Molecular techniques for the study of the interaction between macromolecules

Molecular techniques for the study of the interaction between:

Protein-protein:
Immuprecipitation
GST pull-down

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

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

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

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

Immunoprecipitation

Isolation (enrichment) of antigen/antibody complexes

- Requires: specific antibodies directed toward the target protein or its tagged variant

- Allows: identification of protein or ribonucleoprotein (RNP) complexes

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 efficient purification, allowing to identify multiprotein or RNA/DNA/protein complexes.

1. Tagged proteins can be obtained by cloning into expression vectors (recombinant protein):

   DNA encoding for the protein + DNA encoding for the Tag

2. Tagged protein can be directly expressed within the cell line by using episomal vectors or integration in the genome

Types of TAG

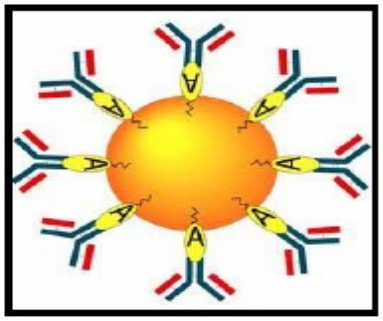
- **GST** - protein of bacterial origin (16 kDa)
- **CBP** - calmodulin binding protein (6 kDa)
- **ProtA** - protein of bacterial origin that binds IgG (20 kDa)
- **TAP** - CBP+ProtA (30 kDa)

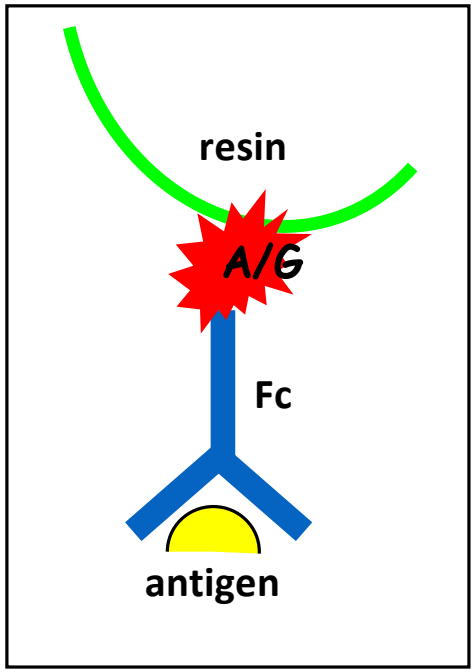
- **c-myc**- 10 aa derived from human c-myc (EQKLISEEDL)
- **HA** - 9 aa derived from ”haemaglutanin protein” of infuenza virus (YPYDVPDYA)

- **6His tag** - synthetic peptide of 6 histidines
- **FLAG** - synthetic peptide of 8 aa (DYKDDDK)

Immunoprecipitation: the role of protein A or G

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

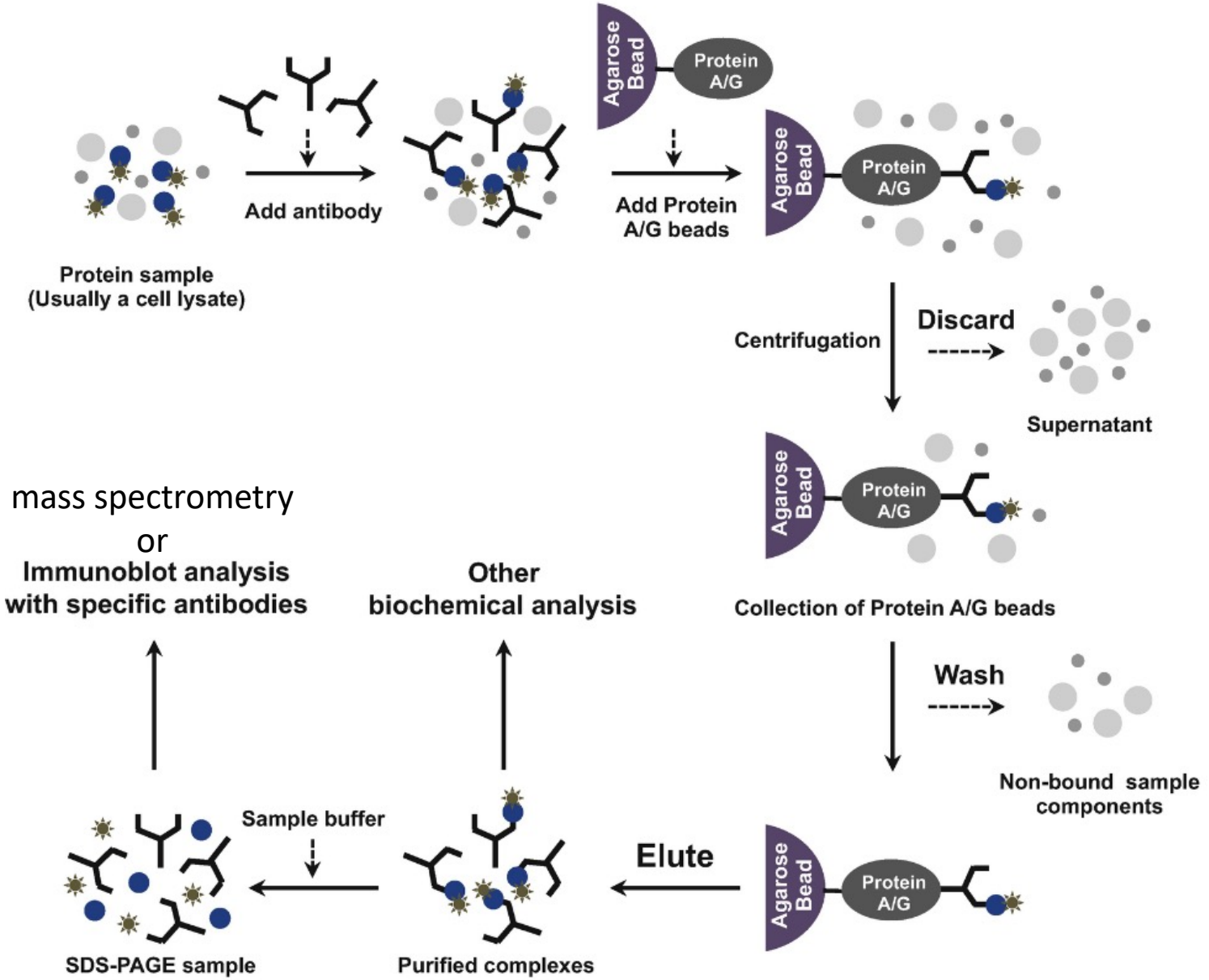




Binding Characteristics of Some Immunoglobulins

Immunoglobulin		Protein A	Protein G
Mouse	IgG1	+	++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+++
	IgM	-	-
	IgA	-	-
	IgE	-	-
Rat	IgG1	+	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++

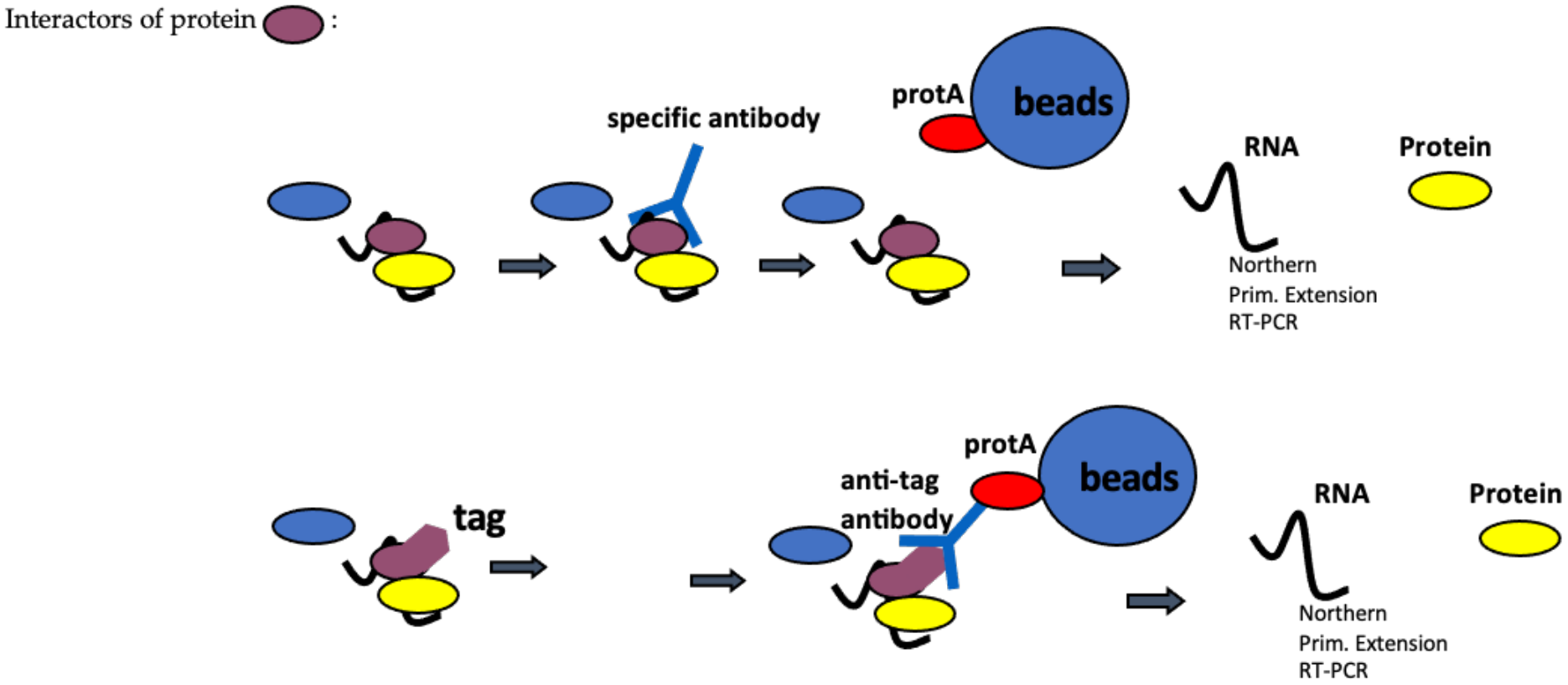
Co-Immunoprecipitation (Co-IP)



