Methods in Protein Analysis

Western Blot

Recombinant Proteins

Immunoprecipitation

Protein Pull Down Assay

October 2020

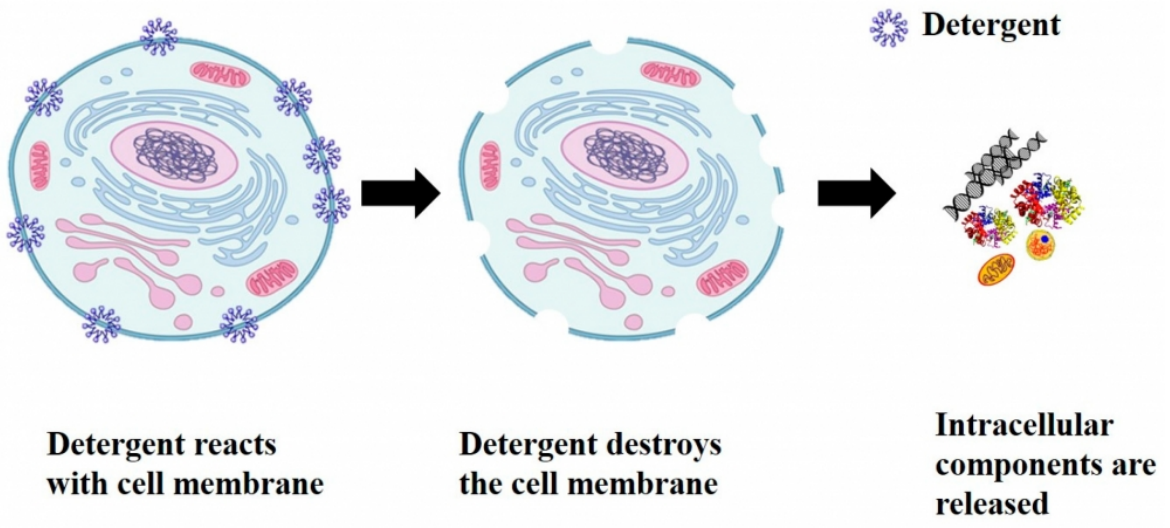
Protein Extraction

- 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 Electrophoresis

Harder than nucleic acid electrophoresis:

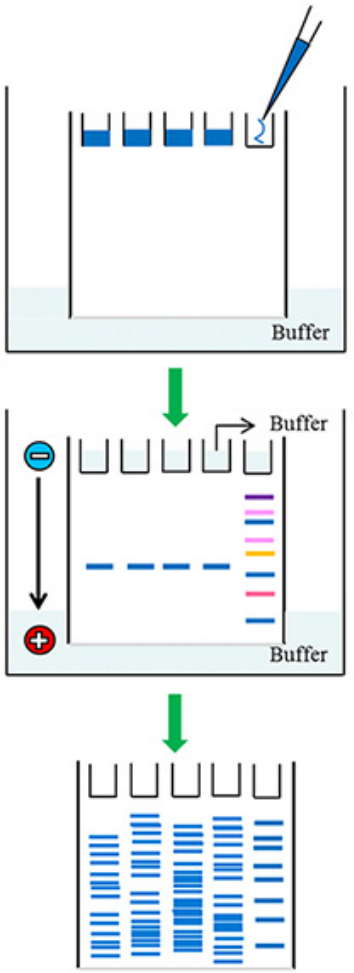
Proteins differ in charge

Proteins differ in conformation

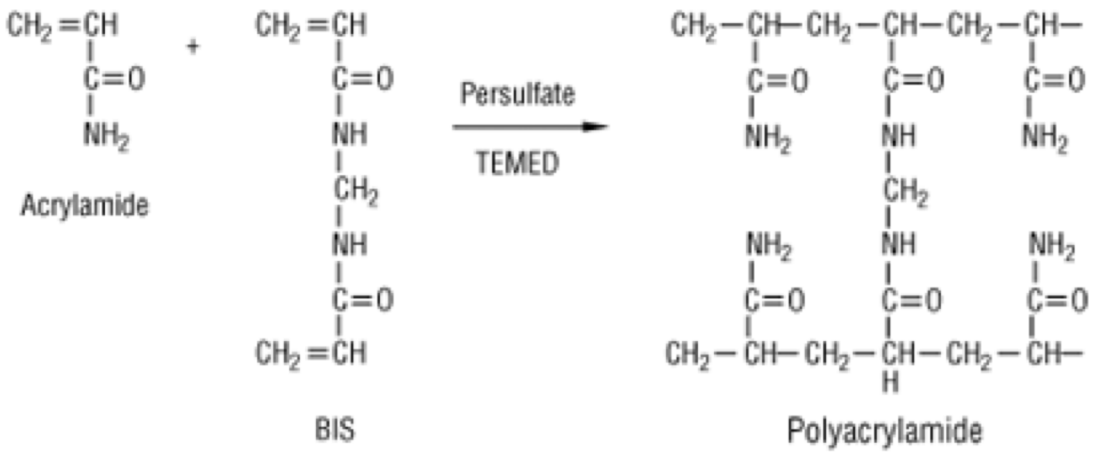
A polyacrylamide gel can be:

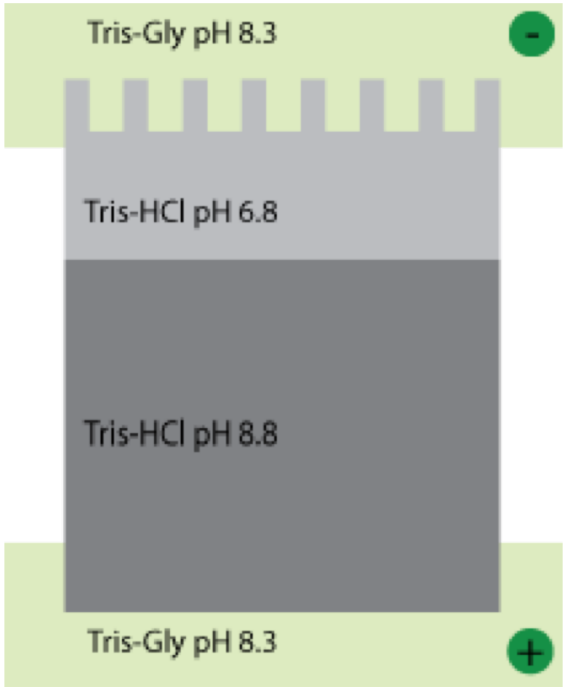
Native
(non denaturing conditions)

SDS-gel
(denaturing conditions)

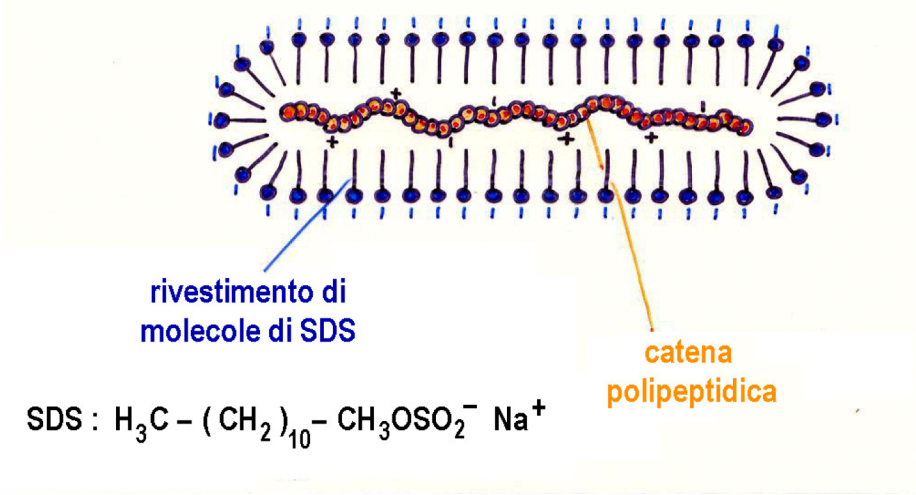


Polyacrylamide Gel



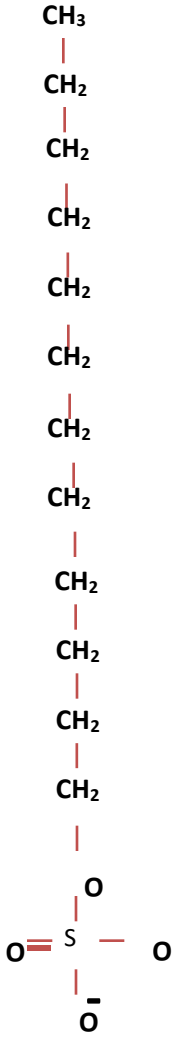


SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Denaturing conditions



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

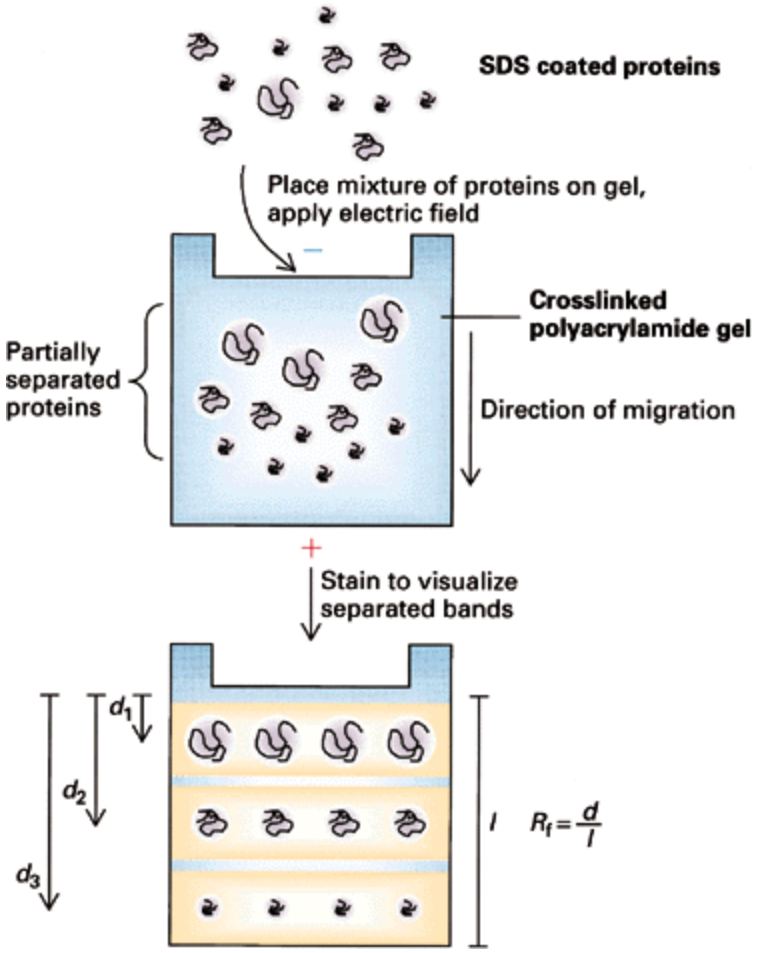
For each SDS denatured protein, the ratio between **m and q is constant**.



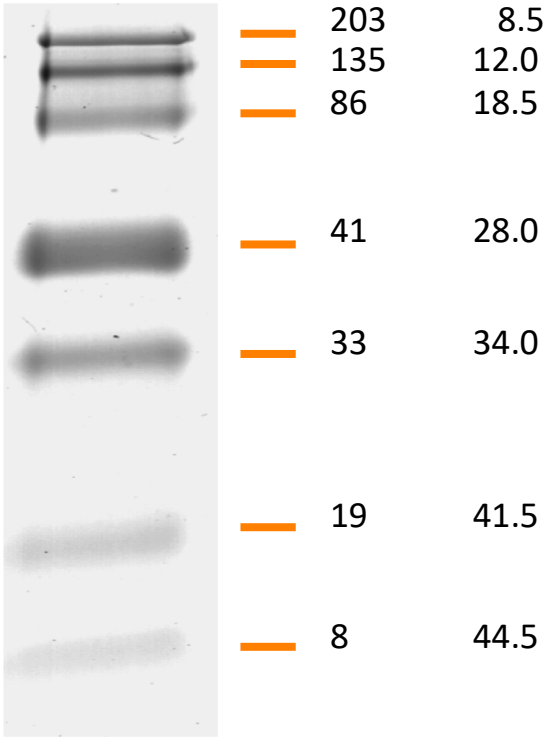
SDS

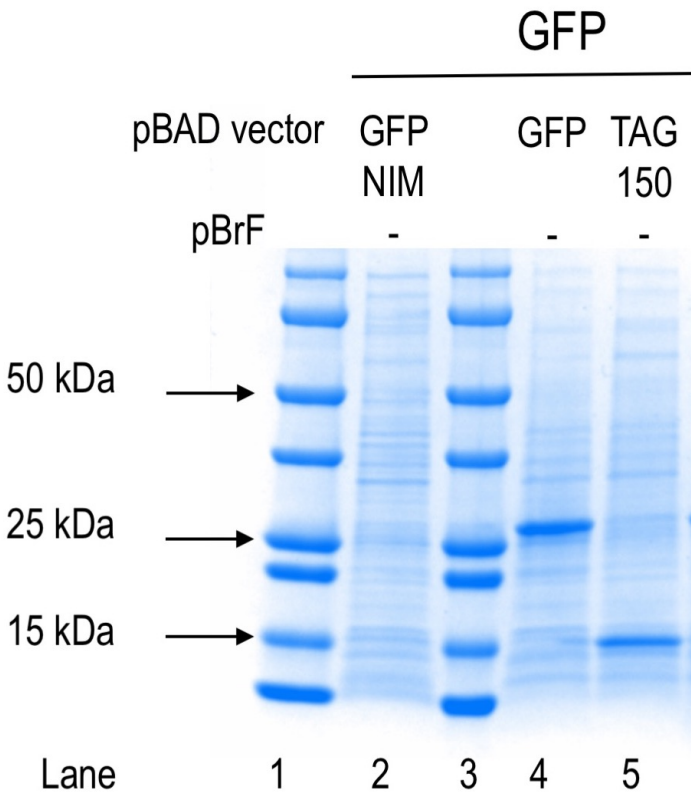
Considerations

- 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



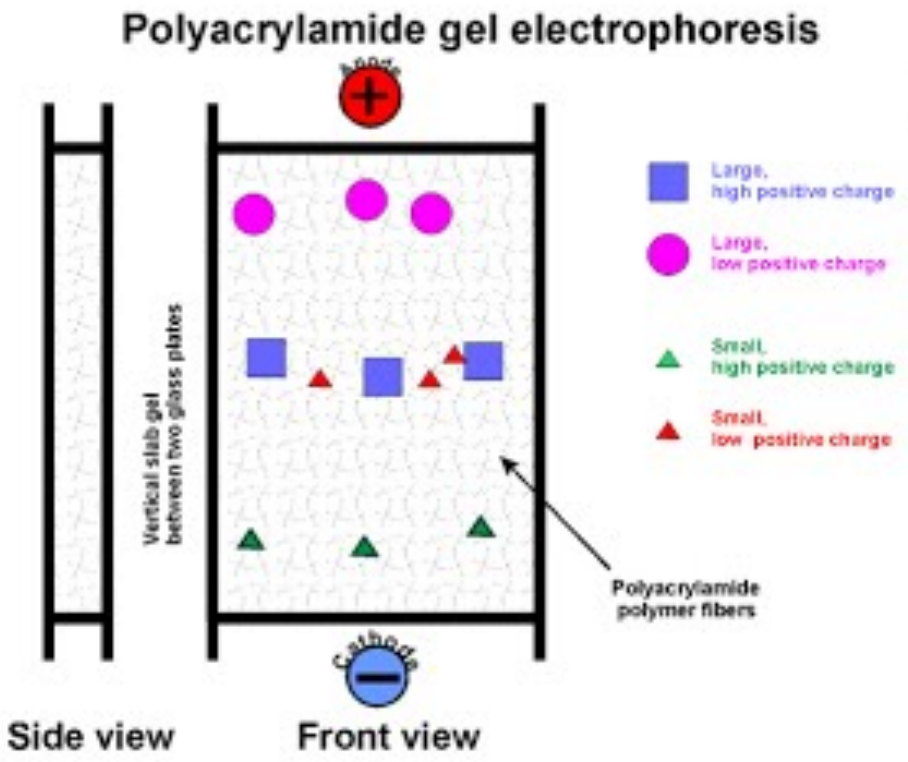
Molecular Weights





- We can separate proteins by mass.

