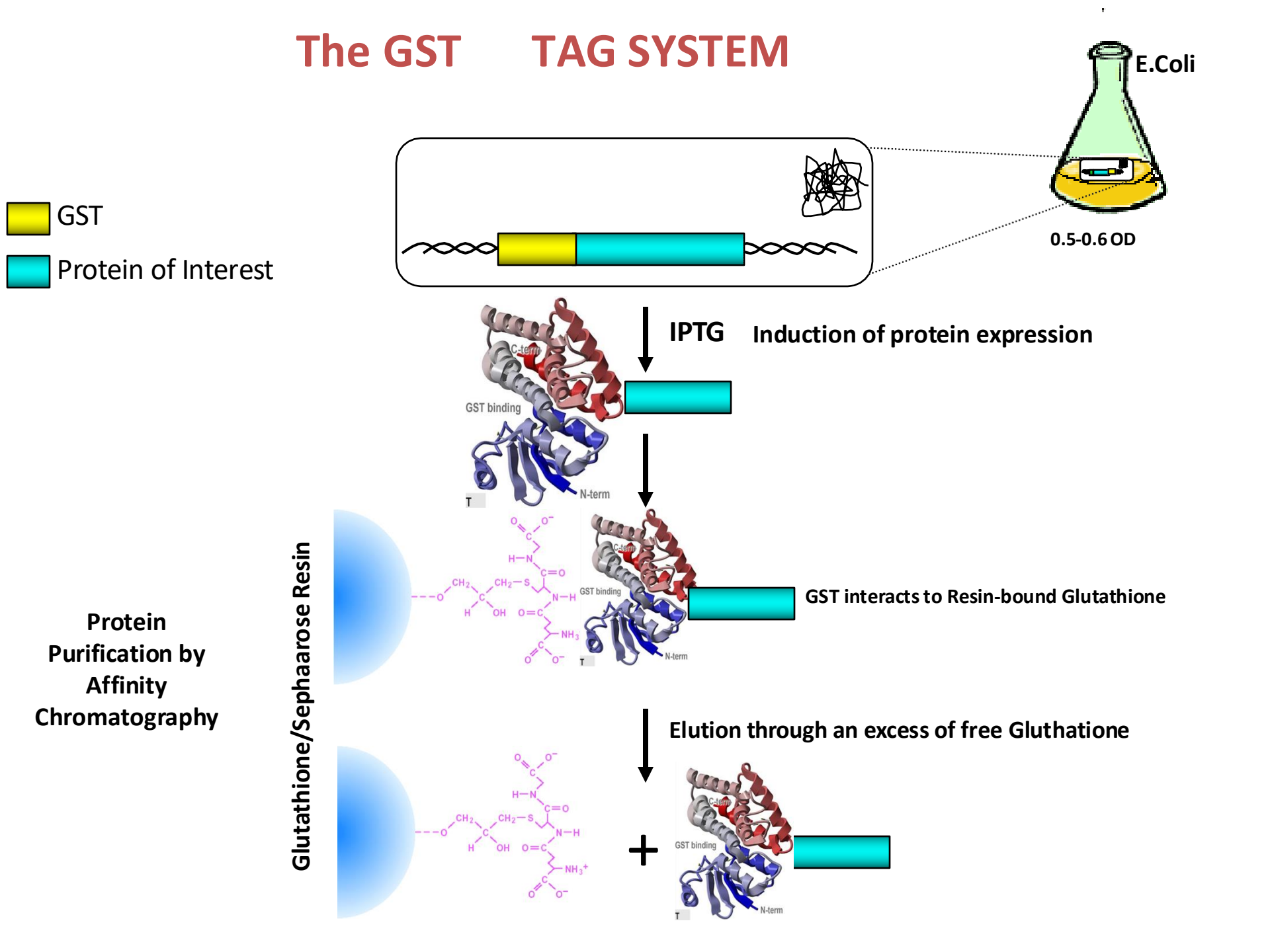
→**Interaction studies**

Purification of proteins: TAGs

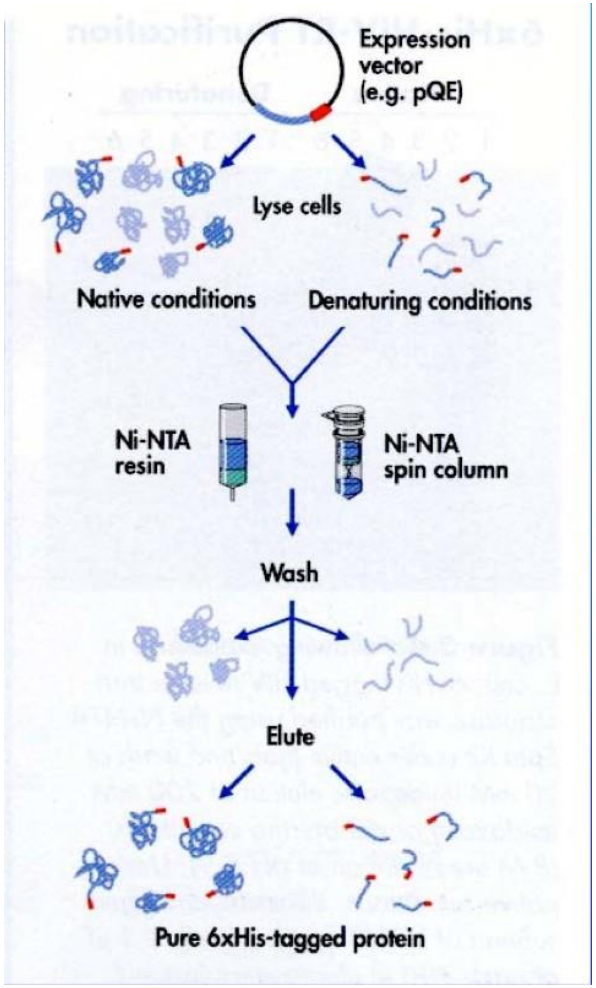
Tag	Description	Affinity ligand
• **His_6**	6 histidines	Ni++, Co++, Cu++
• **GST**	glutathion-S-transferase	glutathion
• **TAP**		
• **FLAG**		
• **HA**		Maltose
• **MBP**	Maltose binding protein	IgG
• **Protein A**	Protein A	Calmodulin
• **CBP** (40kDa)	Calmodulin binding protein	