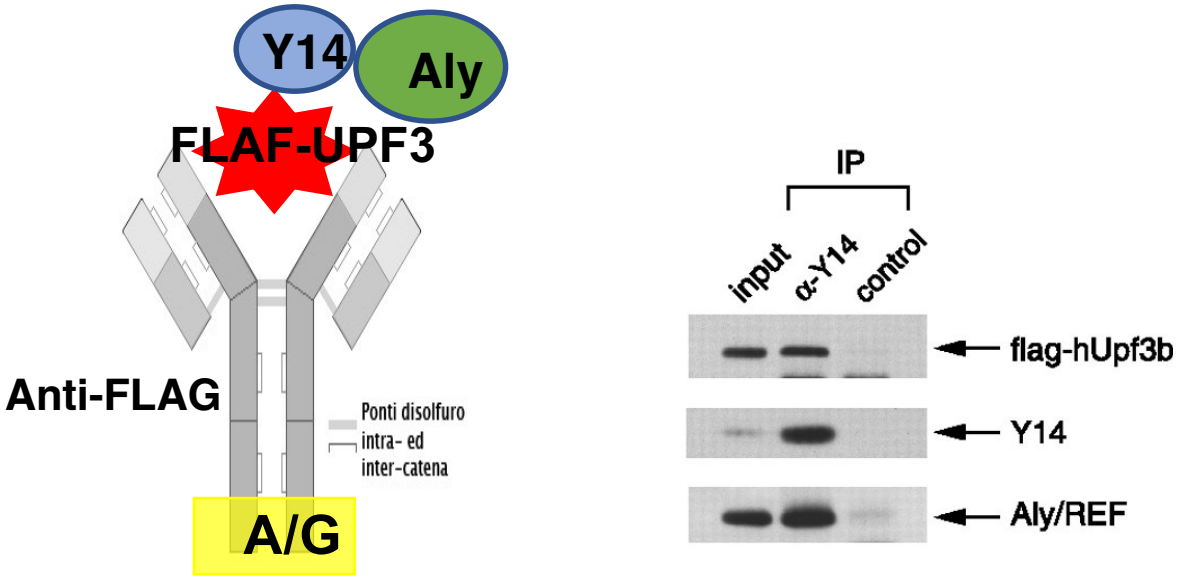
Co-Immunoprecipitation (Co-IP)

Magnetic beads !

Co-immunoprecipitation: experimental variations

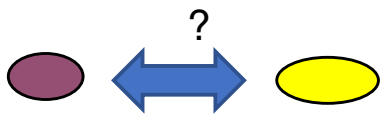


Co-Immunoprecipitation: an example

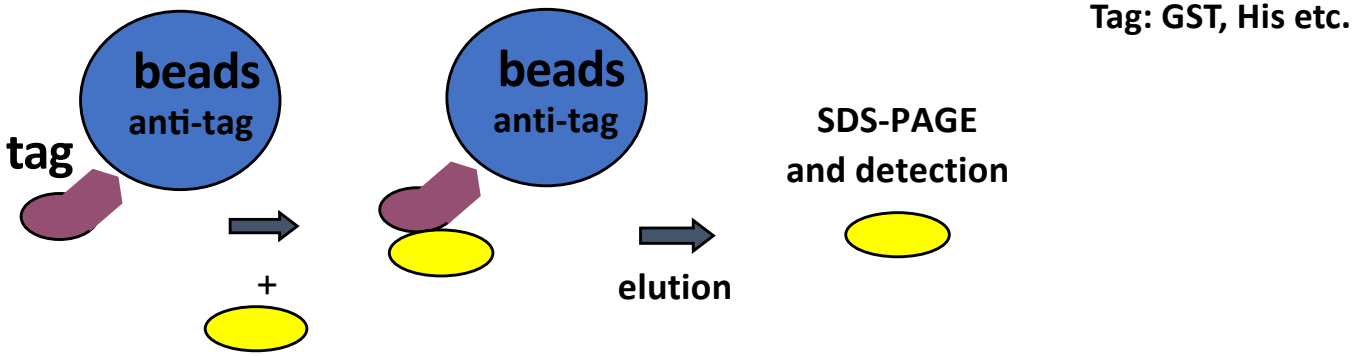


Immunoprecipitation alone does not determine if the interactions are direct !

Protein-protein interaction: experimental variations

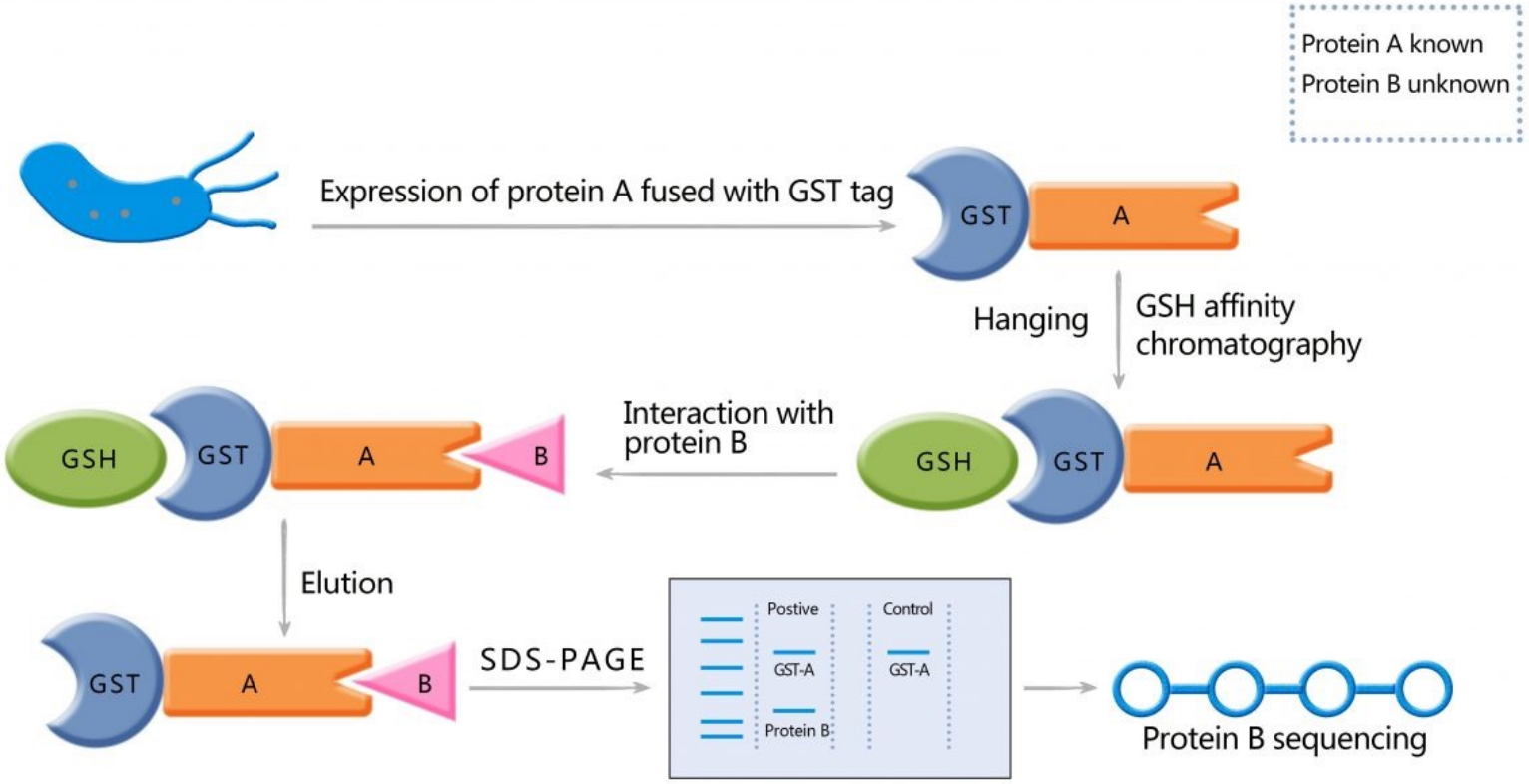


NO protein extract !