Polyacrylamide Gel Electrophoresis (PAGE)



Native gel Electrophoresis

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

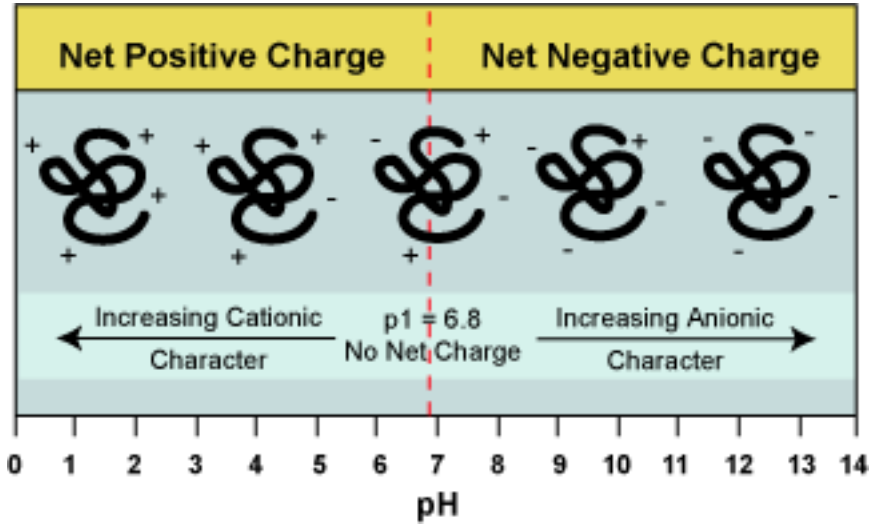
Proteins keep conformation

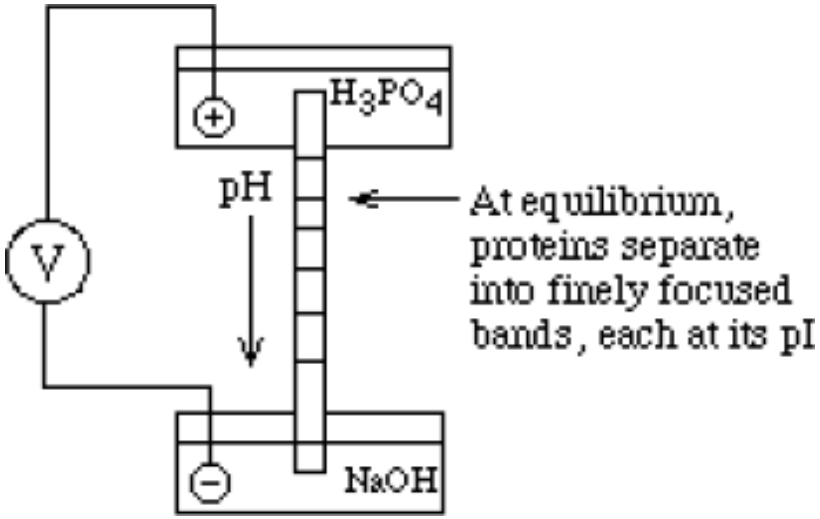
Isoelectric Focusing Electrophoresis (IFE)

-Fractionation based on Isoelectric Point (pI)

-The buffer generates a pH gradient

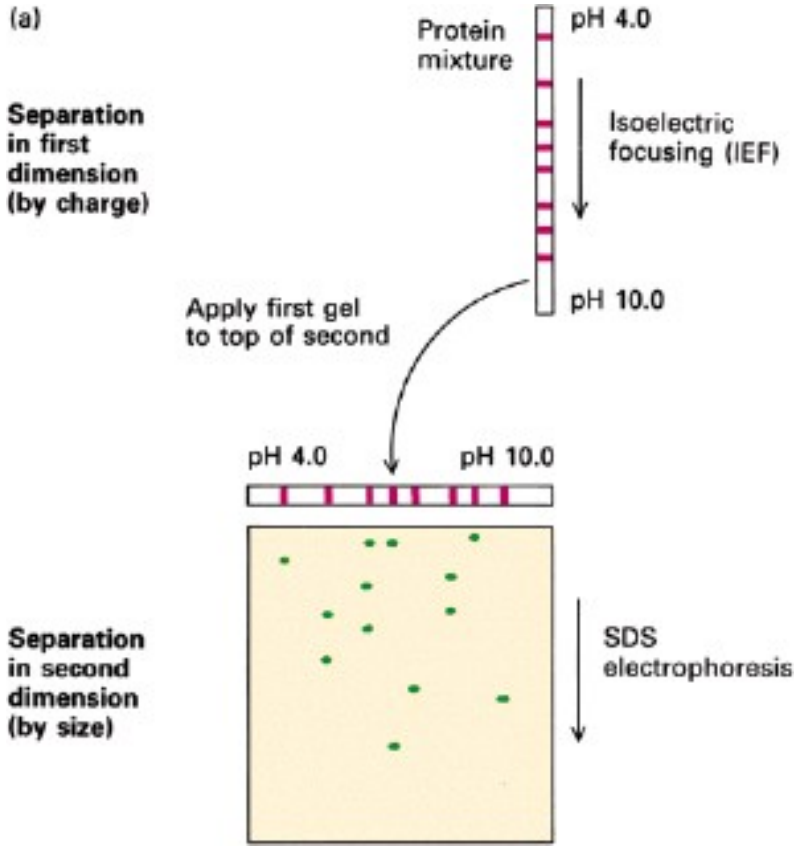
-When reaches the pI, the protein loses its charge (q=0) and stops in the gel