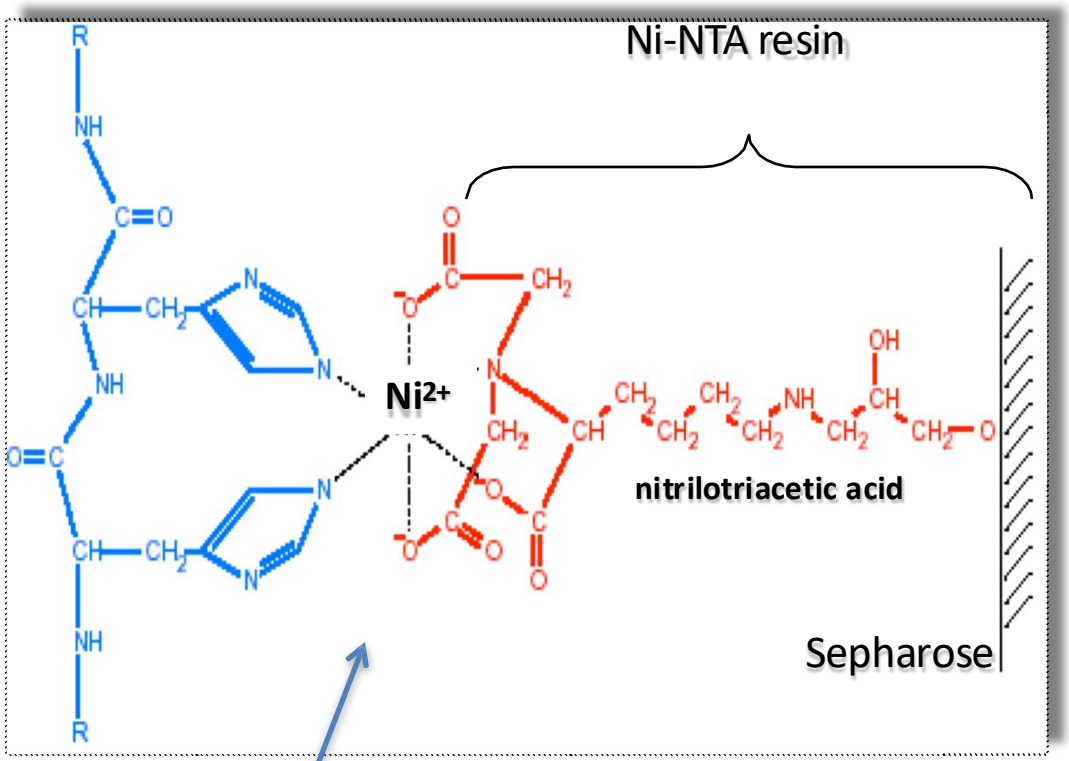
TAGs confer to proteins 2 properties:
specific affinity for a ligand, specific recognition from an antibody

The GST TAG SYSTEM



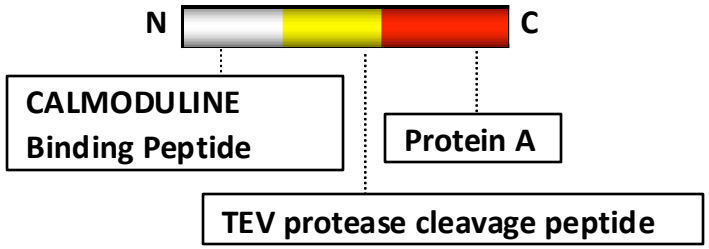
The HIS TAG System

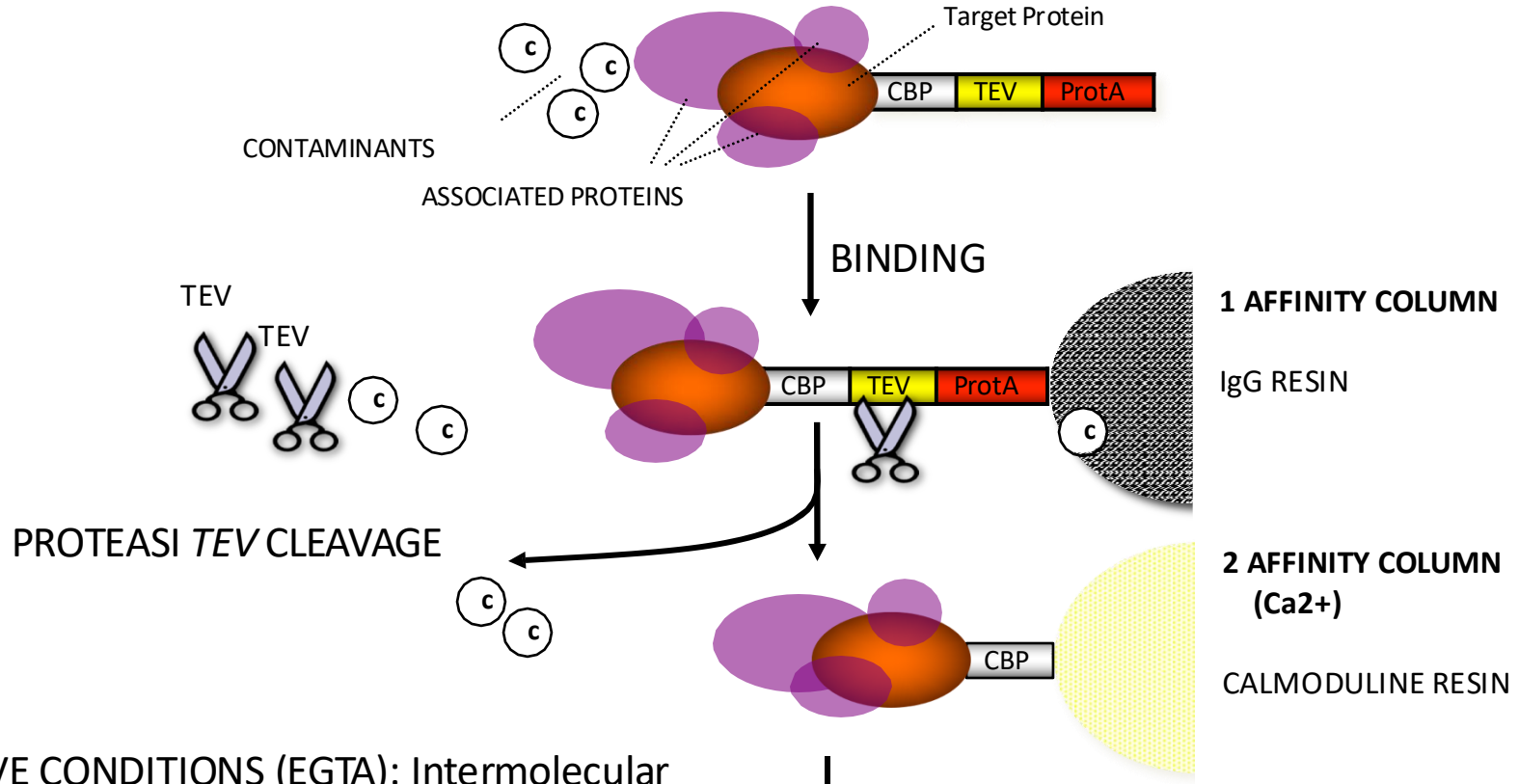




Elution By Imidazole (a histidine analogue)