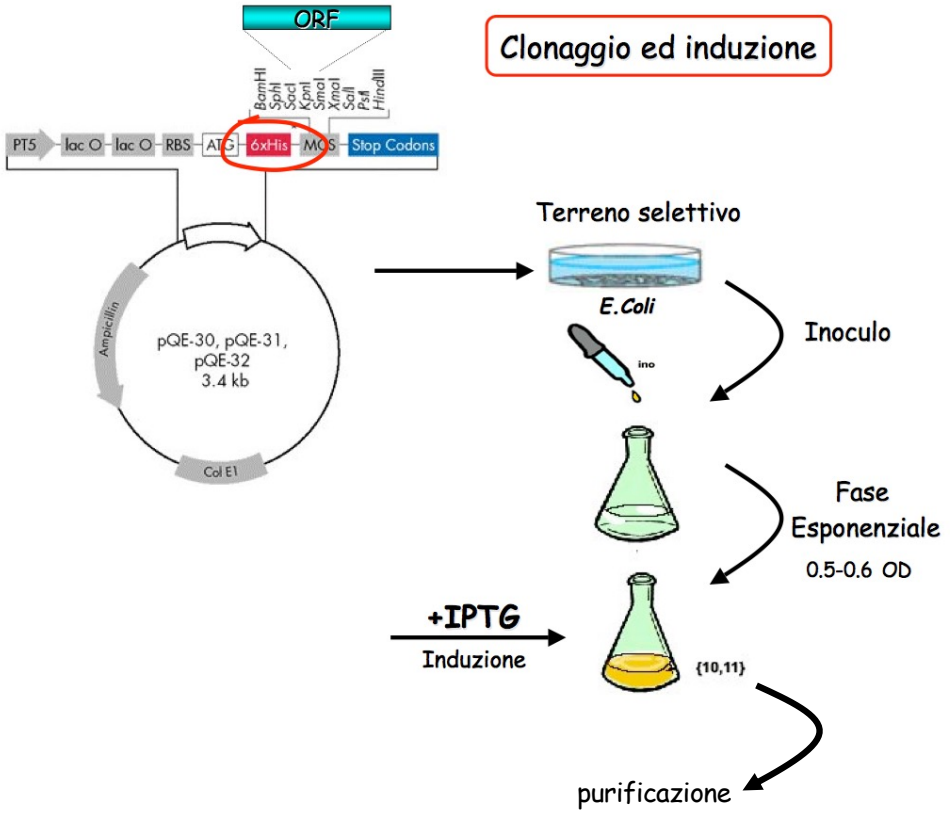
Both proteins must be purified (e.g. recombinant or produce by *in vitro* translation)

GST pull-down

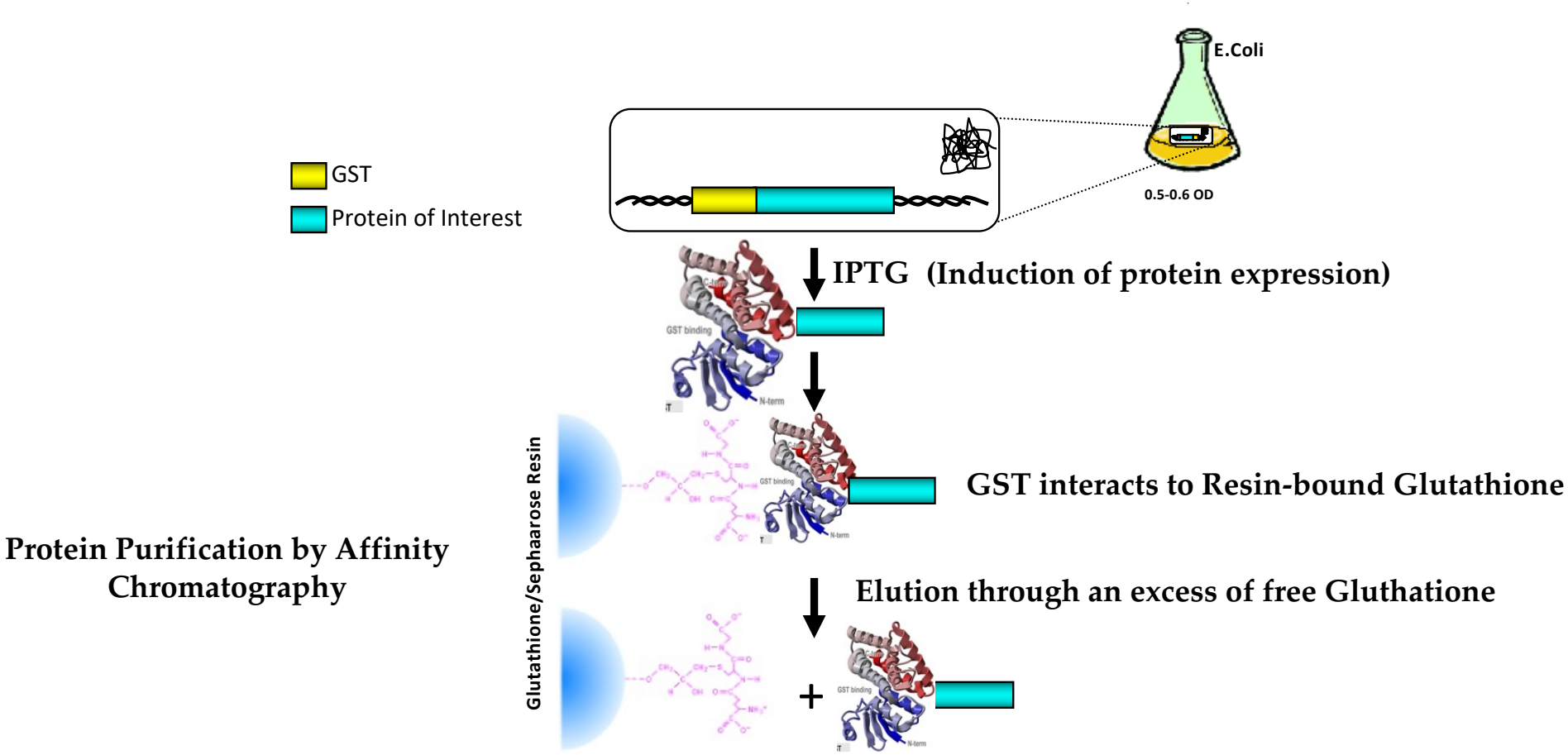


Expression and Purification of a recombinant protein

- **TRANSFORMATION (Expression vector in the expression host).**
- **AMPLIFICATION of the positive bacterial strain.**
- **INDUCTION of the recombinant protein.**
- **PURIFICATION of the recombinant protein.**

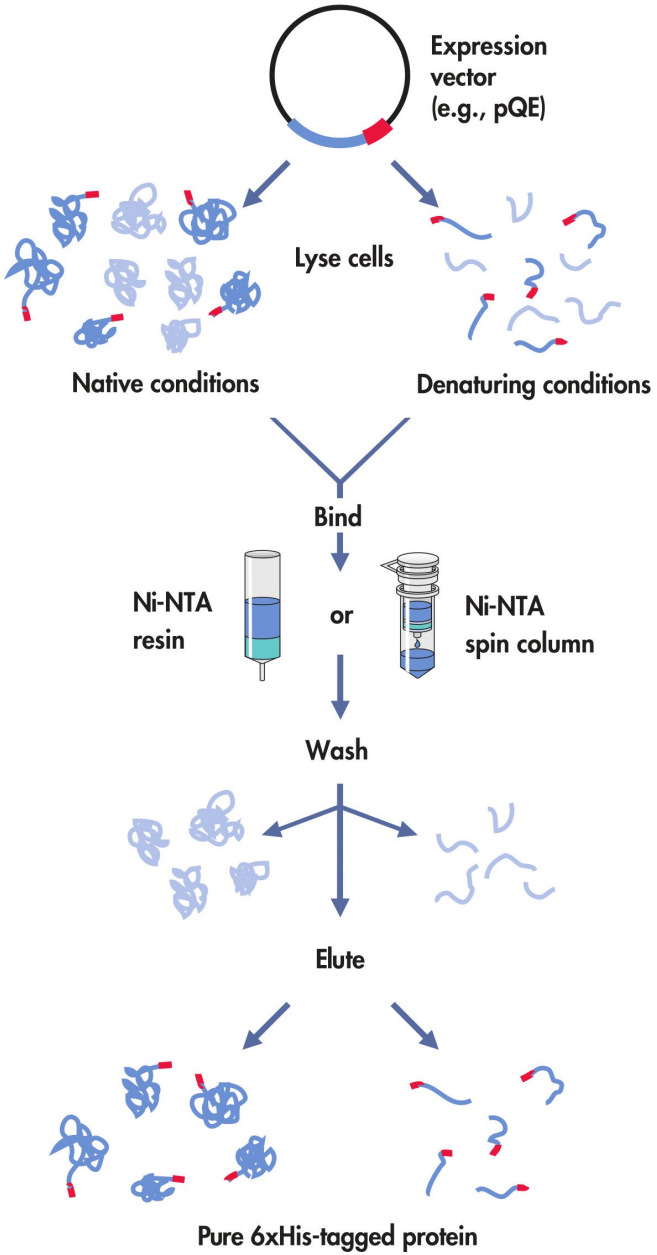


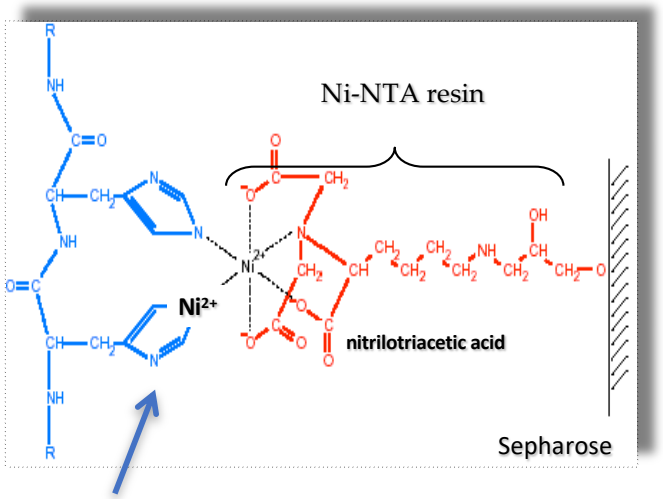
The GST-TAG system



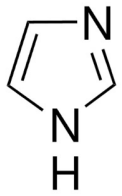
Protein Purification by Affinity Chromatography