2D-PAGE

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

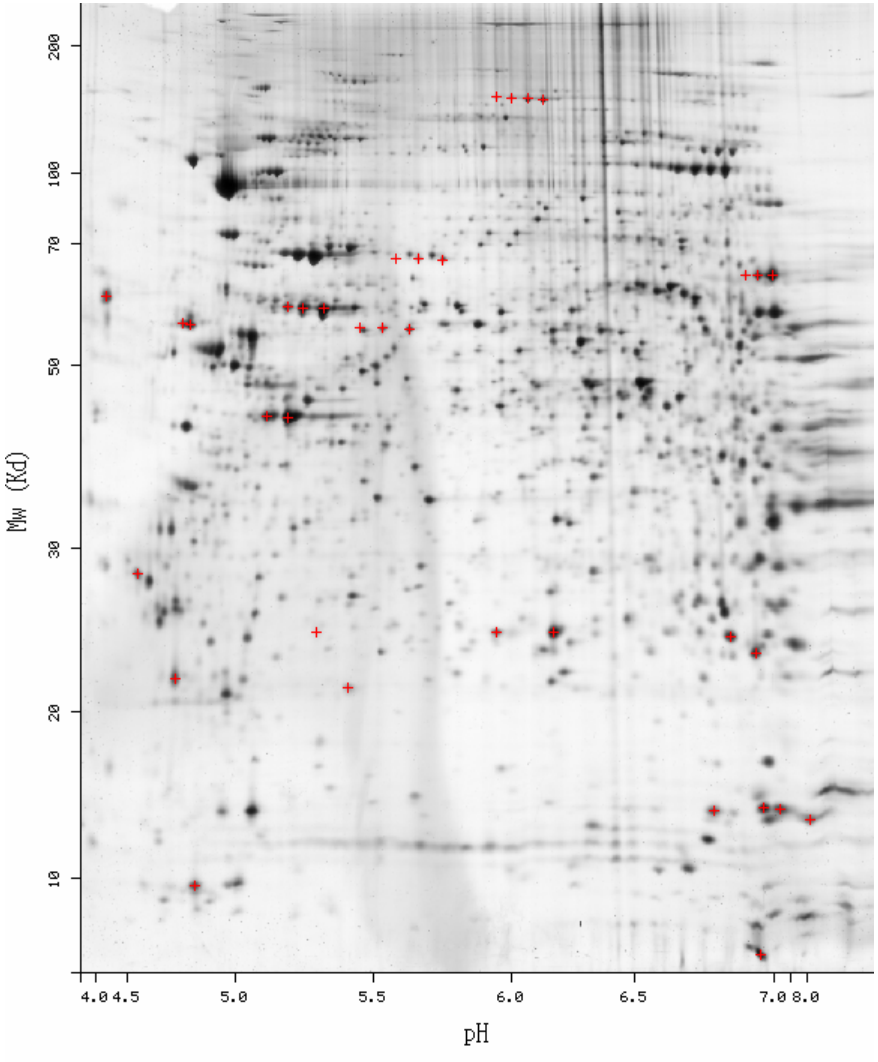


2-dimensional Gel Electrophoresis

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

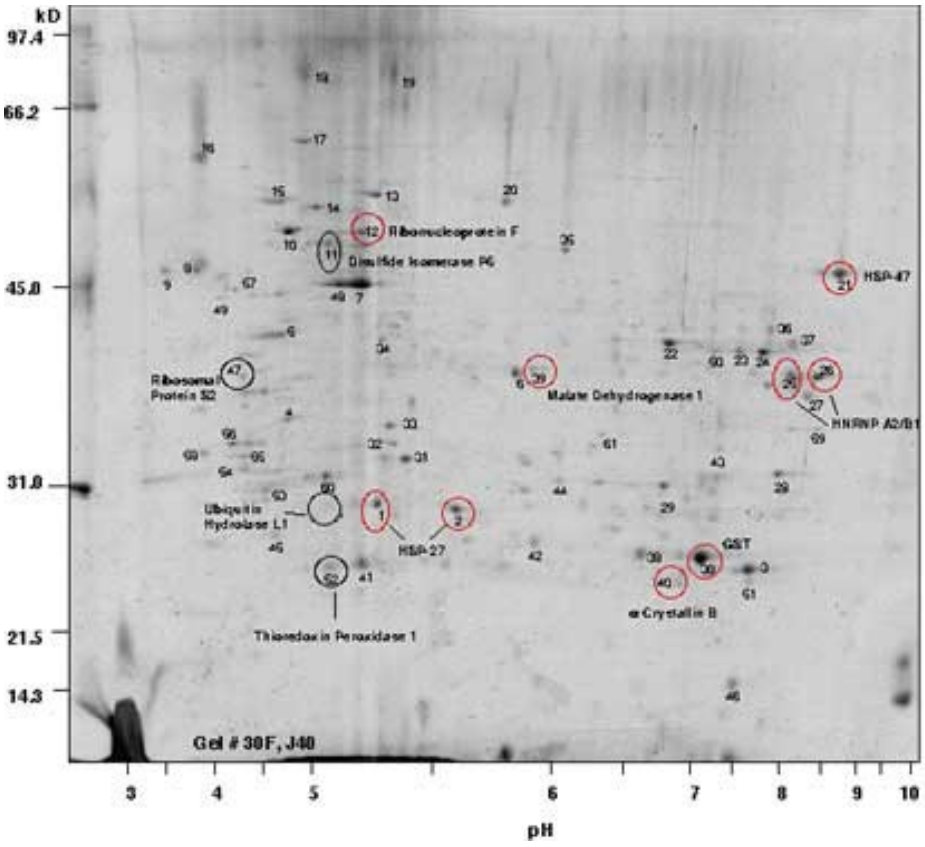
- pI
- MW



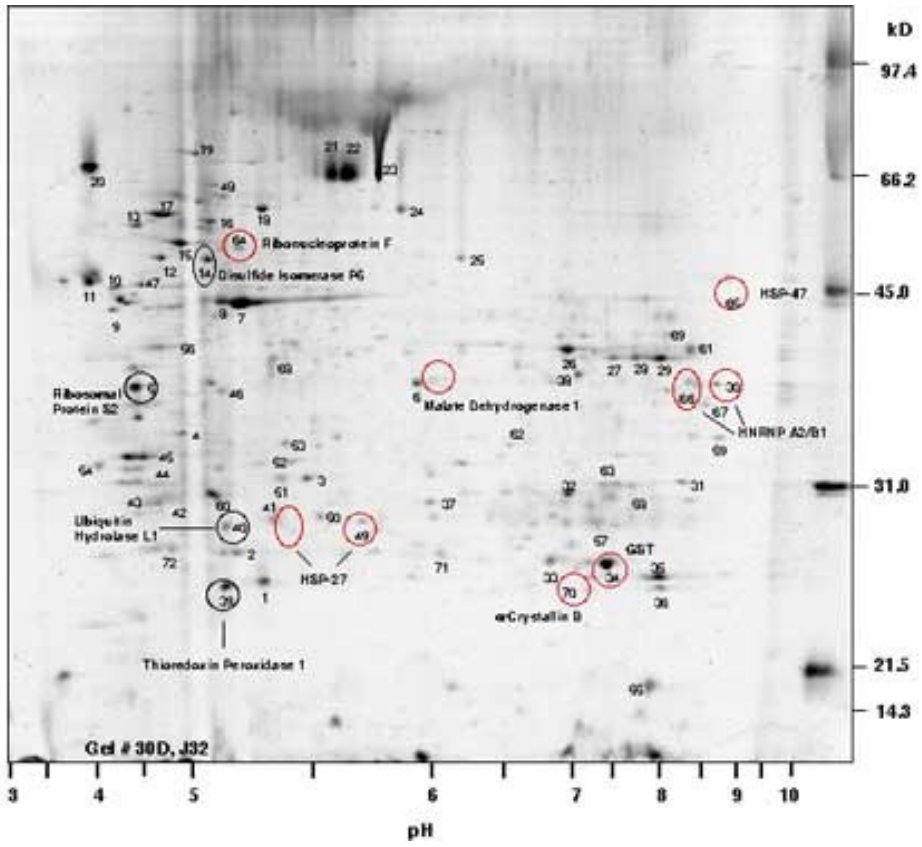
2-dimensional Gel Electrophoresis

Application: Proteomics

Condition A

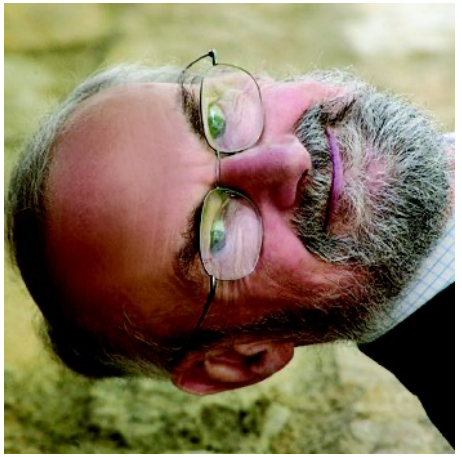


Condition B



How we identify proteins: Western Blot assay

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



Western Blot Assay: steps

–SDS-PAGE

–Blot

–Blocking

–Binding of primary Ab

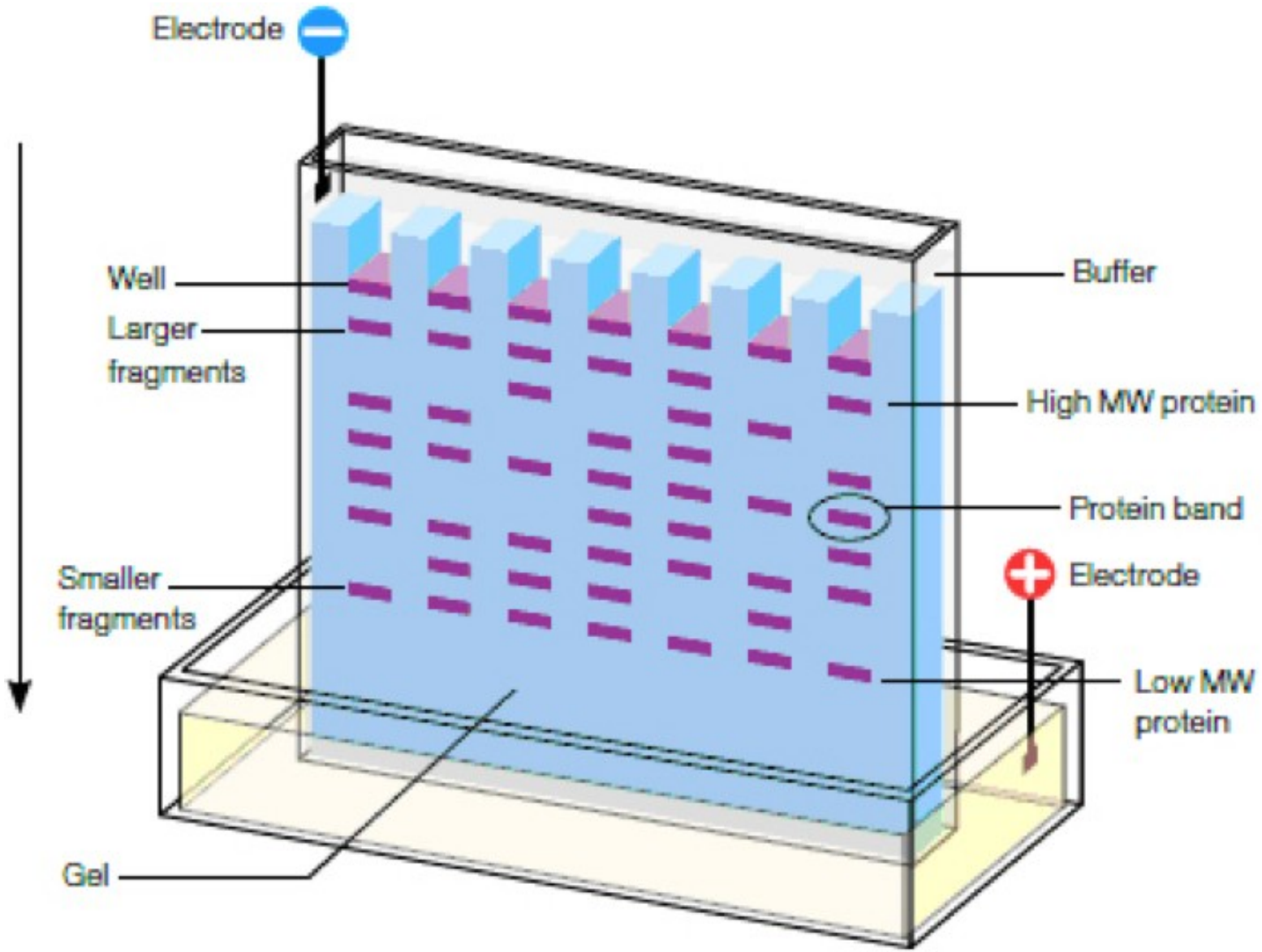
–Wash by buffer

–Binding of secondary Ab

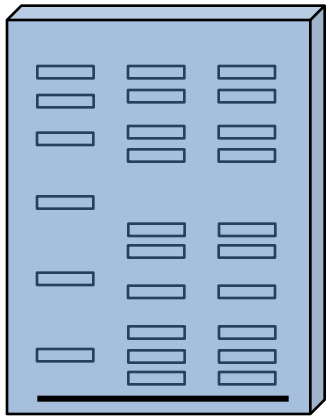
–Wash by buffer

–Signal Detection

SDS-PAGE


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

Blot

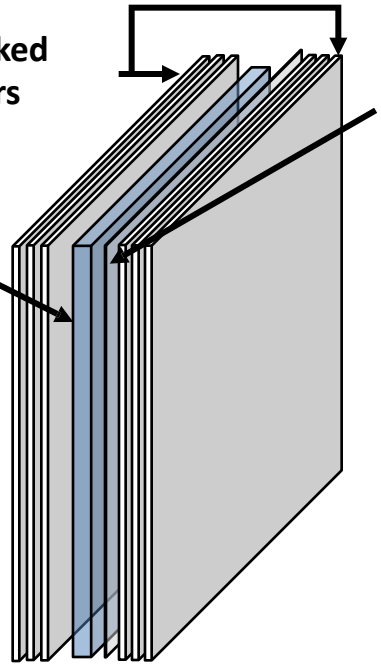


SDS-PAGE

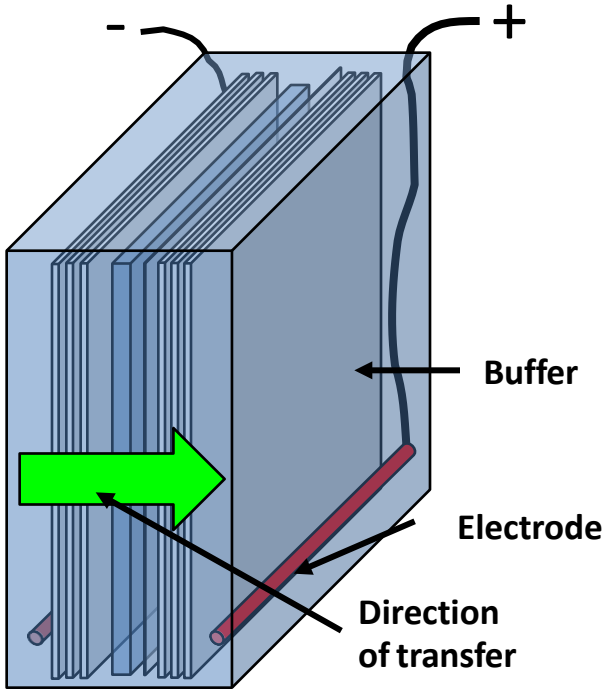
Buffer-soaked filter papers

Nitrocellulose membrane

Gel

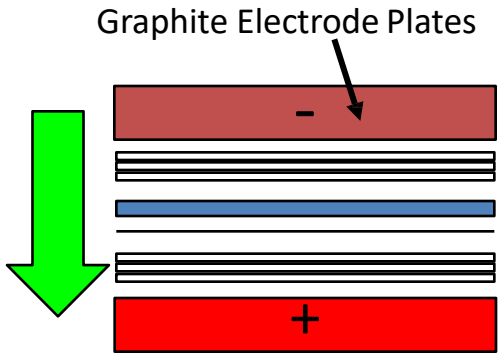


Assemble ‘sandwich’