Tandem Affinity Purification (TAP) System



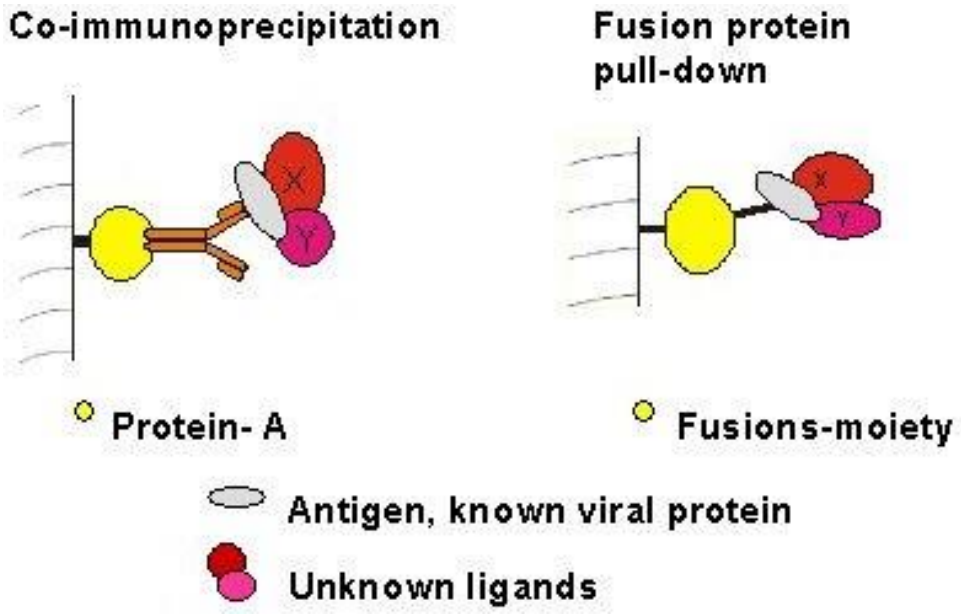


ELUITION IN NATIVE CONDITIONS (EGTA): Intermolecular Interactions are preserved, thus….

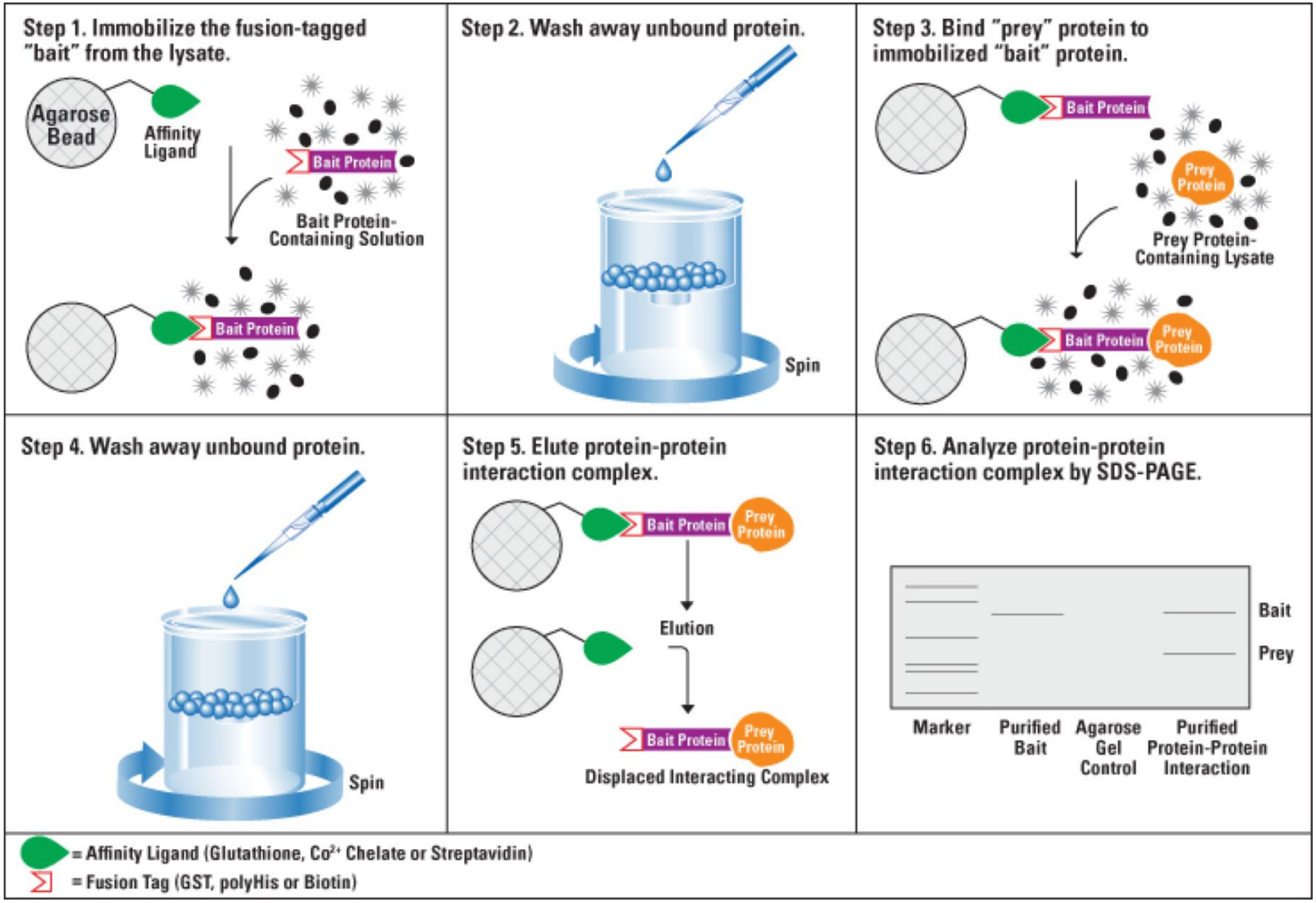
Protein Pull Down Assay

TAGGING (and consequent affinity for a ligand) can be exploited to verify protein interactions