The HIS-TAG System



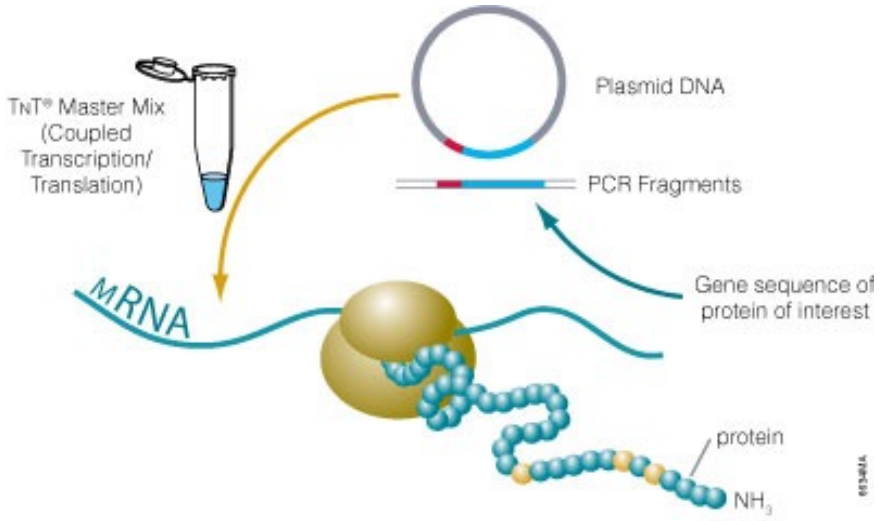


Elution By Imidazole (histidine analogue)



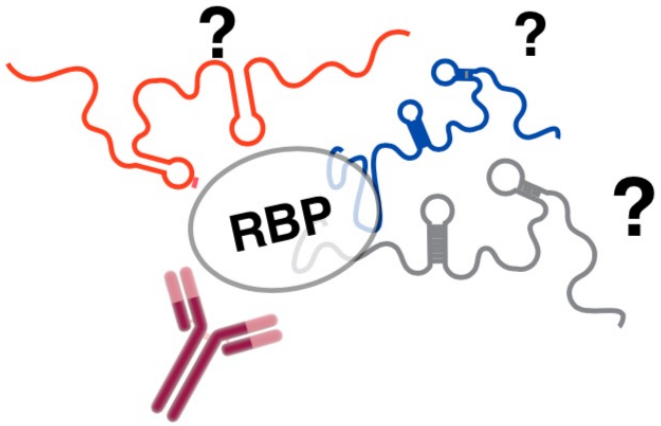
Cell-Free Transcription/Translation Systems

- Rabbit Reticulocyte Lysate or Wheat Germ Extract translation systems use RNA synthesized *in vitro*
- Coupled systems bypass many of these steps by incorporating the reagents needed for transcription directly in the translation mix.
- Traditionally, radioactive **[^{35}S]methionine** has been added to cell-free expression reactions, and the methionine is incorporated into the expressed protein, allowing detection by autoradiography.



RIP -RNA Immunoprecipitation-

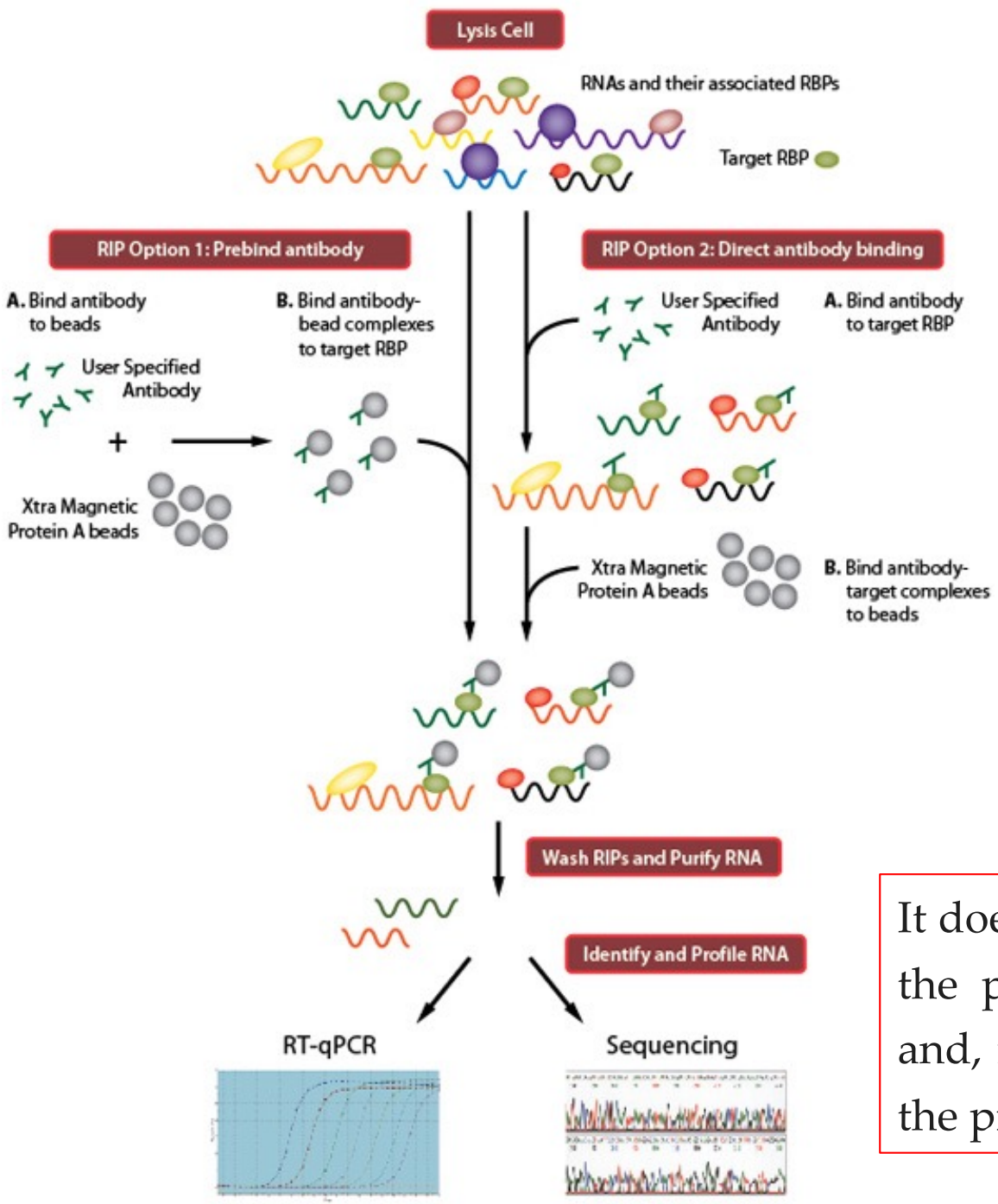
AIM: Identification of the RNAs bound to known proteins



It cannot discriminate between direct and indirect intreactions; the *exact* **binding site** remains unresolved.