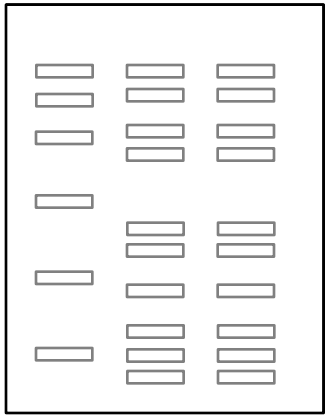


Wet blotting

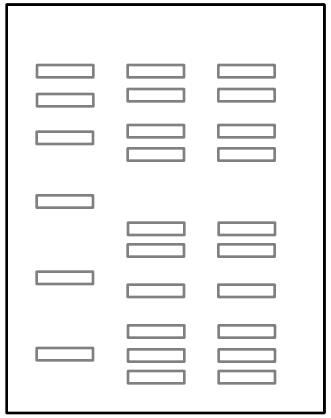
Graphite Electrode Plates



Semi-dry blotting

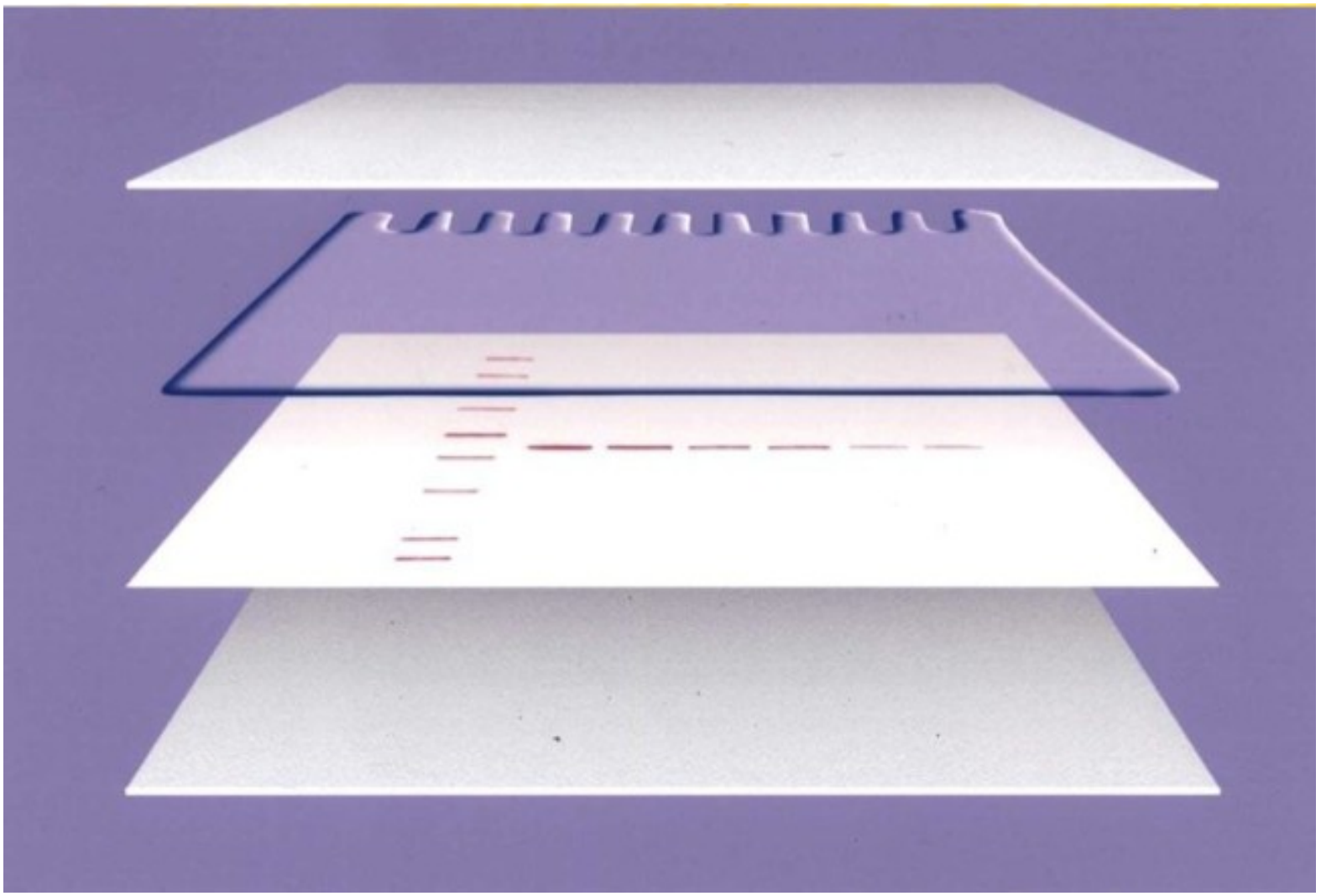


Nitrocellulose with bound proteins



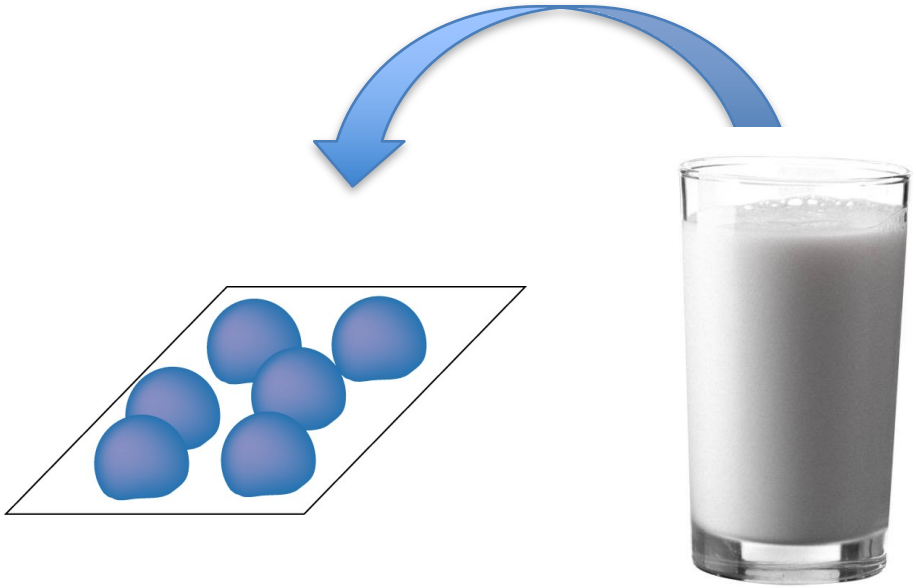
Stained

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 production

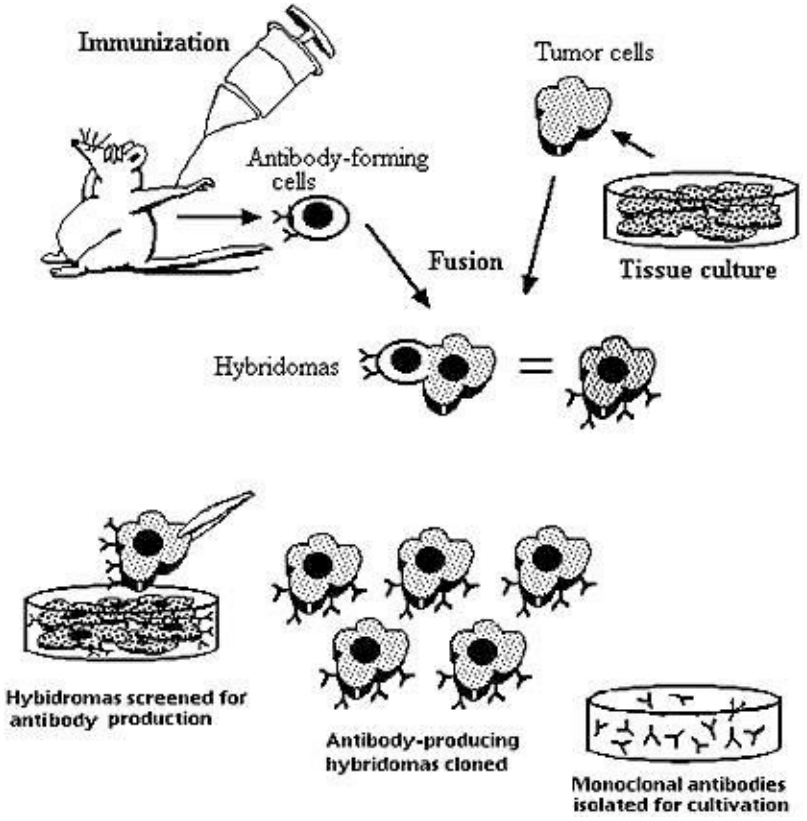
POLICLONAL

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

MONOCLONAL



Antibody Bound

SECONDARY ANTIBODIES

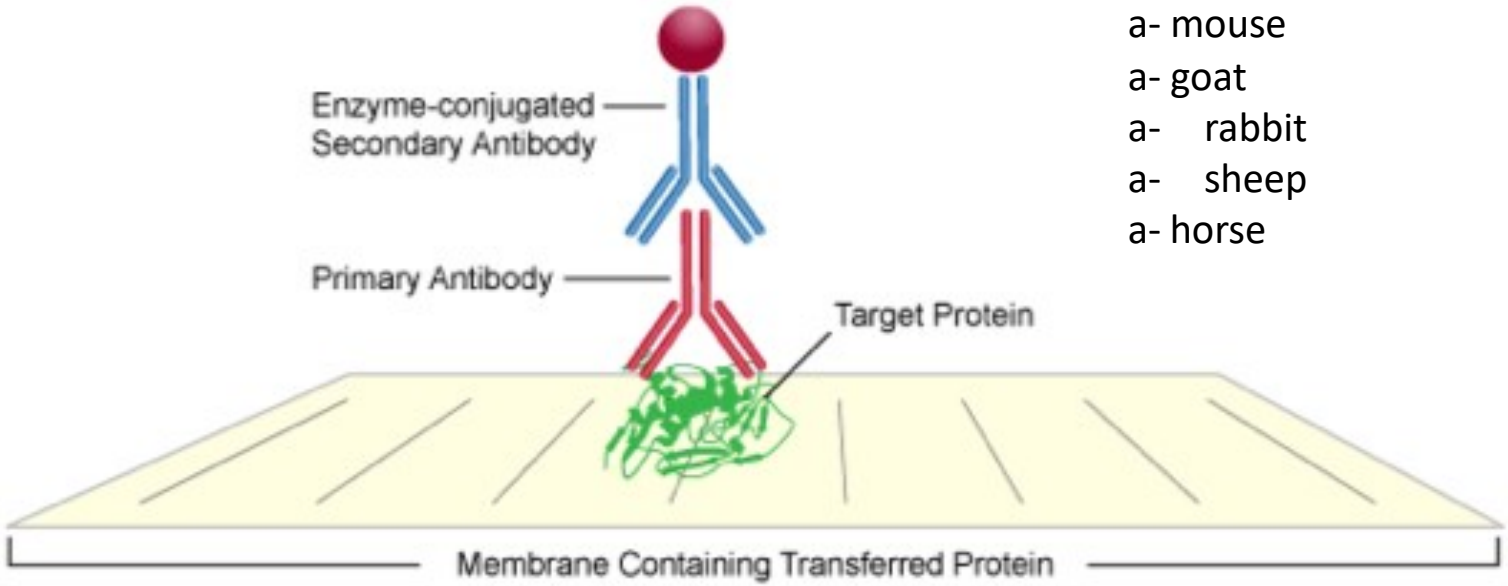
a- mouse

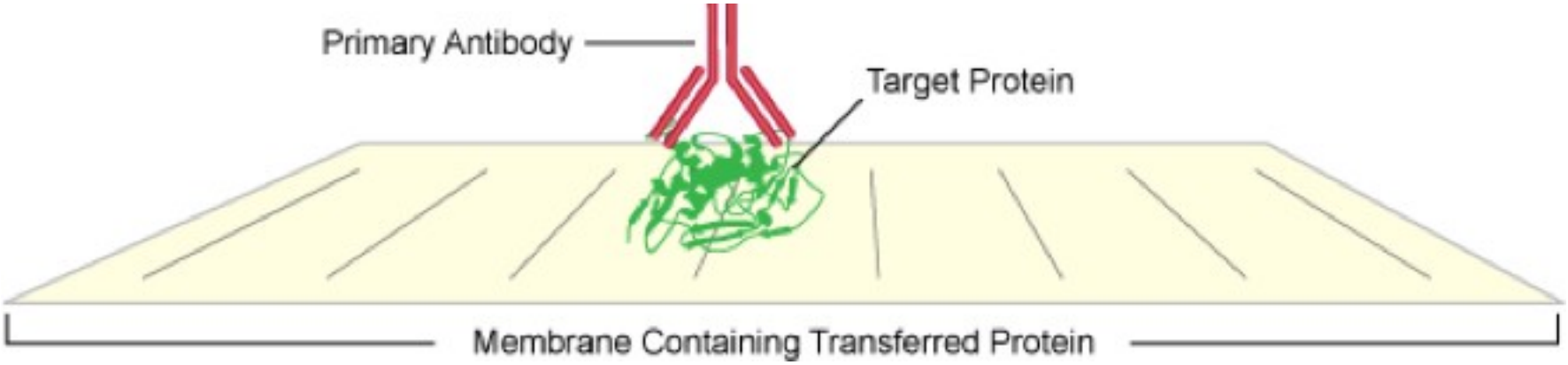
a- goat

a- rabbit

a- sheep

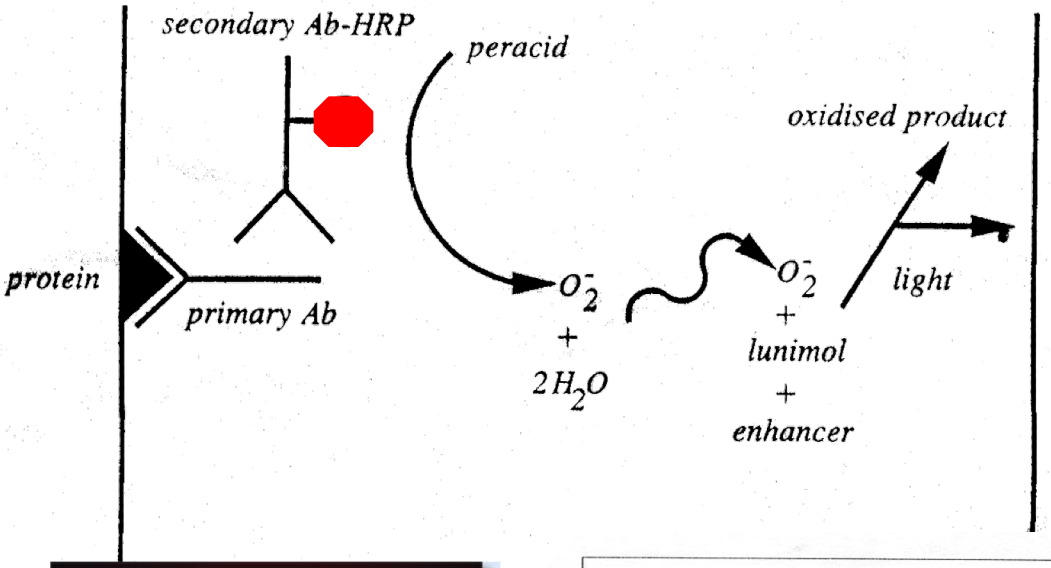
a- horse





ECL (Enhanced Chemio-Luminescence) method

membrane **Autoradiographic film**



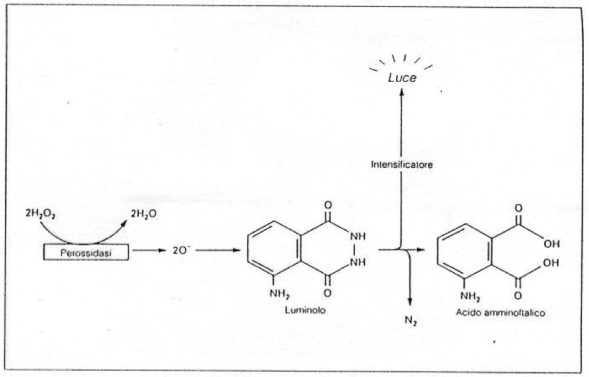
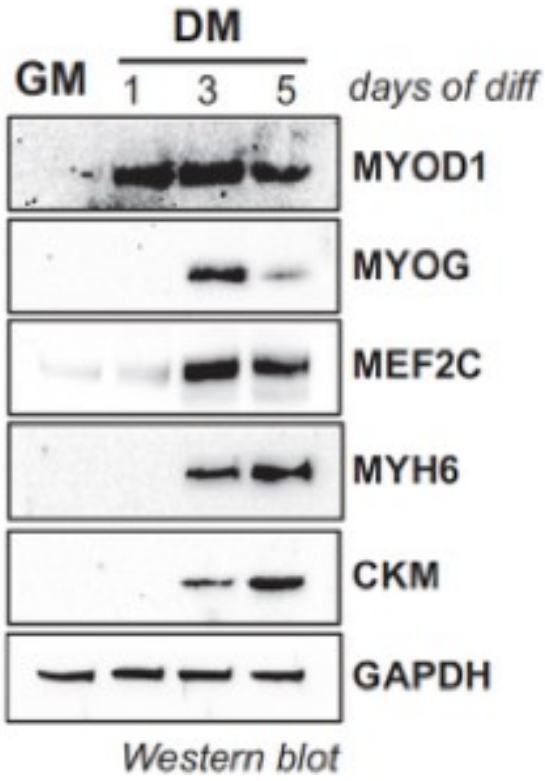


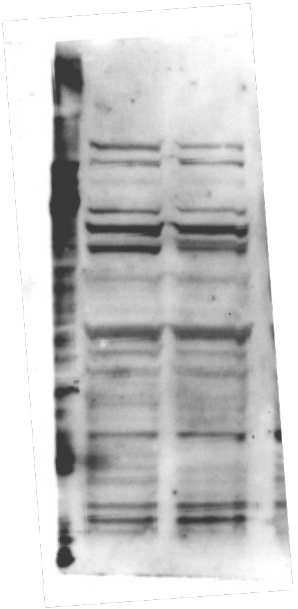
Figura 9.14 Uso della chemiluminescenza intensificata per la rivelazione della perossidasi di rafano.

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?