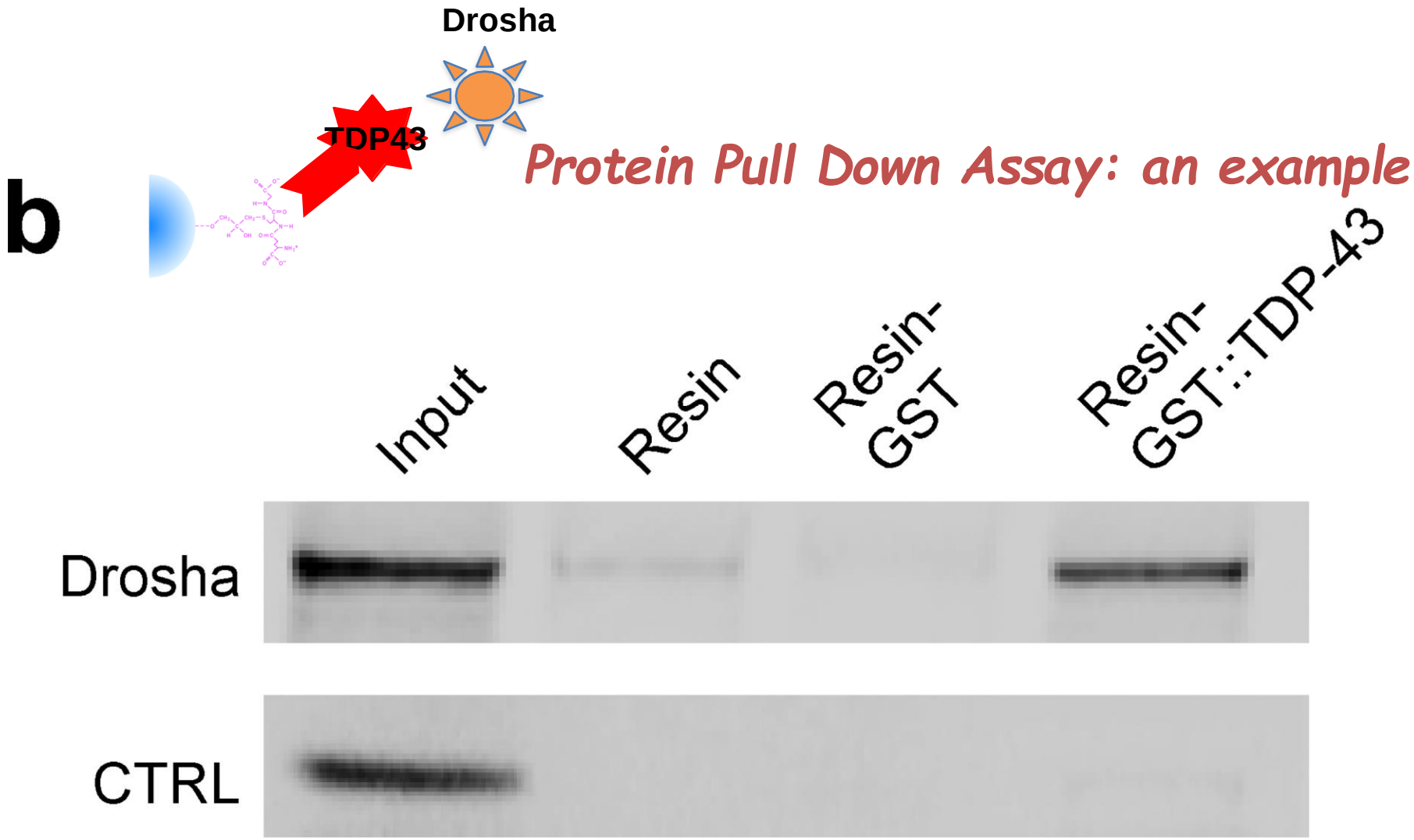
The tagged protein is incubated with an homologous cell extracts and partners can be identified also in the absence of a specific antibody (alternative to co-immunoprecipitation)



Protein Pull Down Assay



Protein Pull Down Assay: an example



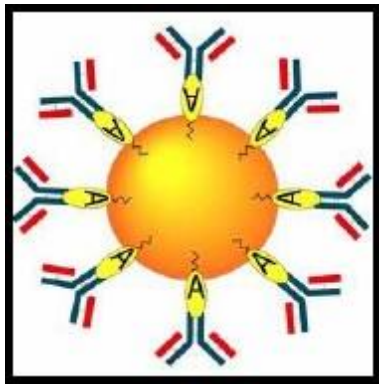
Purification of proteins: Antibody Immunoprecipitation

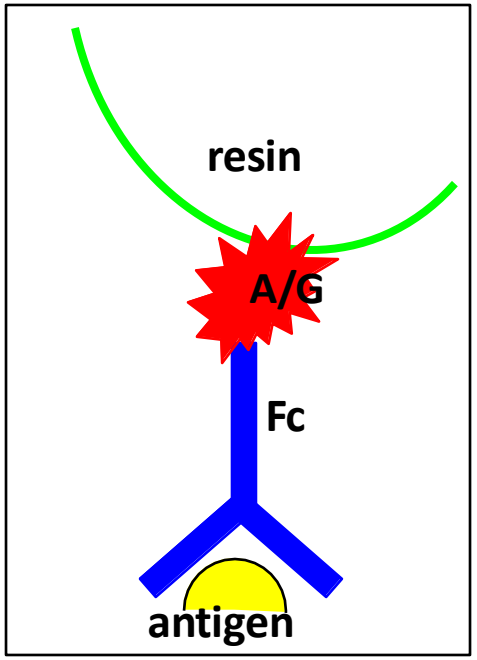
Isolation (enrichment) of antigen/antibody complexes

- **Requires: specific antibodies directed toward the target protein or its recombinant variant**
- **Allows: identification of ribonucleoprotein (RNP complexes)**