Variants: CLIP (UV-RIP), PAR-CLIP, HITS-CLIP (CLIP-seq)

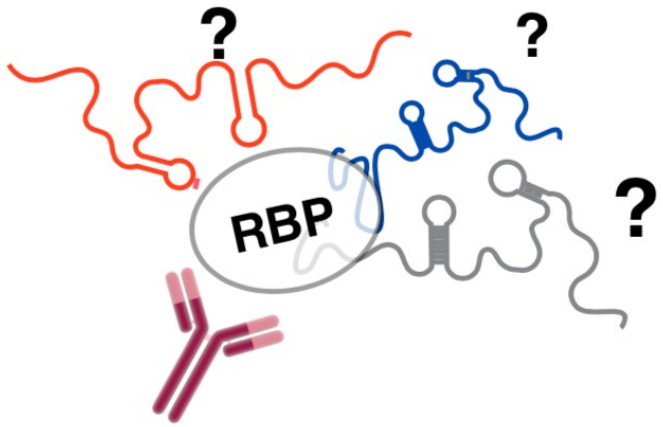
RIP -RNA Immunoprecipitation-



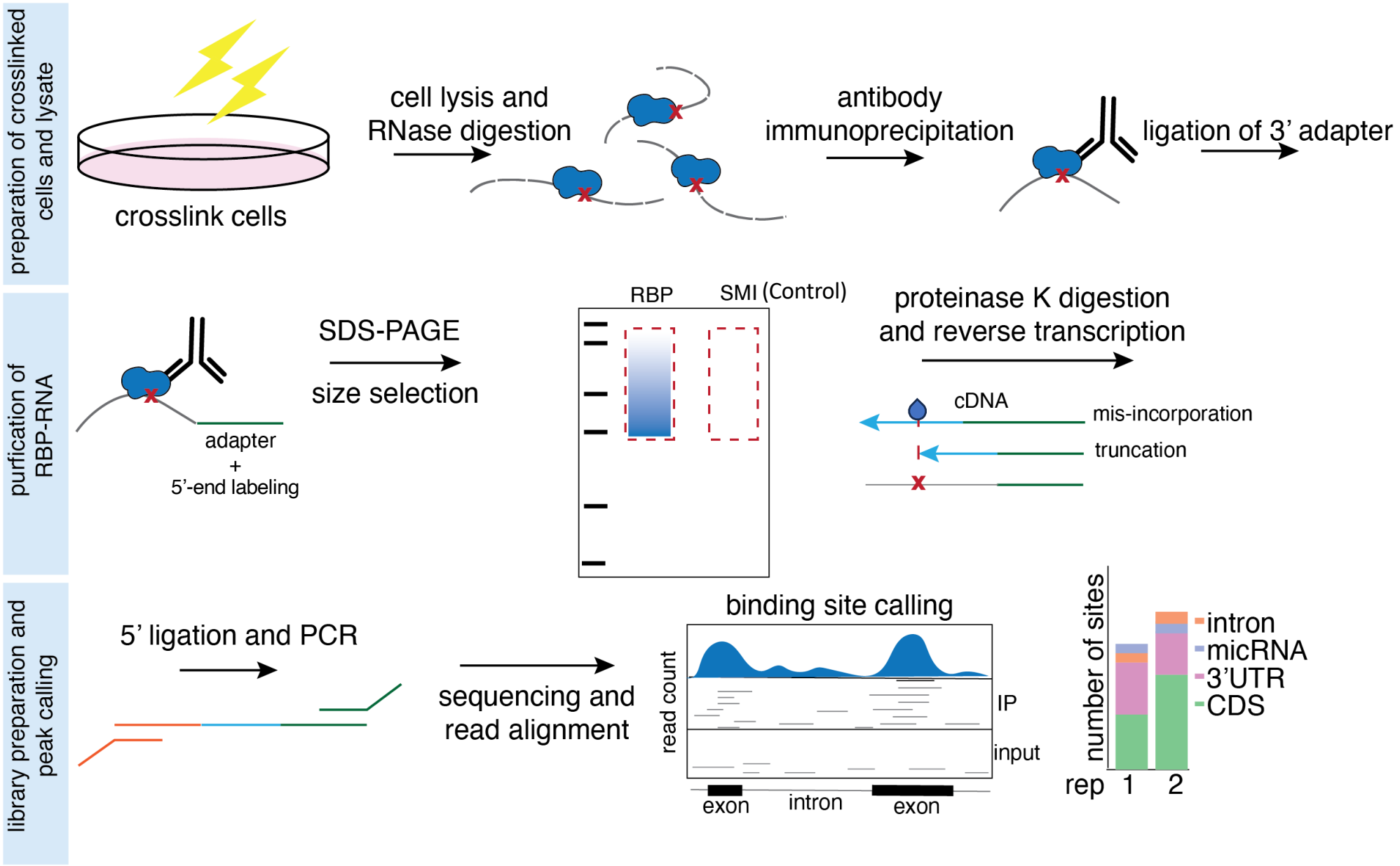
It does not determine whether the protein binding is direct and, more importantly, where the protein binds to RNA.

Crosslinking and Immunoprecipitation (CLIP)

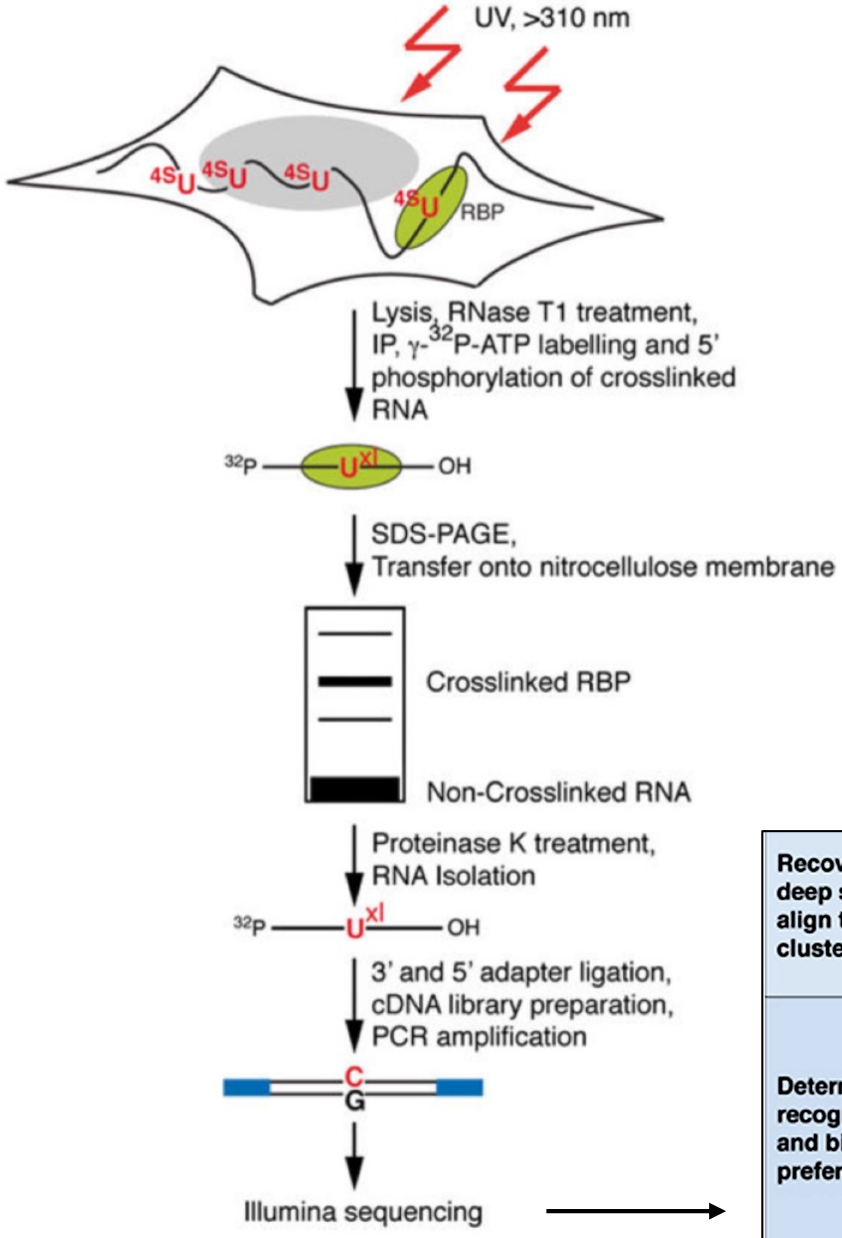
AIM: To identify the positions of protein–RNA interactions with high resolution and specificity

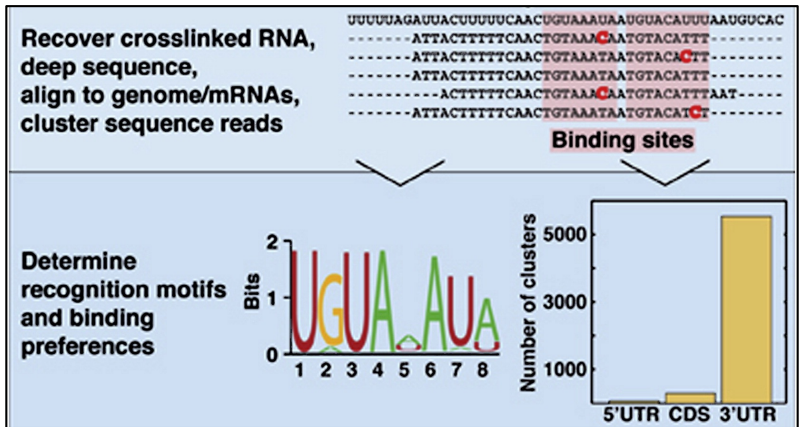


Crosslinking and Immunoprecipitation (CLIP)