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 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**

Recombinant proteins

- Biomedical research
- Commercially relevant factors
- Therapeutic molecules

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

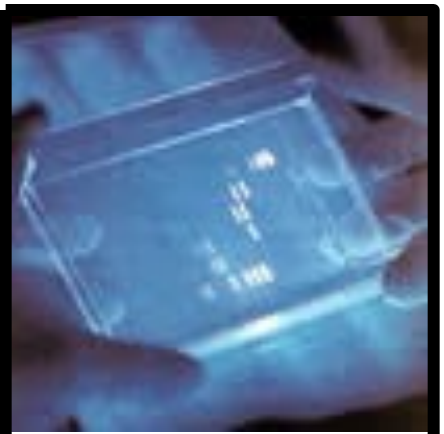
Hormones
ACTH
TSH (Tiretropin)
HGH (Growth hormone)
EPO
Somatotropin
Calcitonin
Glucagon Insulin

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

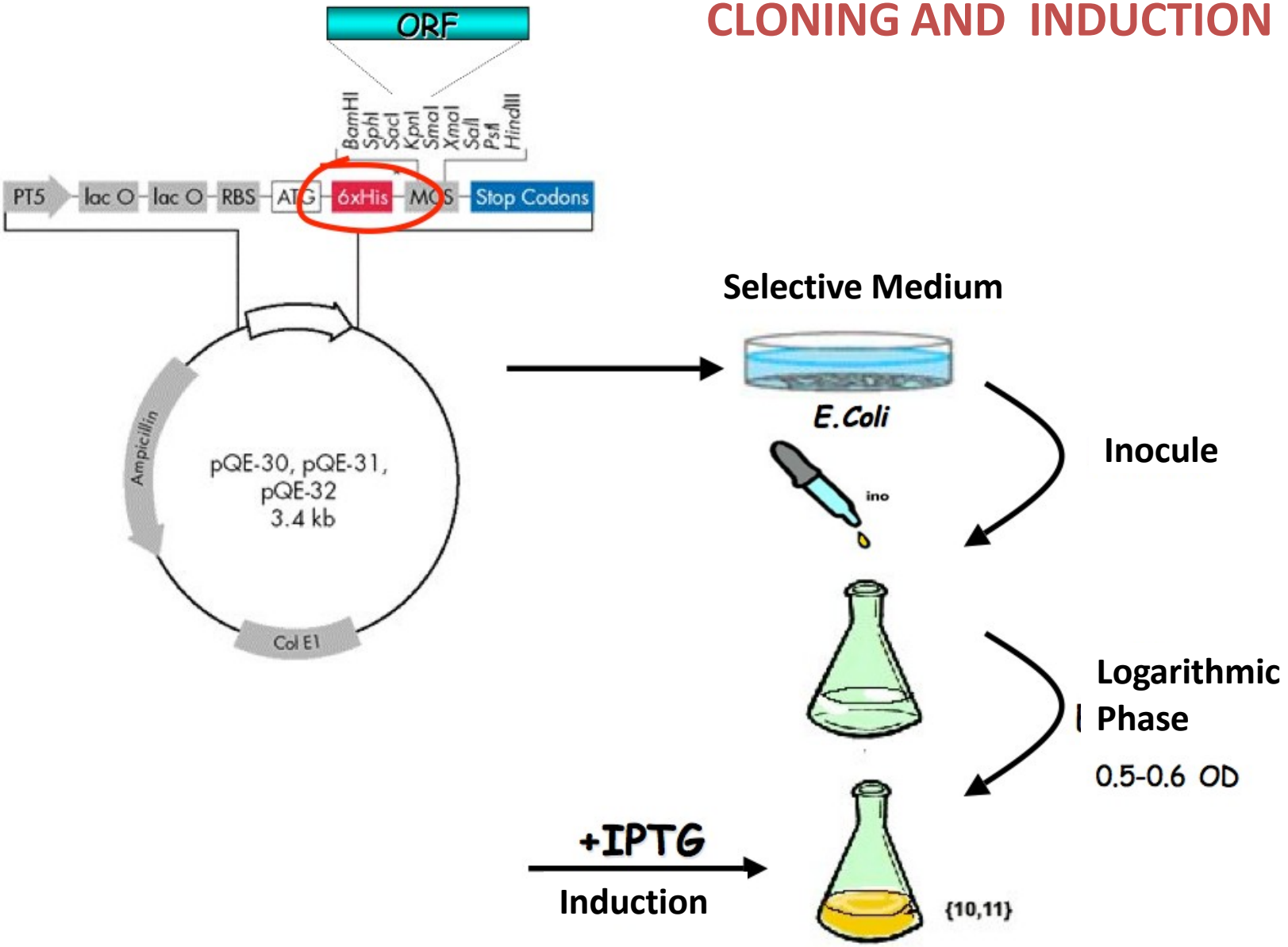
Bacteria	*Escherichia coli* *Bacillus subtilis*	
Fungi	*Sacarocmices cerevisiae* *Aspergillus nidulans*	
Plants	*Arabidopsis thaliana,* *Nicotiana tabacco*	cellule in coltura protoplasti piante transgeniche
Insects	*Dorifera californica* *Drosophila melanogaster*	cellule in coltura *org*anismi interi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

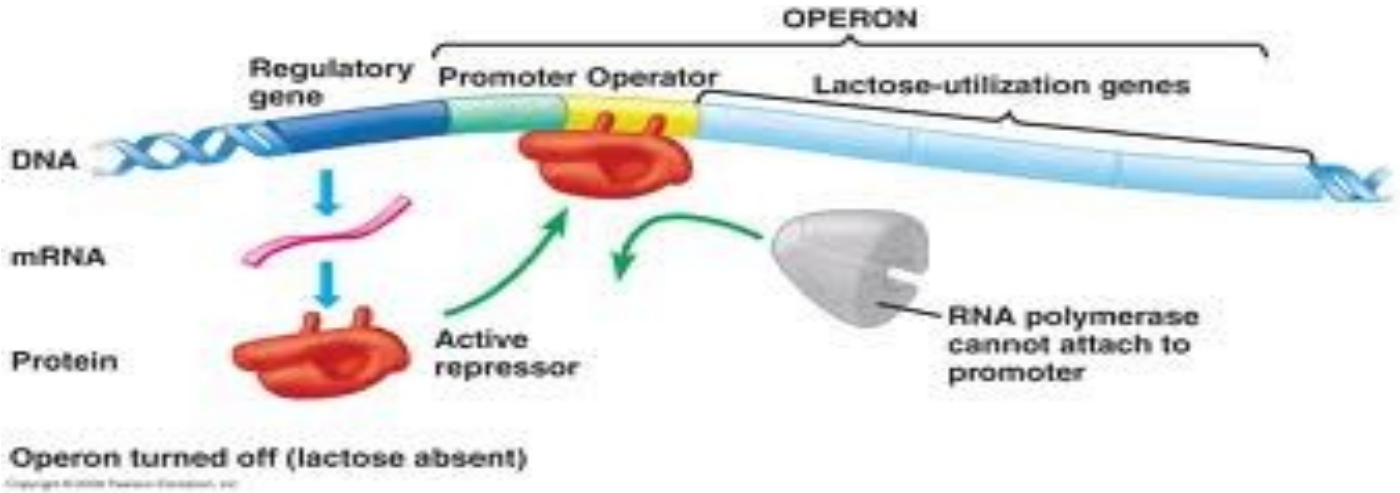
Why do we express proteins in heterologous systems?

- 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

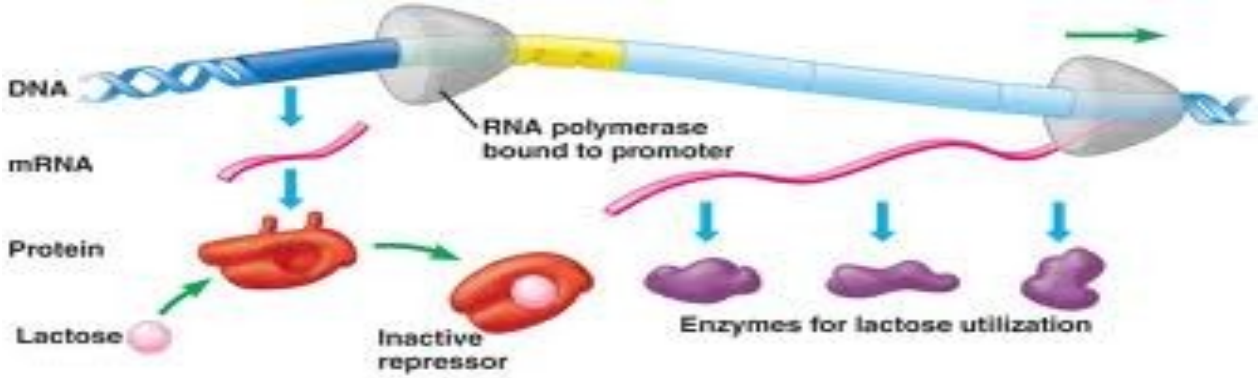
CLONING AND INDUCTION



OPERONE LAC

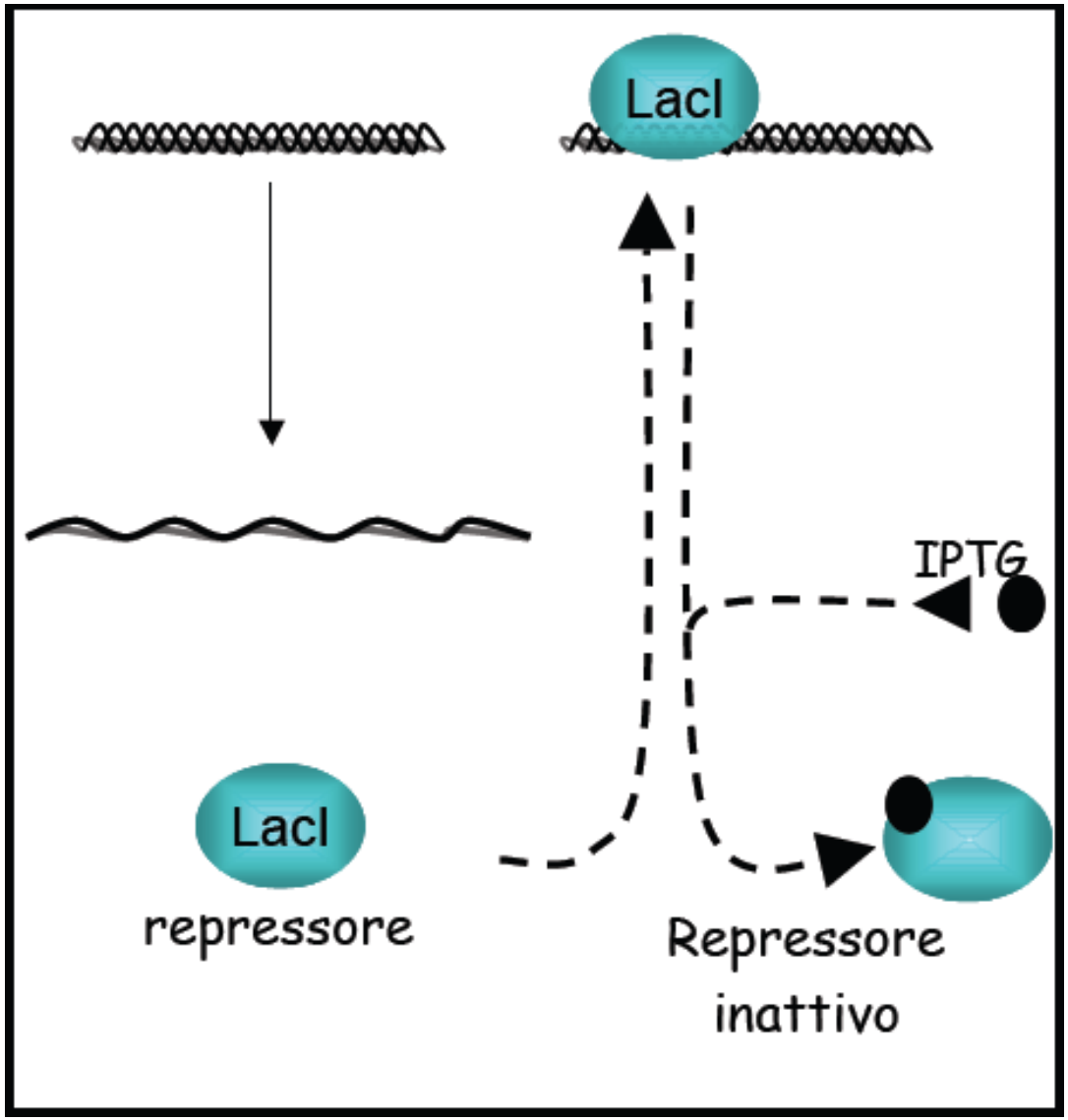


Operon turned off (lactose absent)

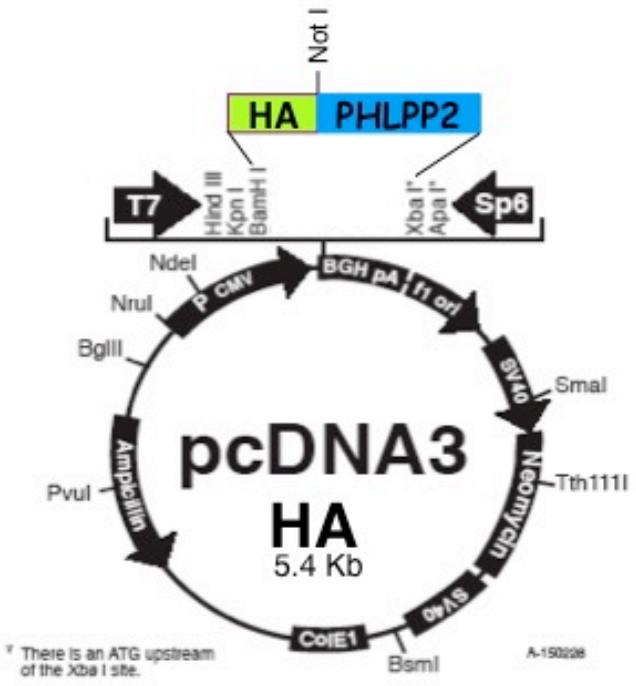


Operon turned on (lactose inactivates repressor)

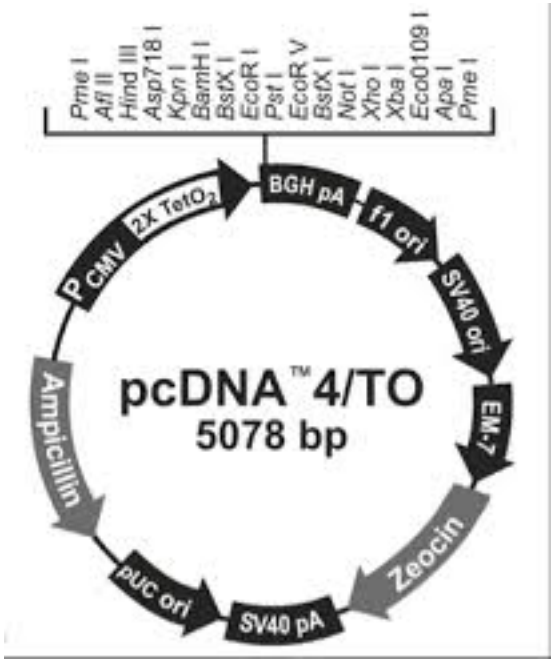
Procariotic gene expression is regulated



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**.