Immunoprecipitation: the role of protein A or G

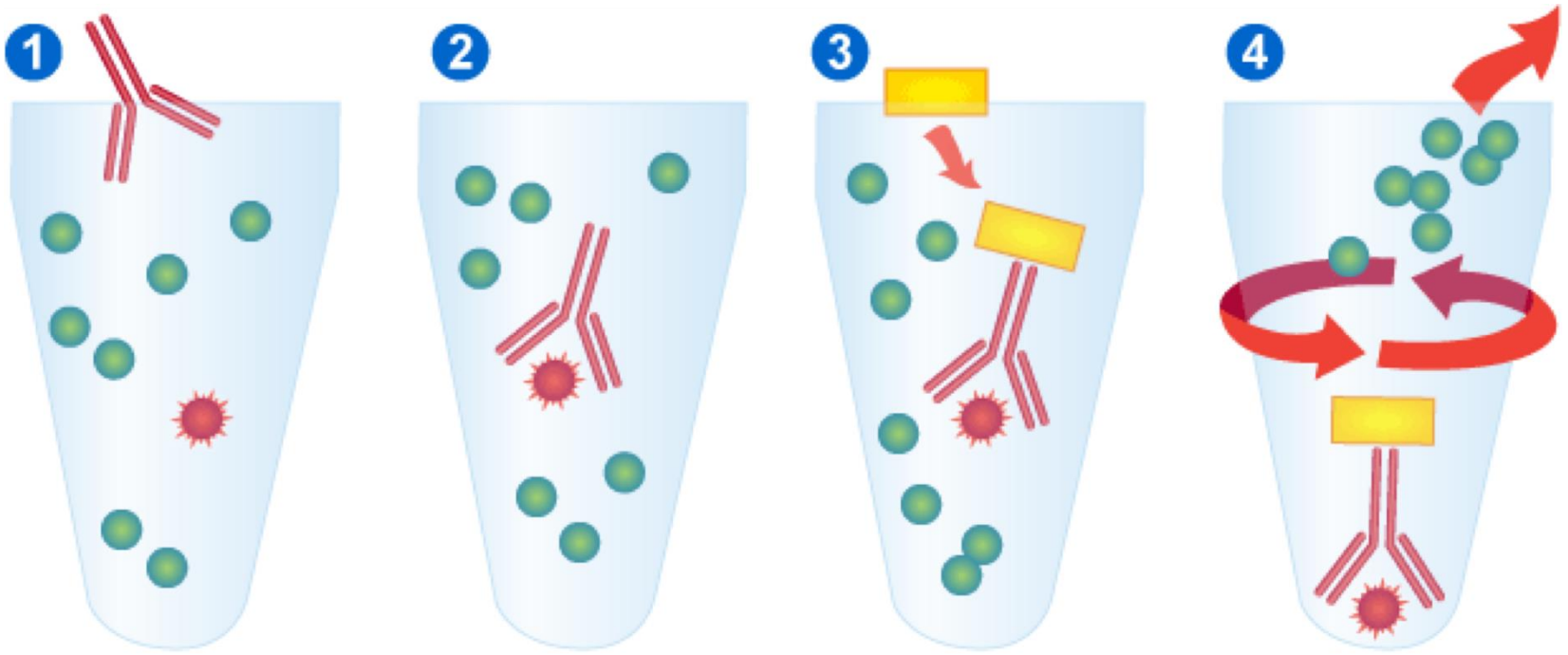
Antibodies specifically bind protein A or G from Staphylococcus, through their Fc region.



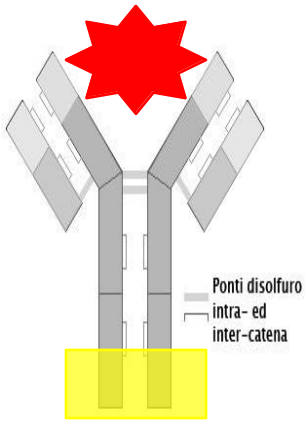


Binding Characteristics of Some Immunoglobulins			
Immunoglobulin		Protein A	Protein G
Mouse	IgG1	+	++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+++
	IgM	-	-
	IgA	-	-
	IgE	-	-
Rat	IgG1	+	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++

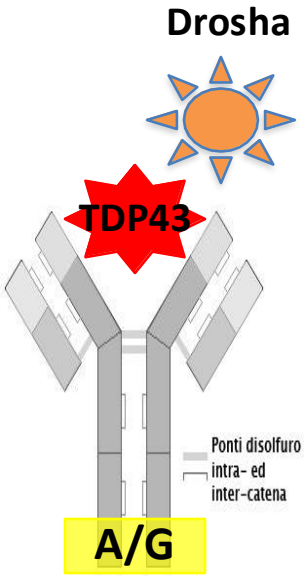
Immunoprecipitation (IP)



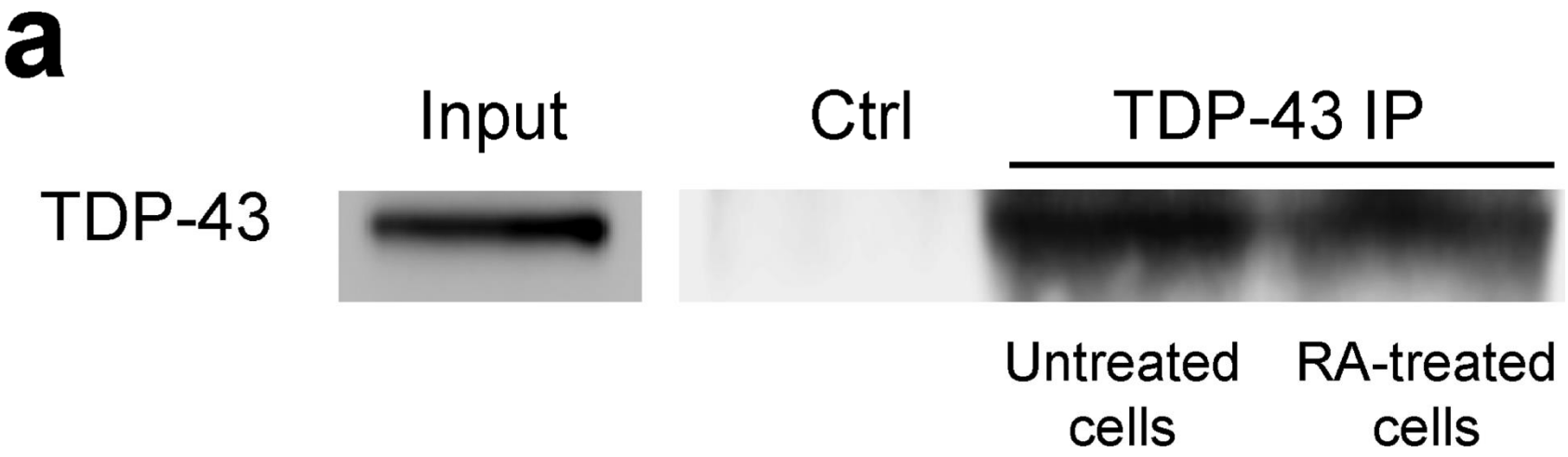
1. Suitable antibody is added.
2. Antibody binds to protein of interest.
3. Protein A or G added to make antibody-protein complexes insoluble.
4. Centrifugation of solution pellets antibody-protein complex. Removal of supernatant and washing.



Magnetic beads !