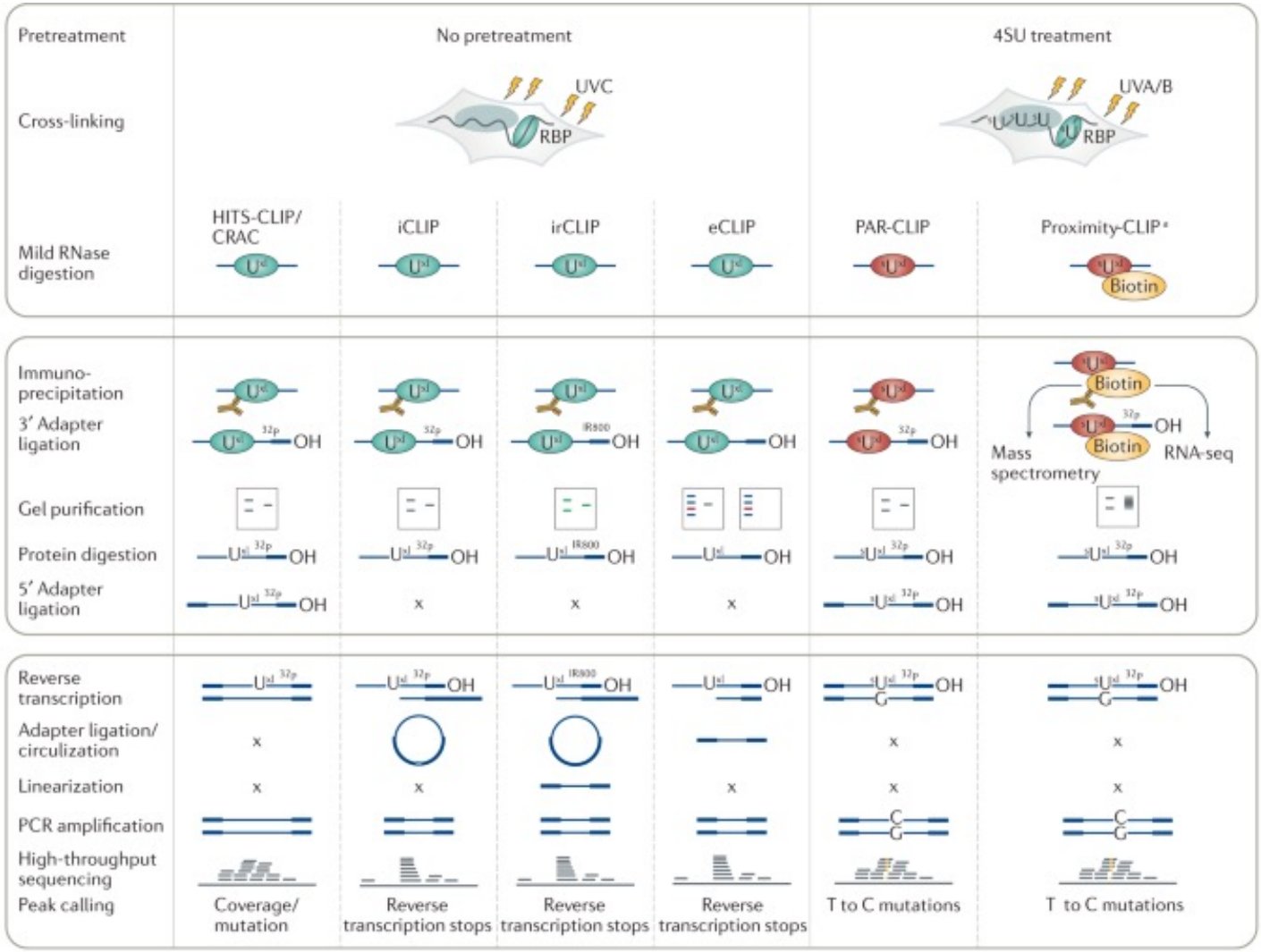
PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation)