Purification of a (recombinant) proteins

1. Purifications of proteins
   Antibodies
   TAG

2. Purifications of proteins

→**Protein production**
→**Interaction studies**

Purification of proteins: TAGs

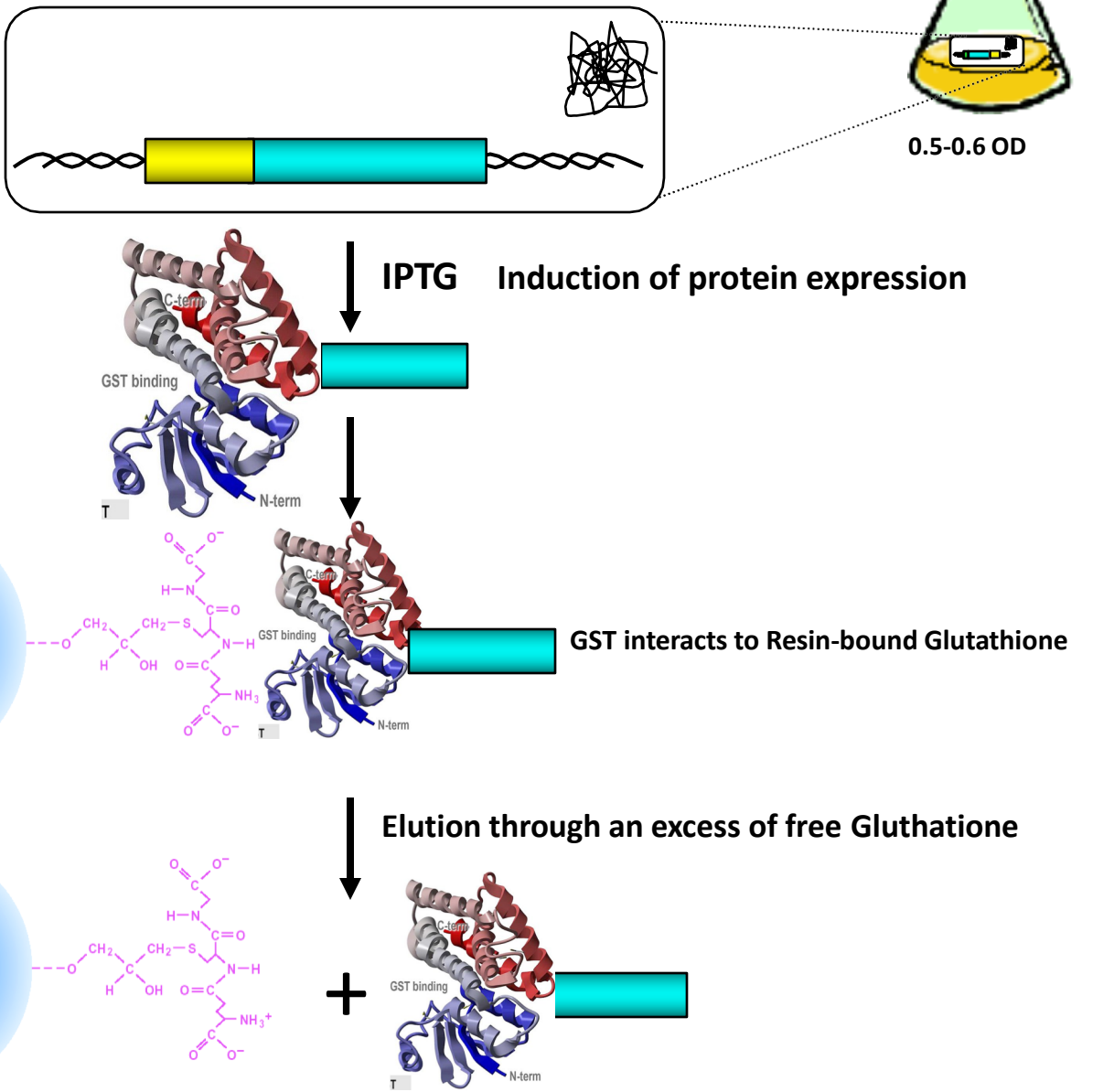
Tag	Description	Affinity ligand
• **His_6**	6 histidines	Ni++, Co++, Cu++
• **GST**	glutathion-S-transferase	glutathion
• **TAP**		
• **FLAG**		
• **HA**		Maltose
• **MBP**	Maltose binding protein	IgG
• **Protein A**	Protein A	Calmodulin
• **CBP** (40kDa)	Calmodulin binding protein	
• **Epitopi biotinilati**		

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

The GST TAG SYSTEM

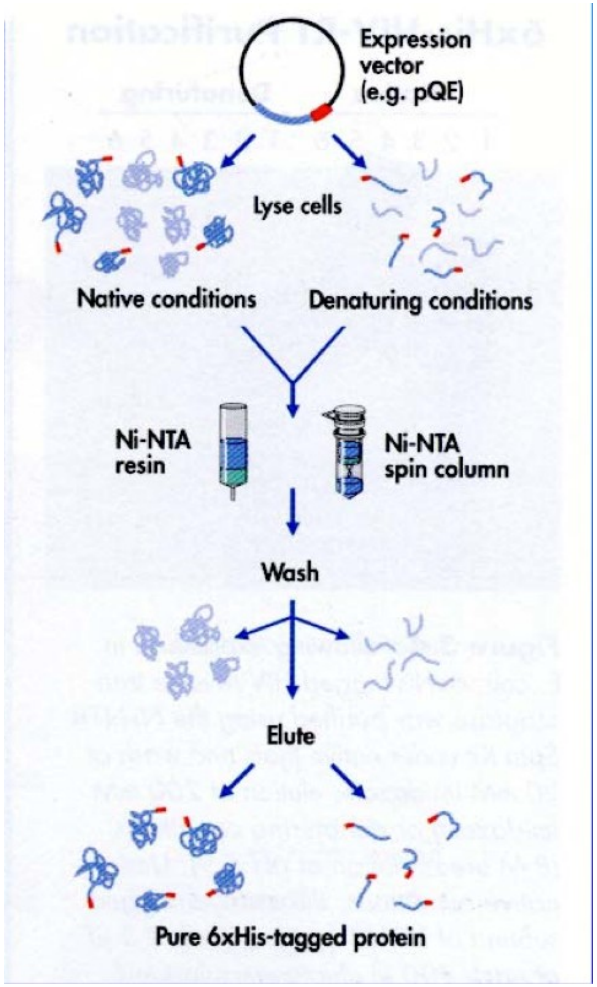
GST

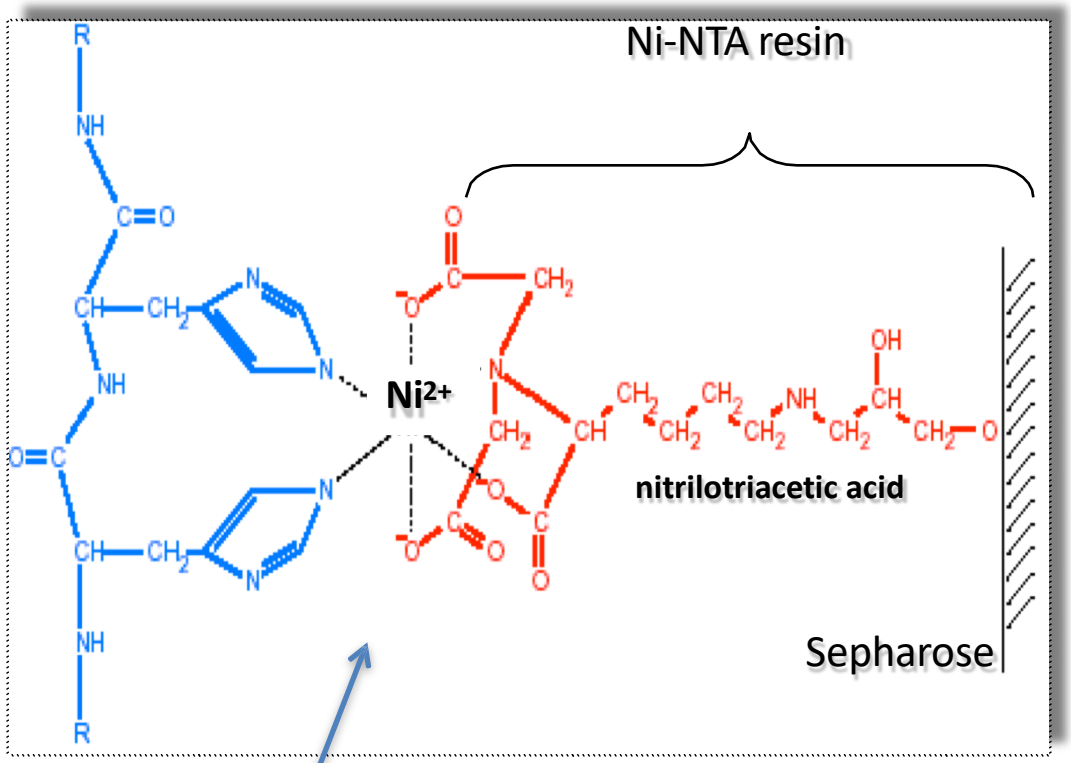
Protein of Interest



Protein Purification by Affinity Chromatography

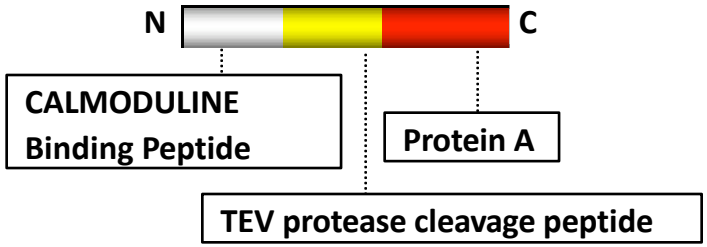
The HIS TAG System

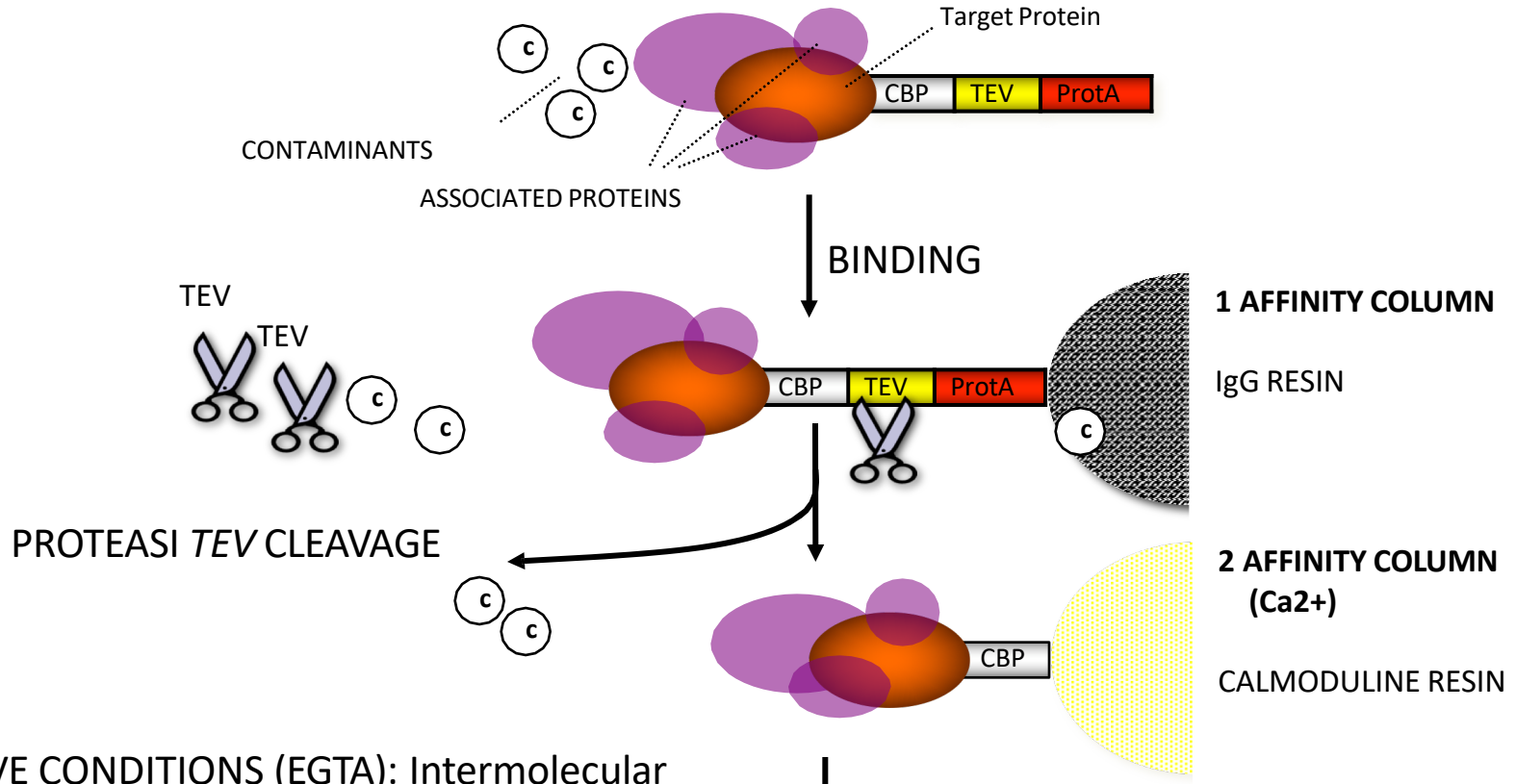




Elution By Imidazole (a histidine analogue)

Tandem Affinity Purification (TAP) System



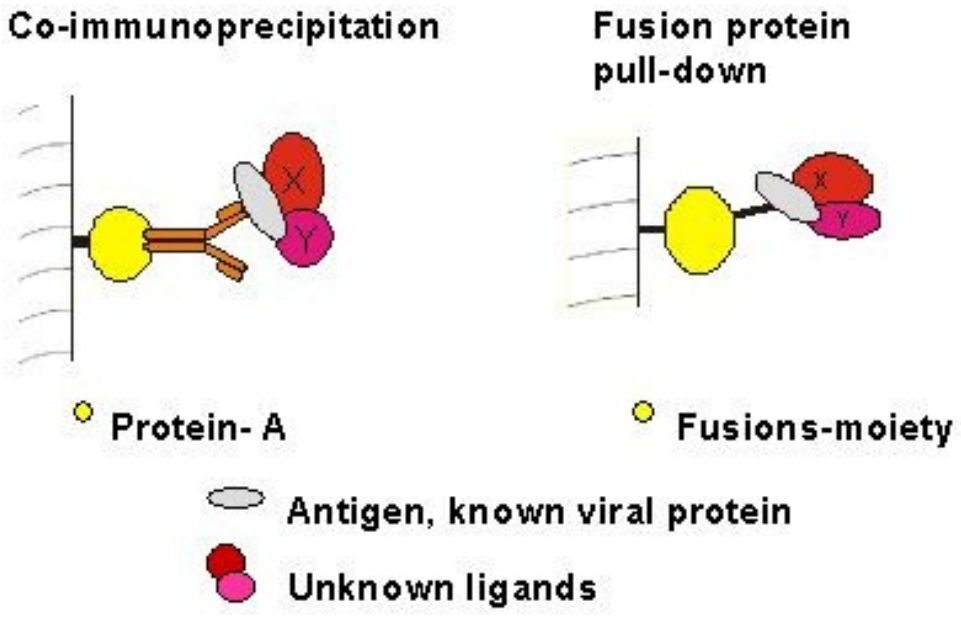


ELUITION IN NATIVE CONDITIONS (EGTA): Intermolecular Interactions are preserved, thus….