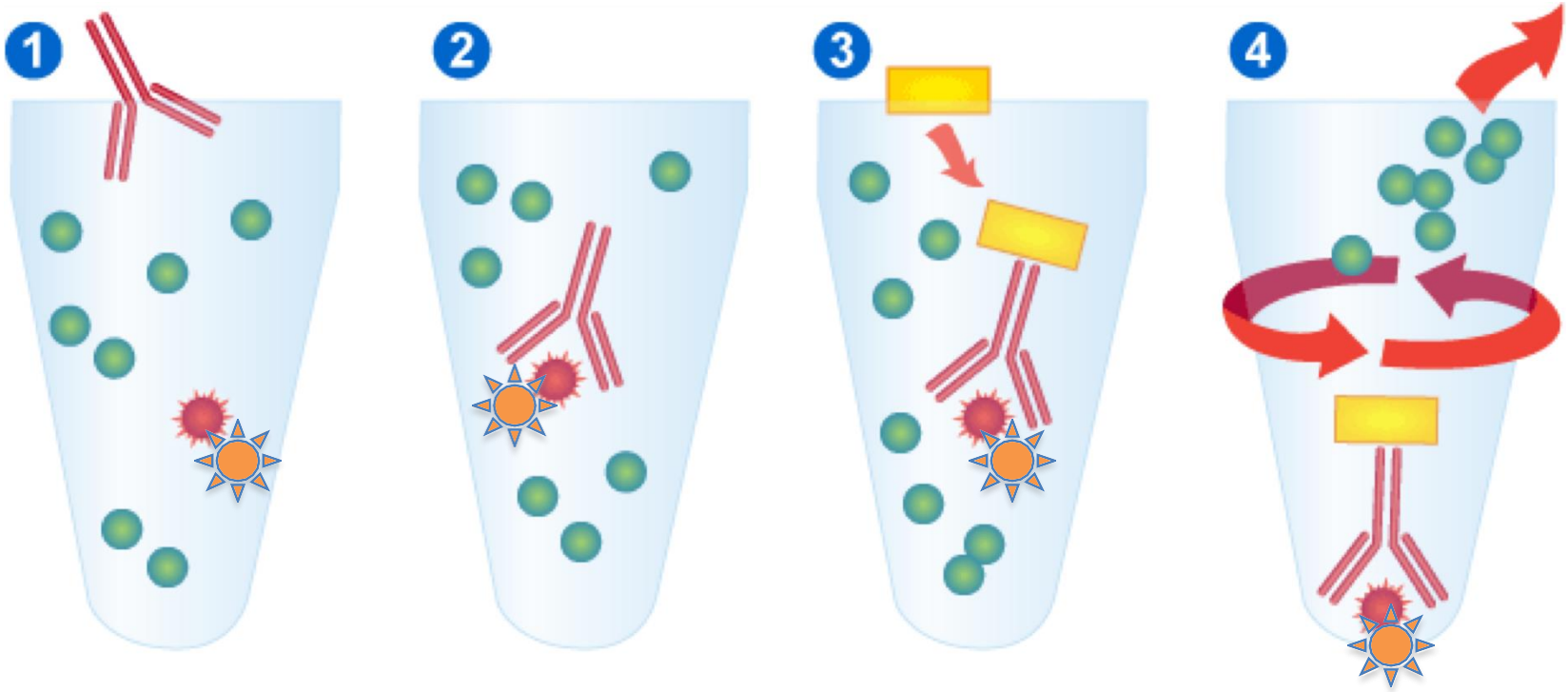


Immunoprecipitation: an example



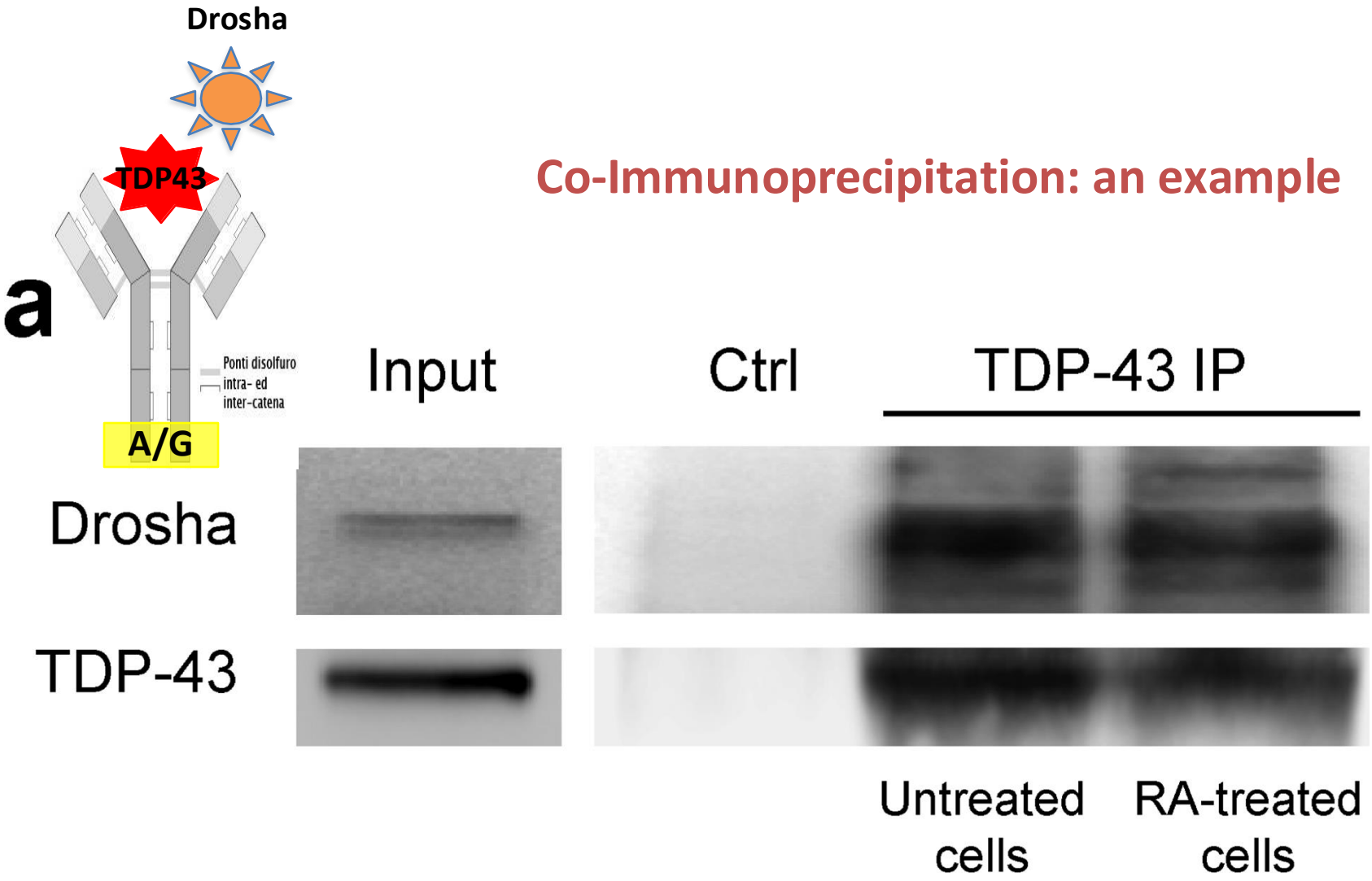
Di Carlo V. et al, 2013

Co-Immunoprecipitation (Co-IP)