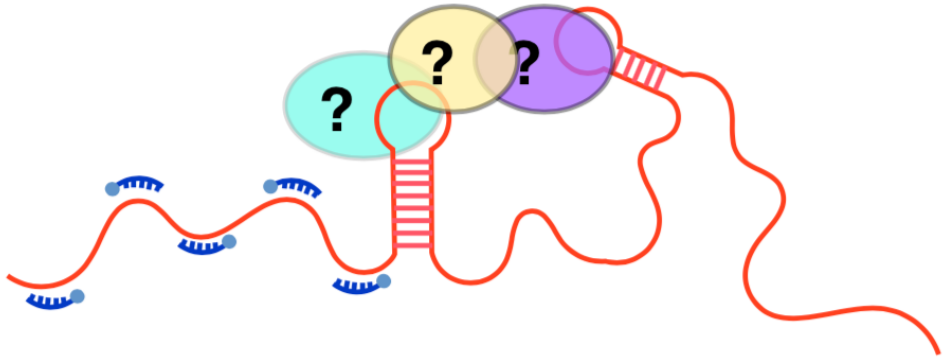
CLIP methodologies

The principle is the same, but the methodology for preparing the library varies



RNA pull-down

AIM: Identification of the protein interactors of an RNA



Endogenous RNA pull down

WORK FLOW

1.Probe Design

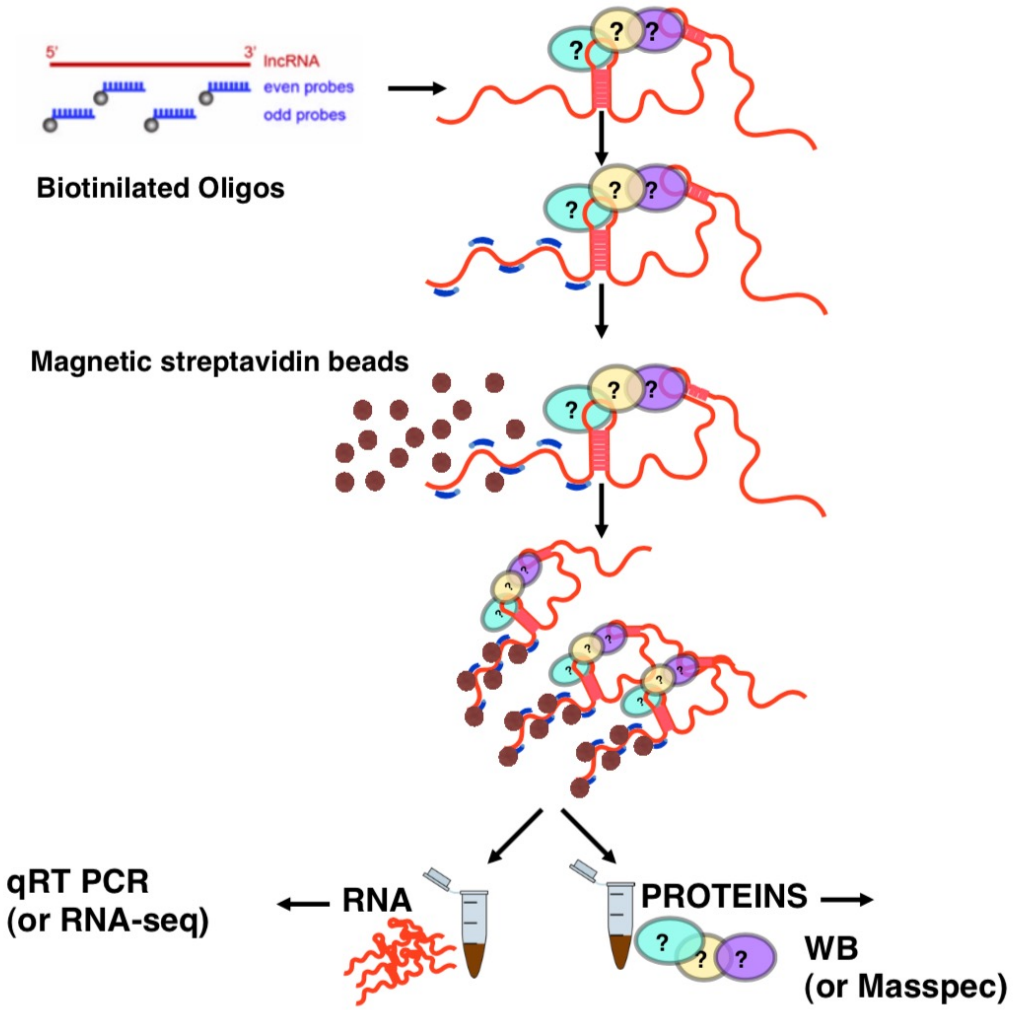
2 Collect cell extract

3. Binding step

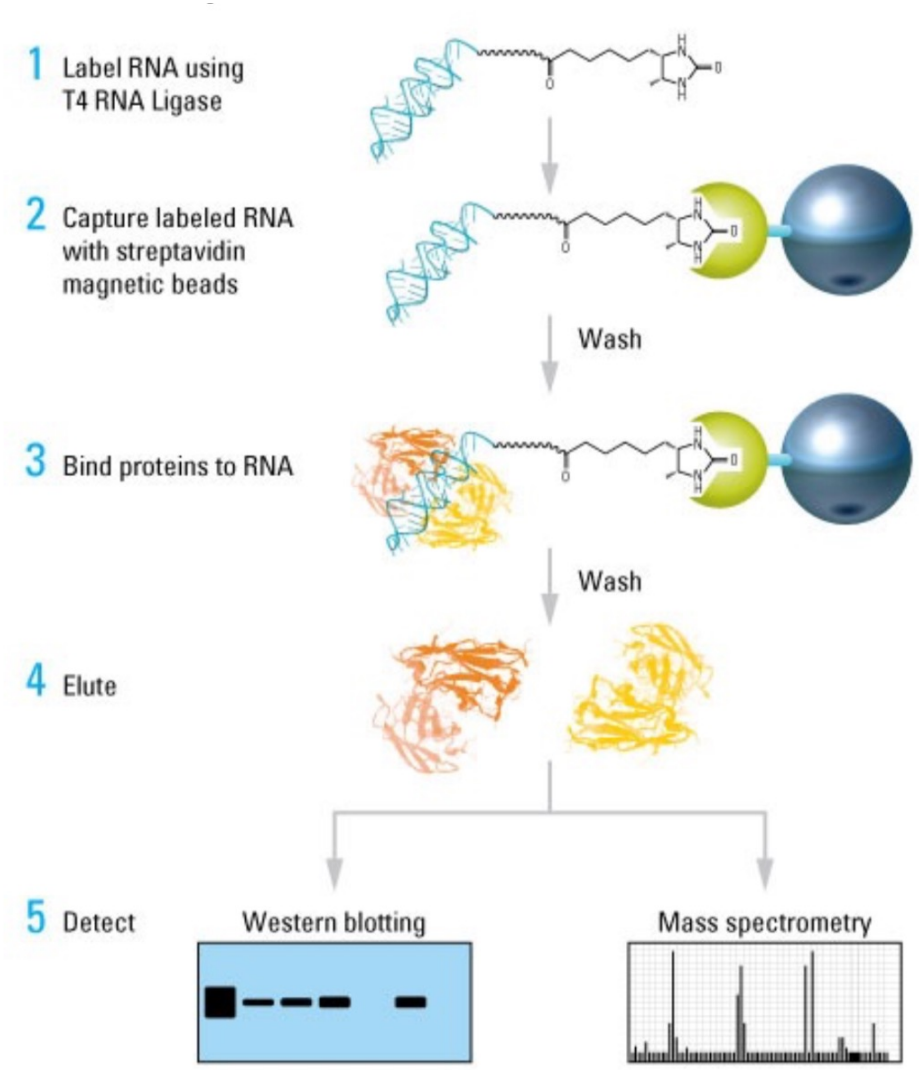
4. Introduction of Streptavidin-magnetic beads

5. Pull down

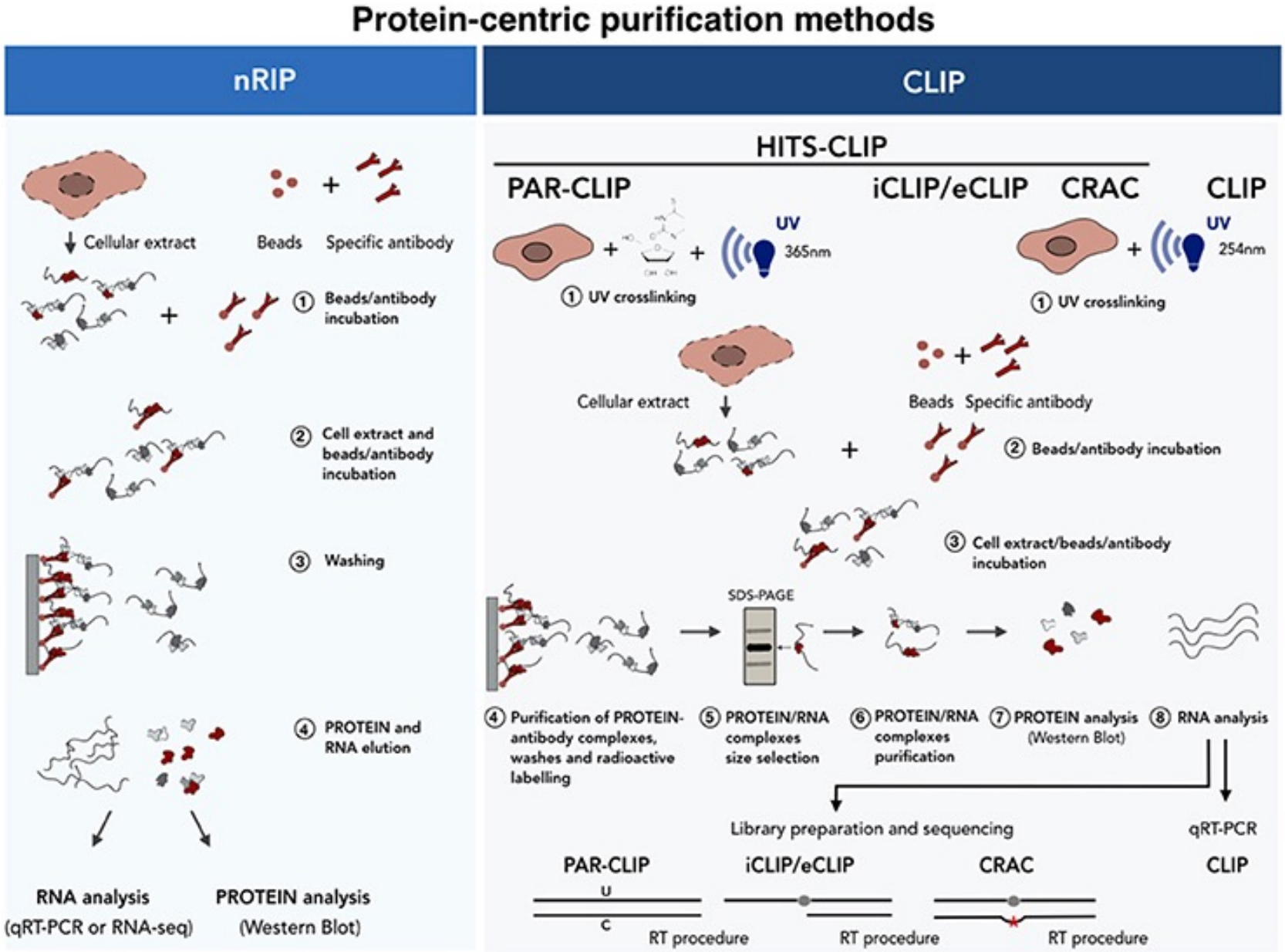
6. Protein and RNA analysis



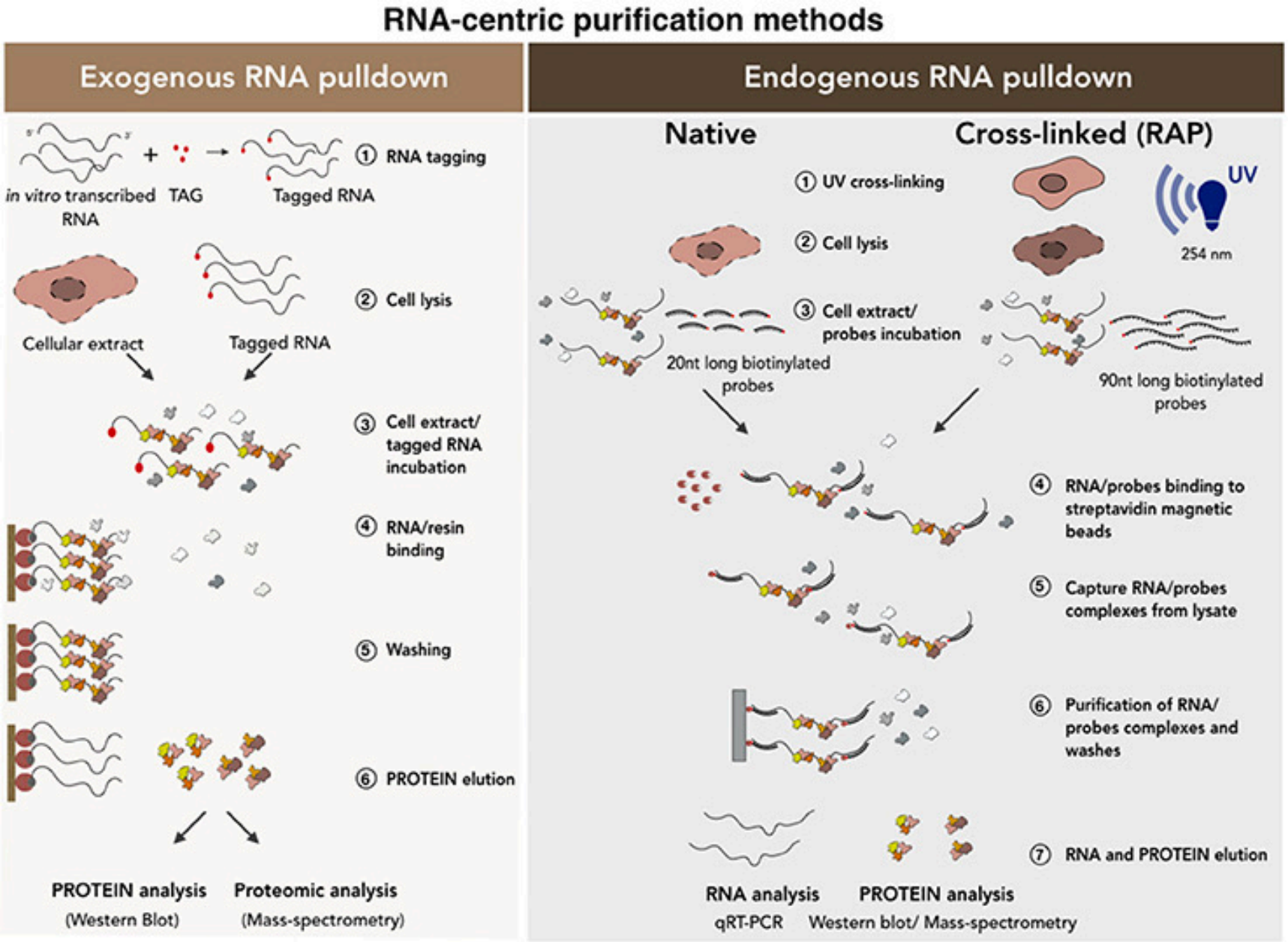
Exogenous RNA pull down



Methods for the identification of RNAs that interact with specific proteins



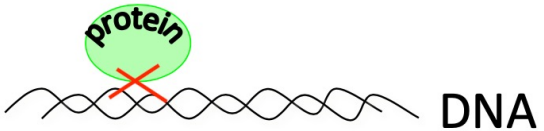
Methods for the identification of lncRNAs interactors



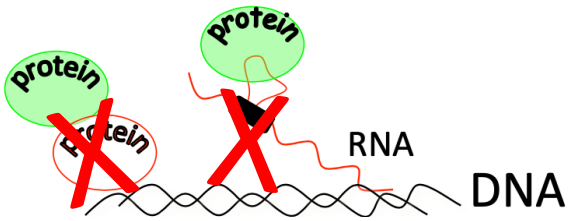
Chromatin Immunoprecipitation (ChIP)

AIM: Identification of the genomic loci bound to a DNA binding protein.