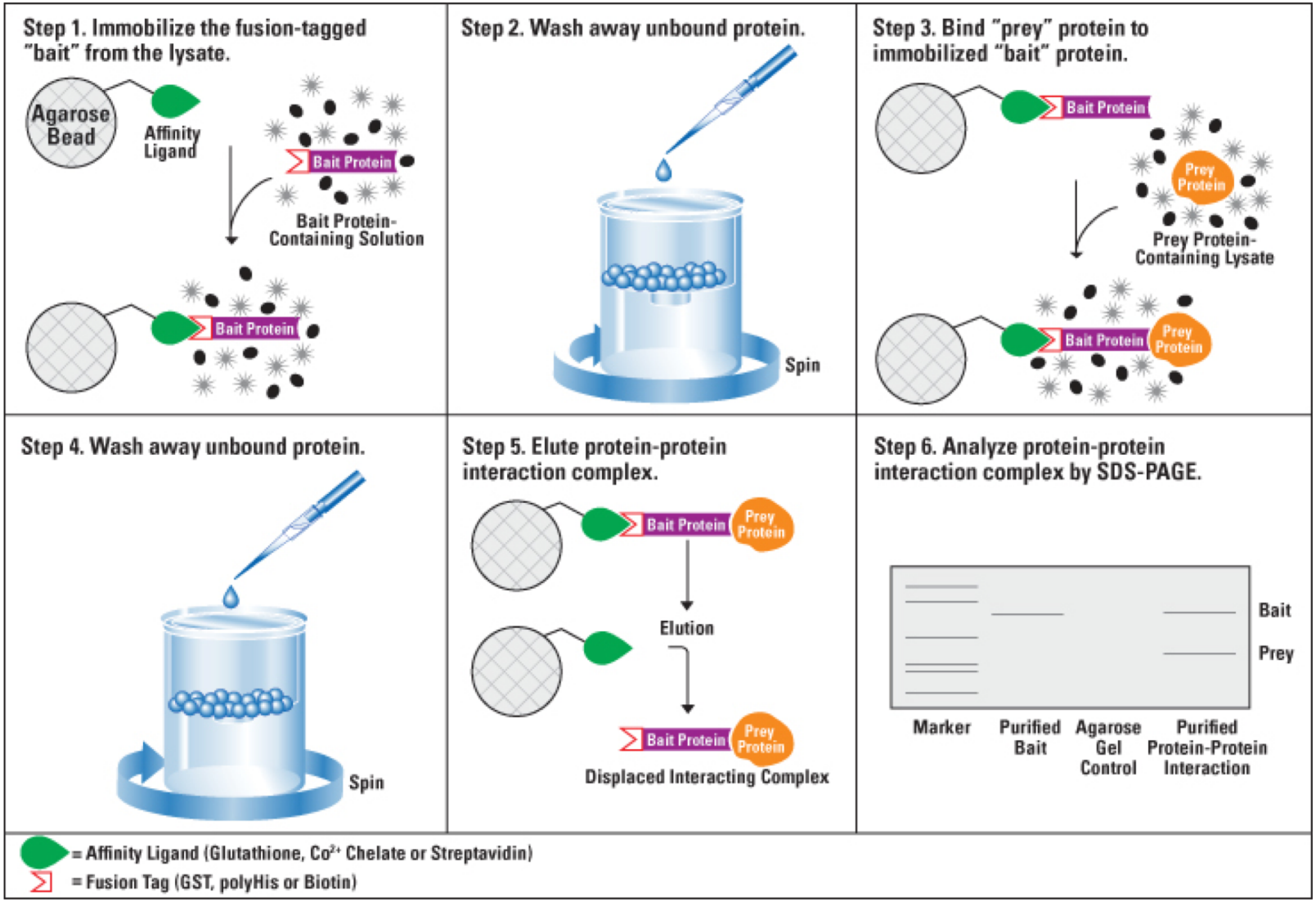
Protein Pull Down Assay

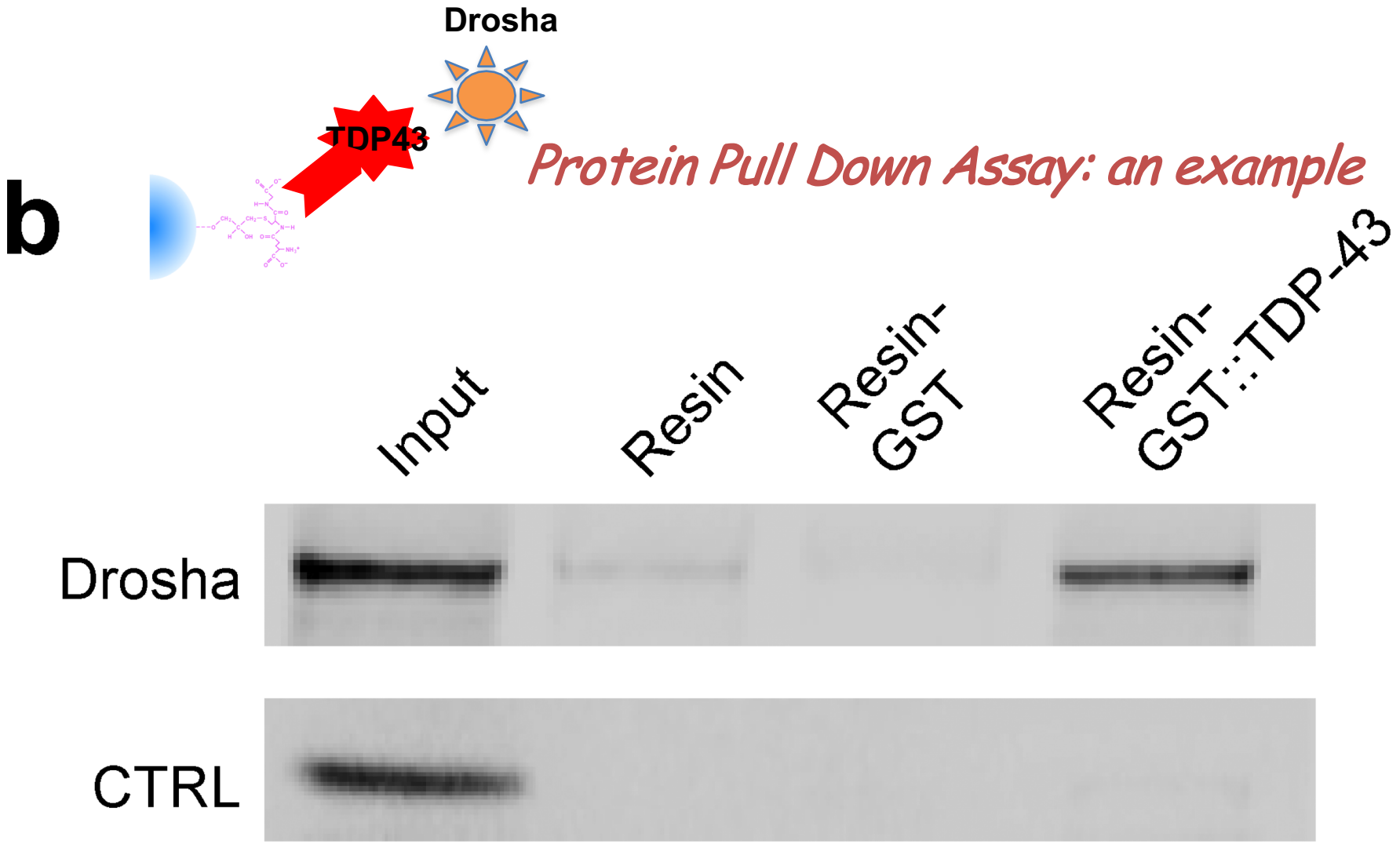
TAGGING (and consequent affinity for a ligand) can be exploited to verify protein interactions

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




Drosha
TDP43
b
Protein Pull Down Assay: an example
Input
Resin
Resin-GST
Resin-GST::TDP-43
Drosha
CTRL

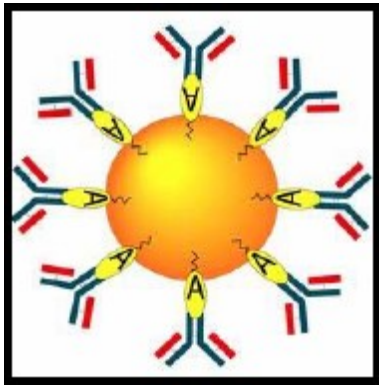
Purification of proteins: Antibody Immunoprecipitation

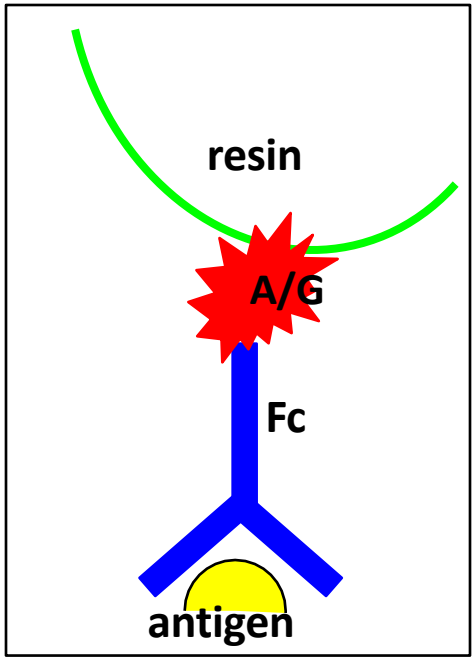
Isolation (enrichment) of antigen/antibody complexes

- **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

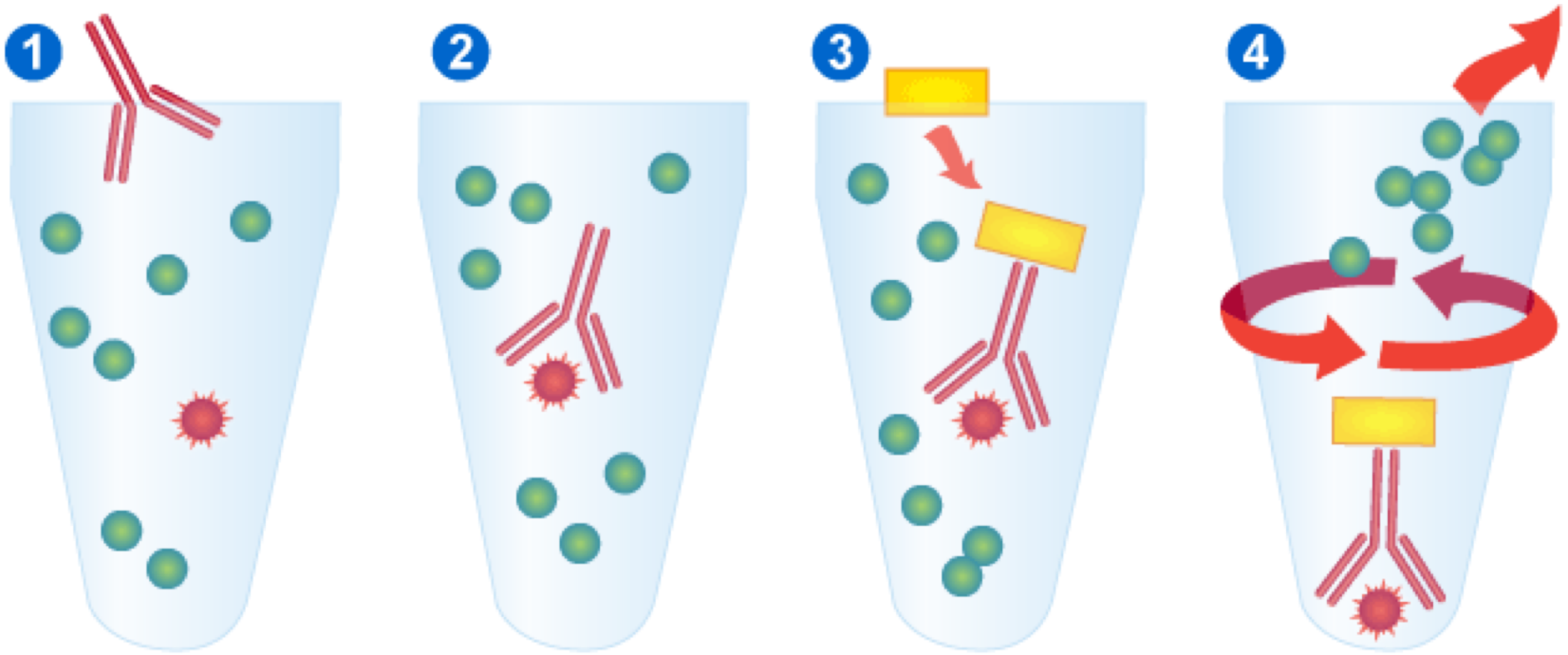
Antibodies specifically bind protein A or G from Staphylococcus, through their Fc region.



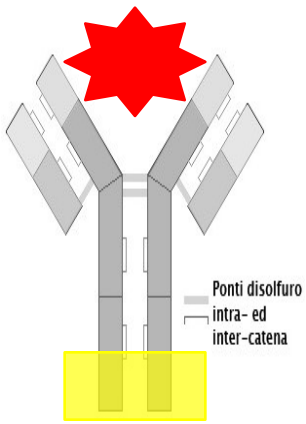


Binding Characteristics of Some Immunoglobulins			
Immunoglobulin		Protein A	Protein G
Mouse	IgG1	+	++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+++
	IgM	-	-
	IgA	-	-
	IgE	-	-
Rat	IgG1	+	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++

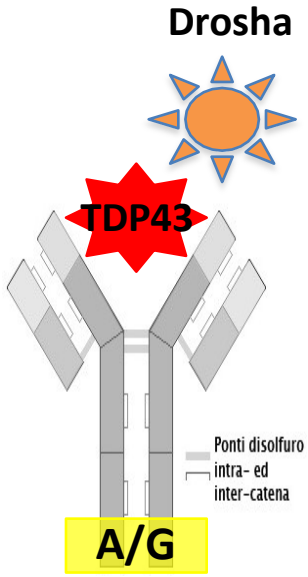
Immunoprecipitation (IP)



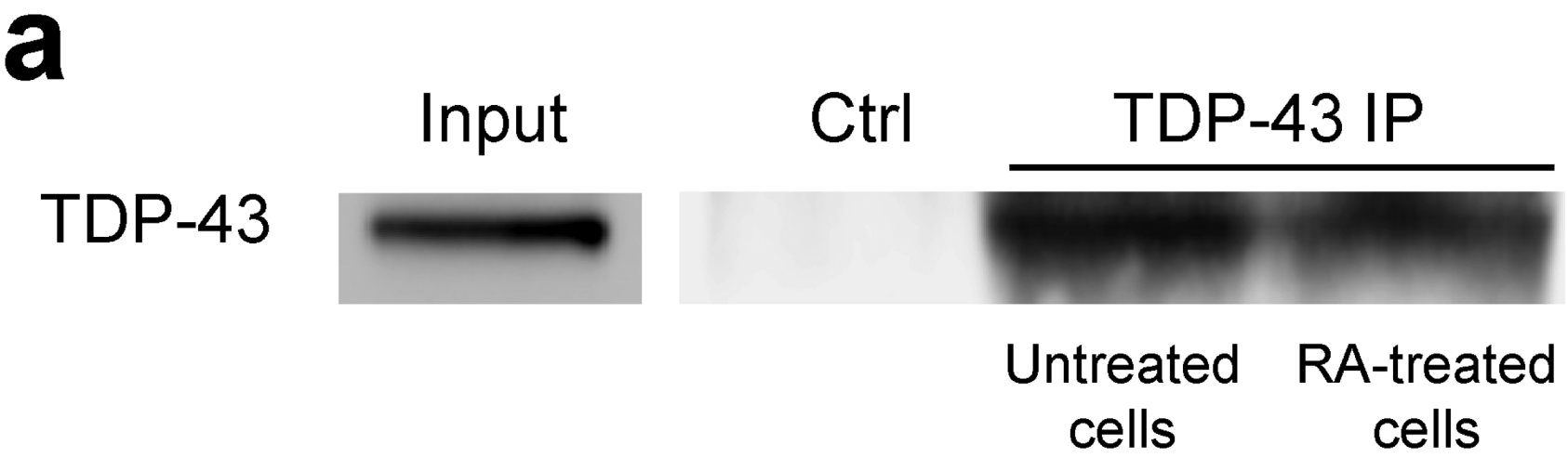
1. Suitable antibody is added.
2. Antibody binds to protein of interest.
3. Protein A or G added to make antibody-protein complexes insoluble.
4. Centrifugation of solution pellets antibody-protein complex. Removal of supernatant and washing.