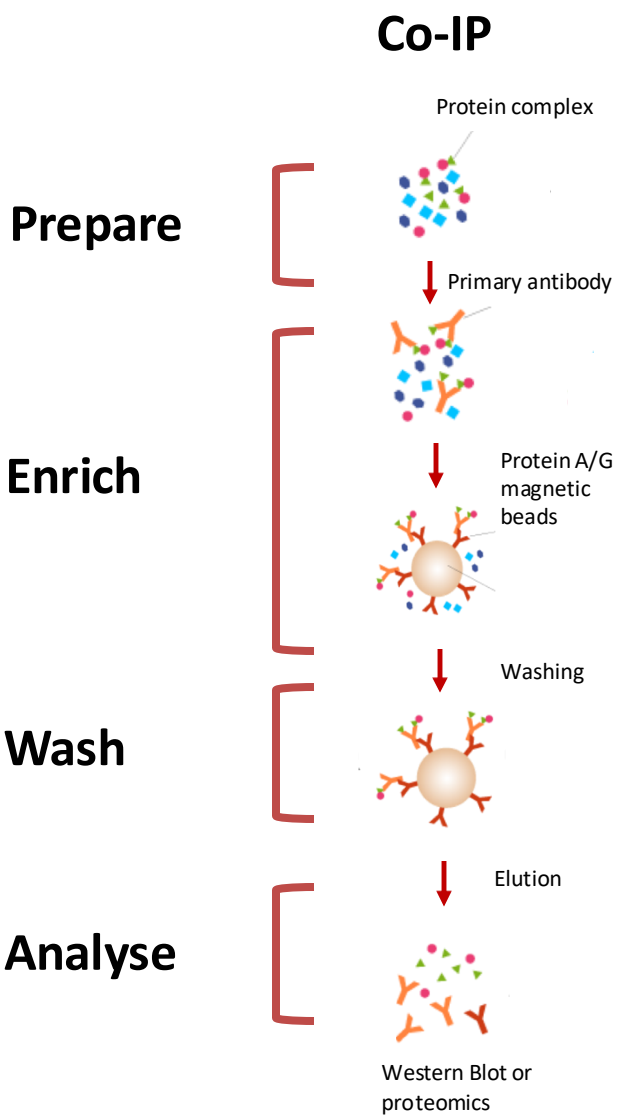
1. Suitable antibody is added.
2. Antibody binds to protein of interest.
3. Protein A or G added to make antibody-protein complexes insoluble.
4. Centrifugation of solution pellets antibody-protein complex.
   Removal of supernatant and washing.

Co-Immunoprecipitation: an example



Di Carlo V. et al, 2013

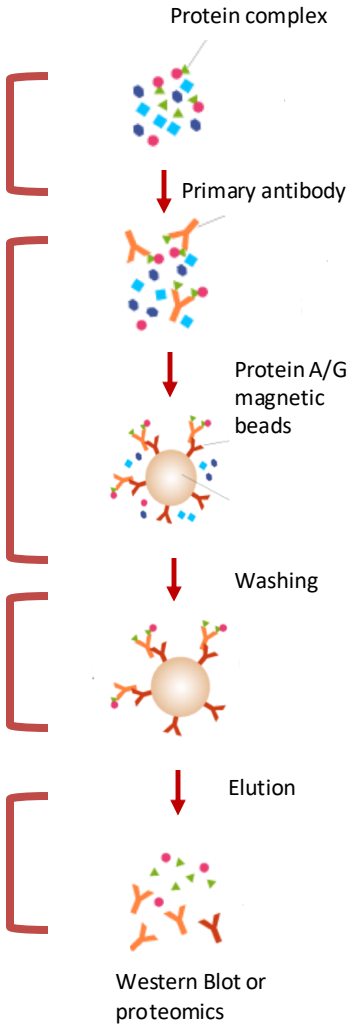
Immunoprecipitation (IP)



Immunoprecipitation (IP)

Finding molecular parterns:

Co-IP



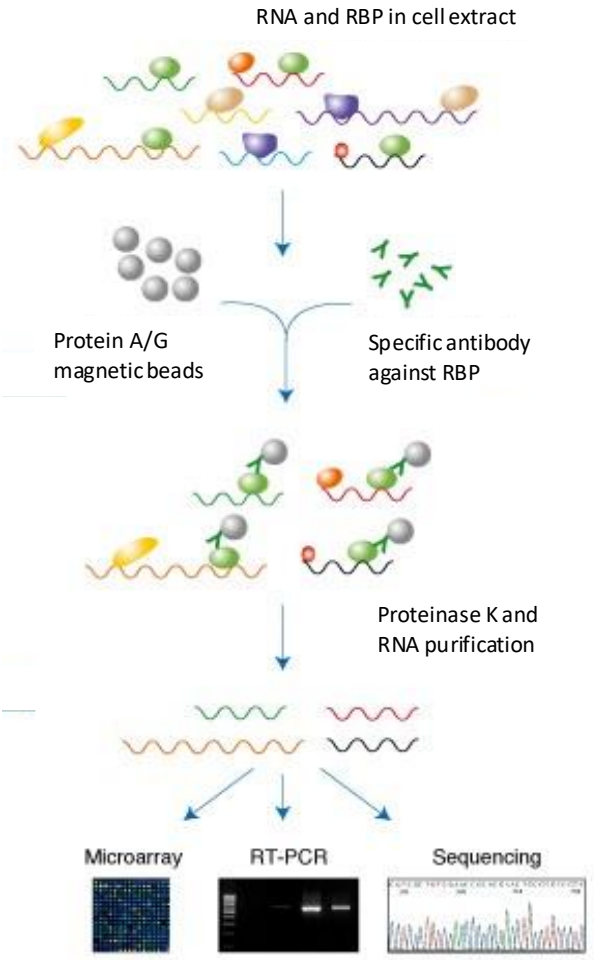
Prepare

Enrich

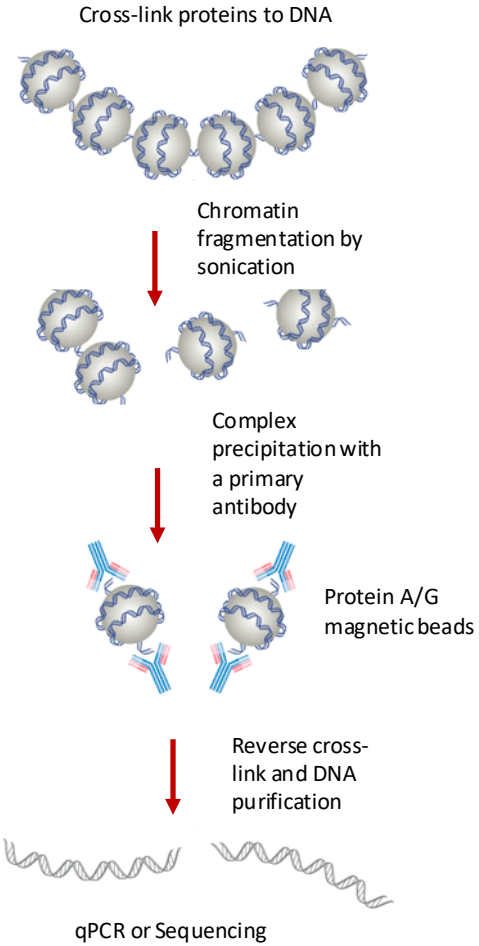
Wash

Analyse

RIP/CLIP

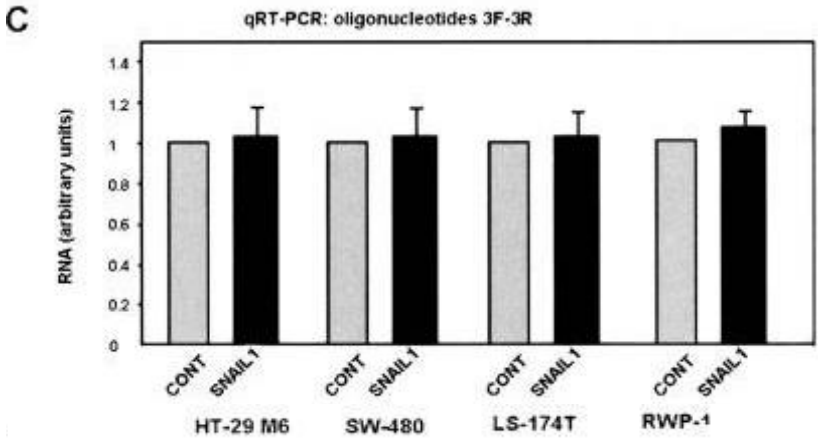


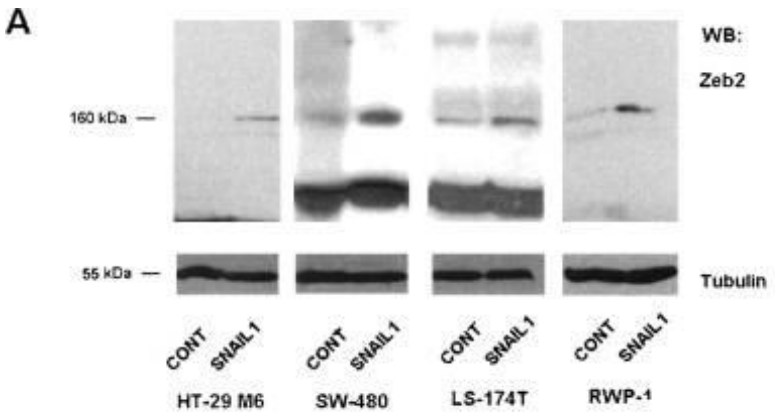
CHIP



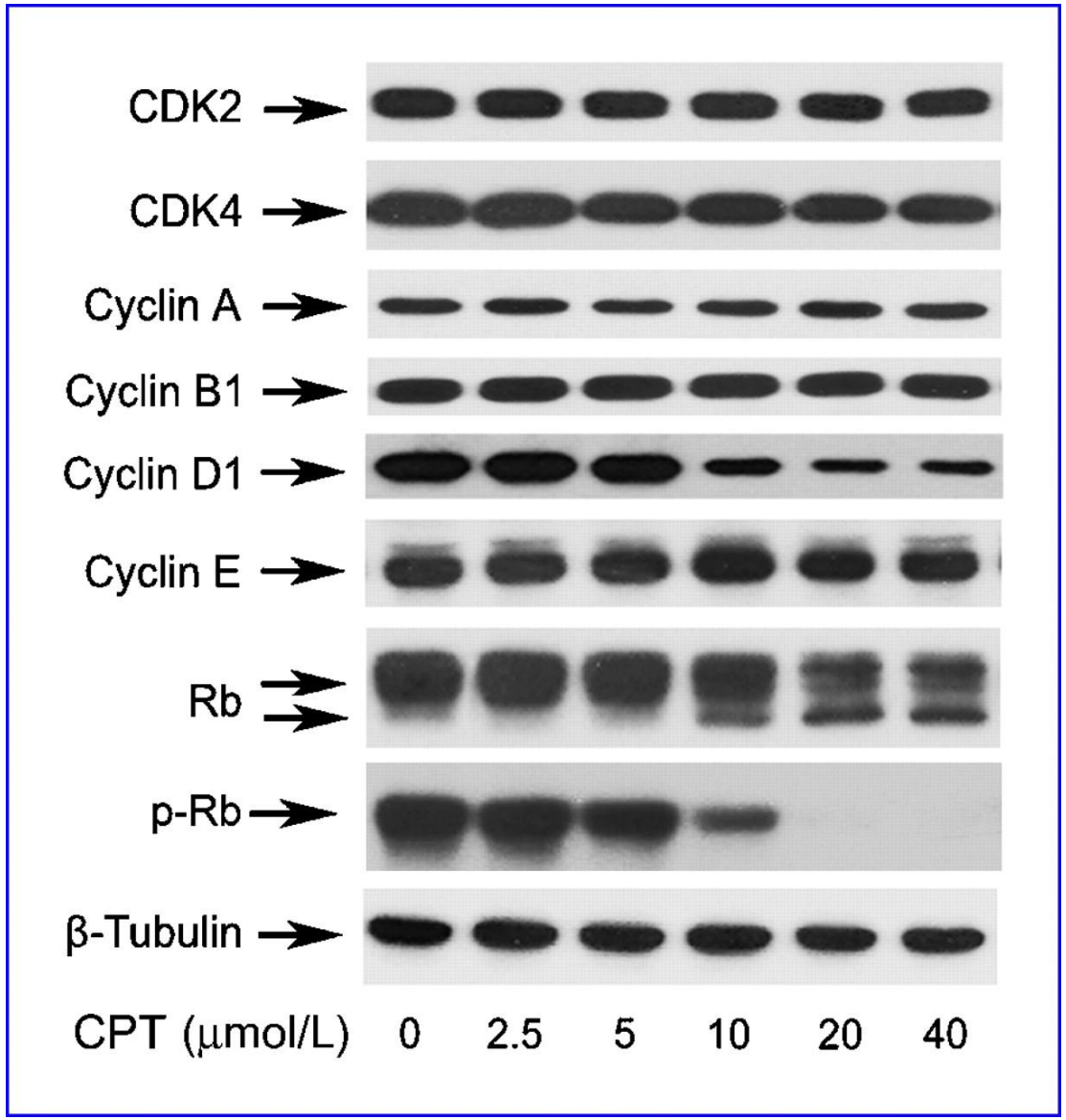
Exercise I

Zeb2 protein and cancer; RT-PCr shows no change in mRNA level during tumor progression but….





Exercise II



CTP is a drug to treat cancer cell cells ….

Exercise III

JAZF1-SUZ12 is an oncogenic fusion product in some cancers, How this fusion affects its molecular partners?