Magnetic beads !

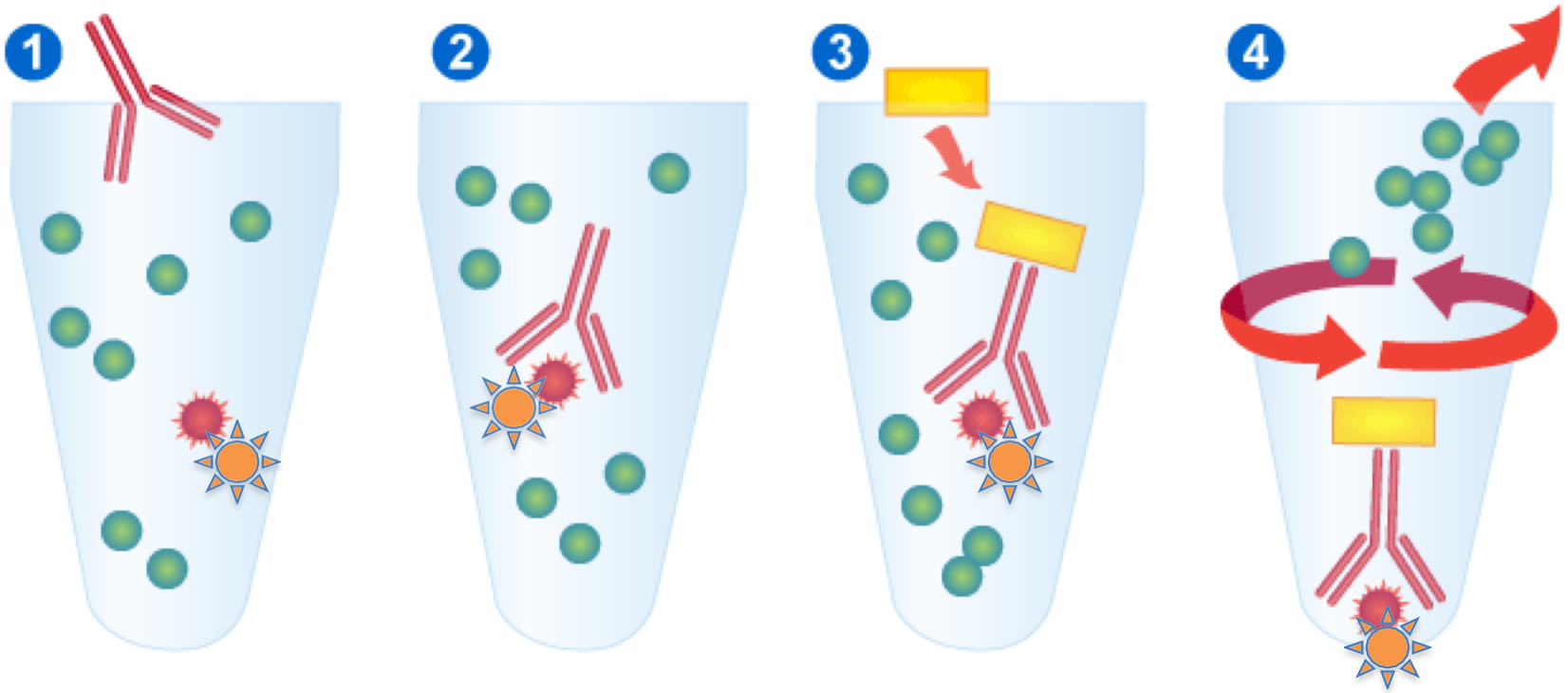


Immunoprecipitation: an example



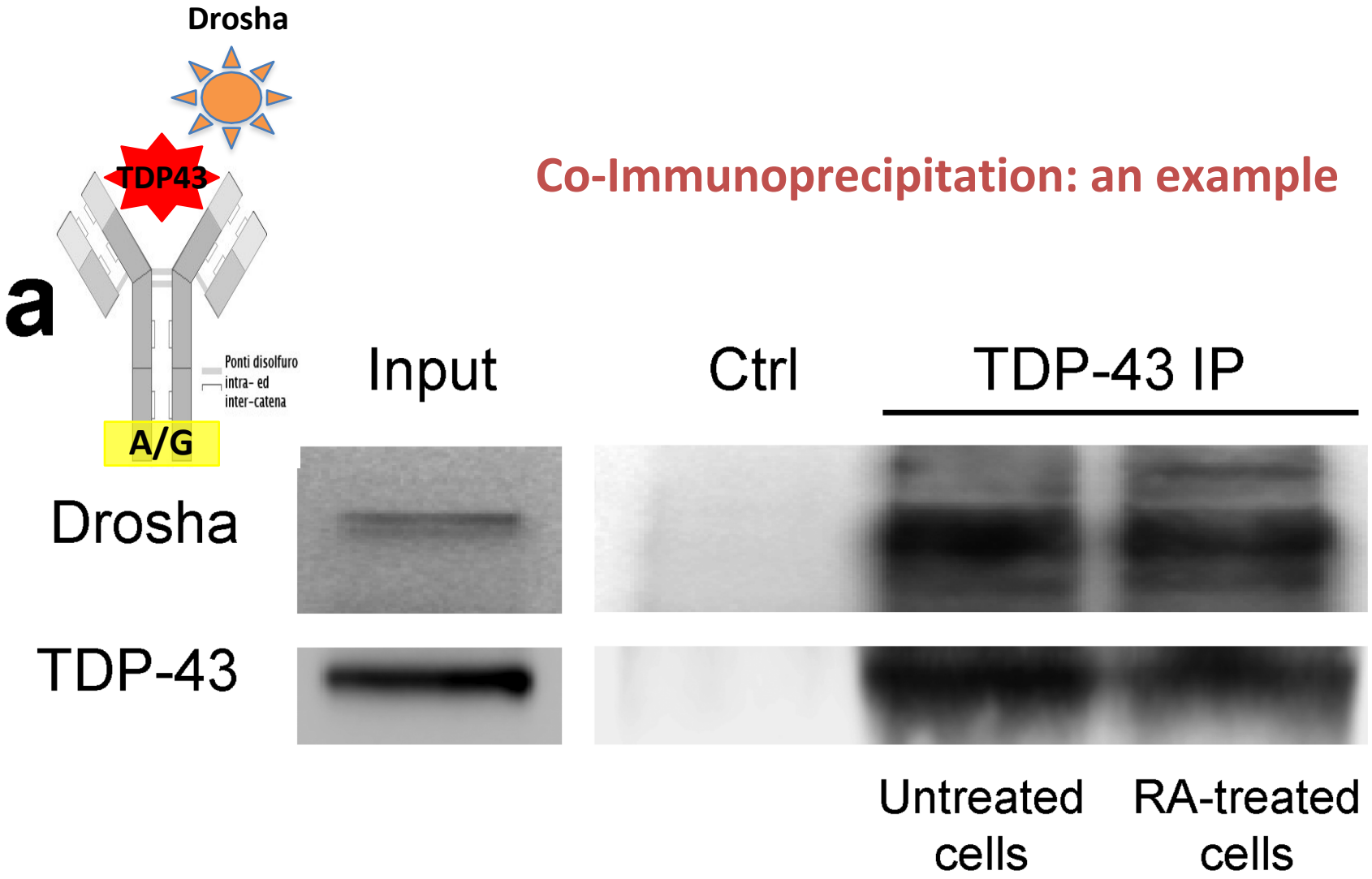
Di Carlo V. et al, 2013

Co-Immunoprecipitation (Co-IP)



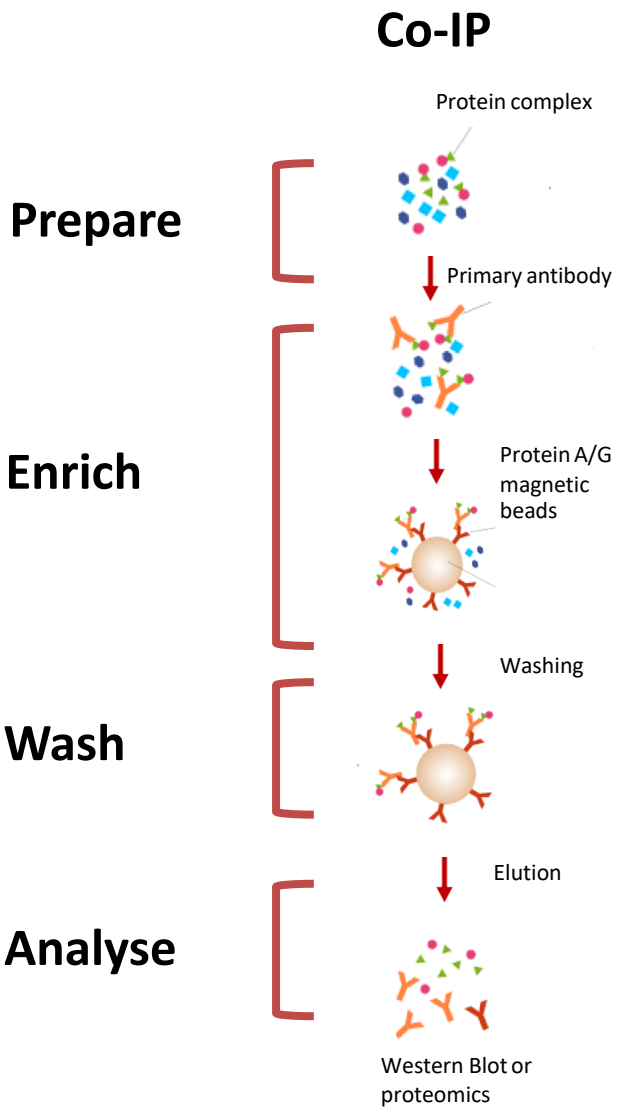
1. Suitable antibody is added.
2. Antibody binds to protein of interest.
3. Protein A or G added to make antibody-protein complexes insoluble.
4. Centrifugation of solution pellets antibody-protein complex.
   Removal of supernatant and washing.

Co-Immunoprecipitation: an example



Di Carlo V. et al, 2013

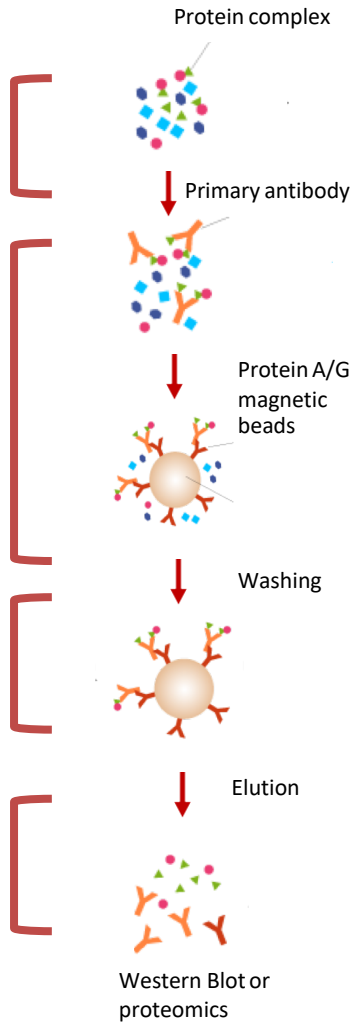
Immunoprecipitation (IP)



Immunoprecipitation (IP)

Finding molecular parterns:

Co-IP



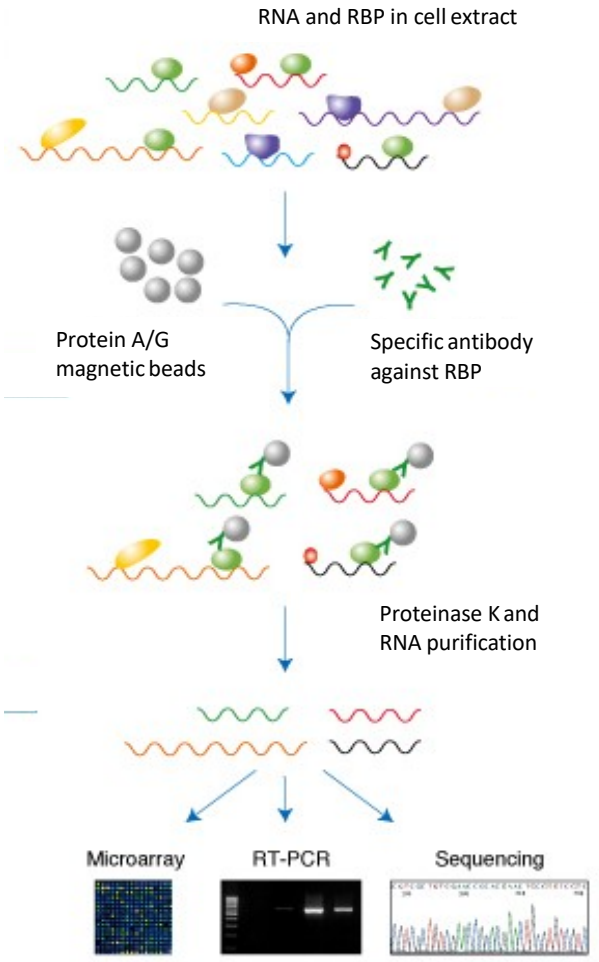
Prepare

Enrich

Wash

Analyse

RIP/CLIP



CHIP