Chromatin Immunoprecipitation (ChIP) is an immunoprecipitation technique used to investigate the interaction between proteins and DNA in the cell after they have been chemically crosslinked. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factors on promoters. It can also be utilized for proteins **not directly** bound to DNA but "close" to chromatin.



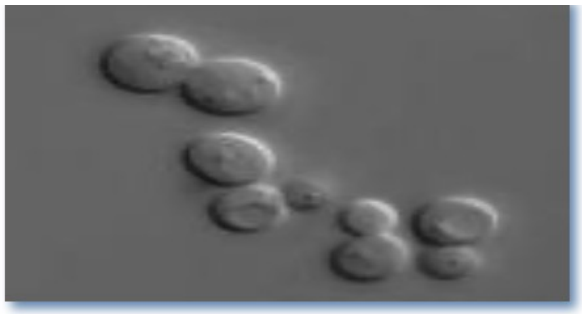
direct



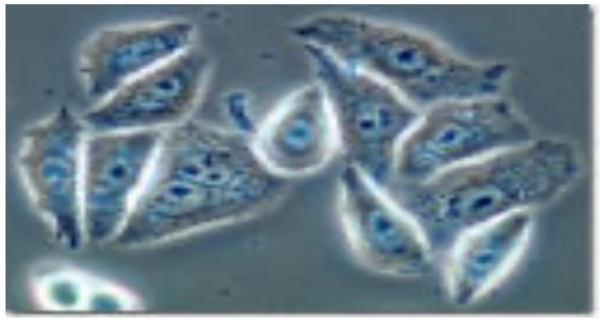
indirect

Chromatin Immunoprecipitation (ChIP)

Chromatin from most sources is a suitable substrate for ChIP:



yeast



cell cultures

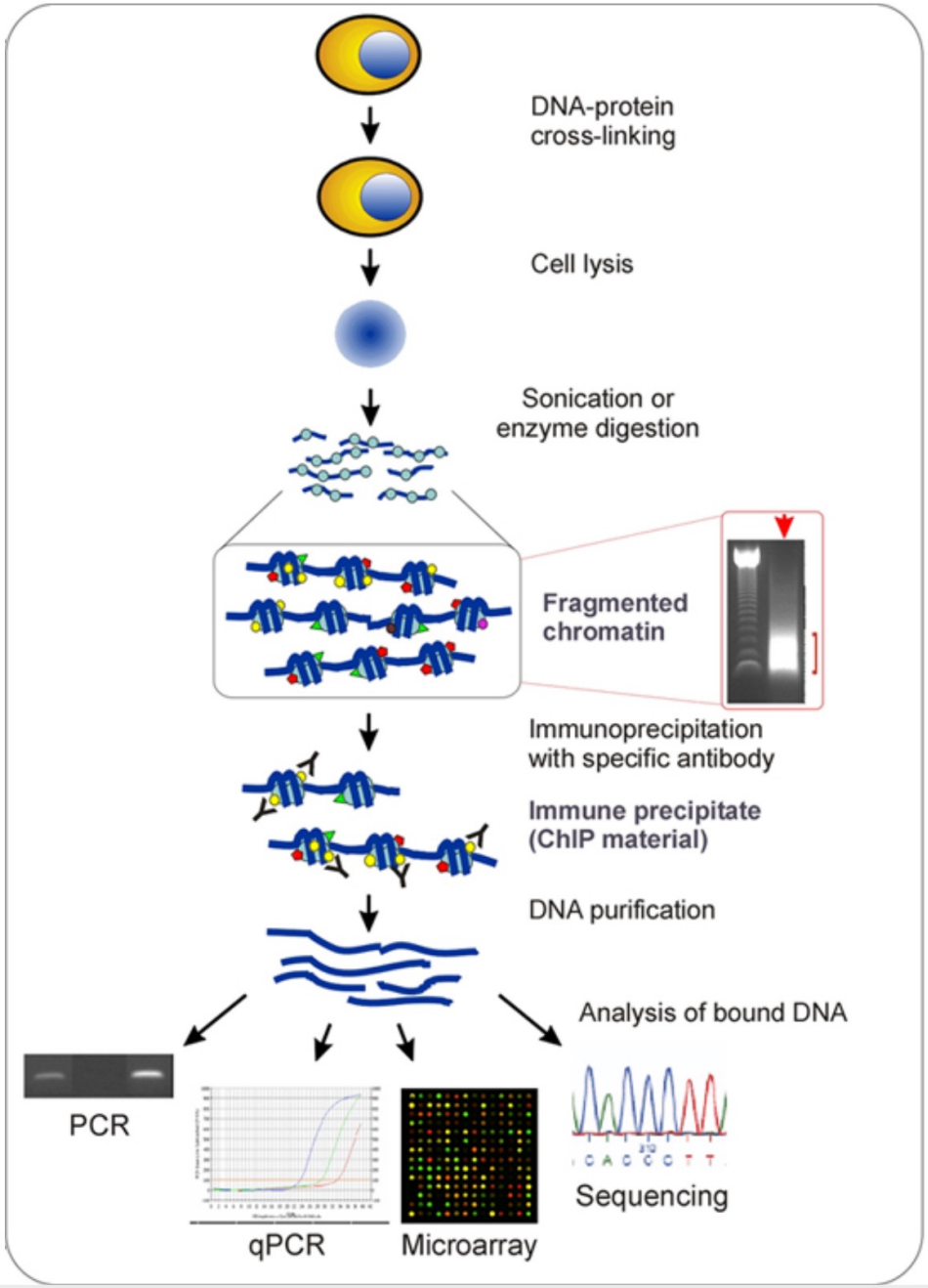
tissues

- For analysis of recovered IP material at least 10-50 ng of DNA is needed
- Each “starting ChIP sample” should contain equivalent of 25-50 µg DNA (millions of cells)

Chromatin Immunoprecipitation (ChIP)

Work flow:

1. **Cell Crosslinking**
2. **Chromatin Sonication**
3. **Antibody -Extract incubation**
4. **Binding between Beads and Antibody**
5. **Reverse crosslink and DNA purification**
6. **DNA analysis**



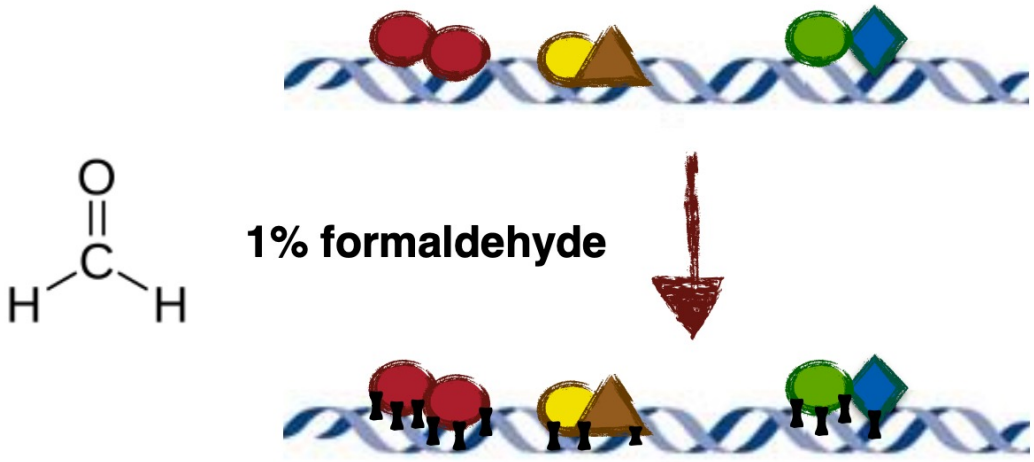
Chromatin Immunoprecipitation (ChIP)

1. **Cell Crosslinking**

The cross-linking is an experimental procedure that convert in **covalent** all the weak and non-covalent interactions between DNA - PROTEINS and PROTEIN-PROTEIN

Crosslinking strategies:

Formaldehyde
Glutaraldehyde



Chromatin Immunoprecipitation (ChIP)

1. Cell Crosslinking

Formaldehyde Cross-linking

- Formaldehyde is an organic compound. It is water soluble and penetrates biological membranes. It targets **primary aminogroups** (i.e. lysine in proteins, side chains of A,C,G in DNA)

- It crosslinks both **protein-nucleic acids**, **nucleic acids- nucleic acids** and **protein-protein**

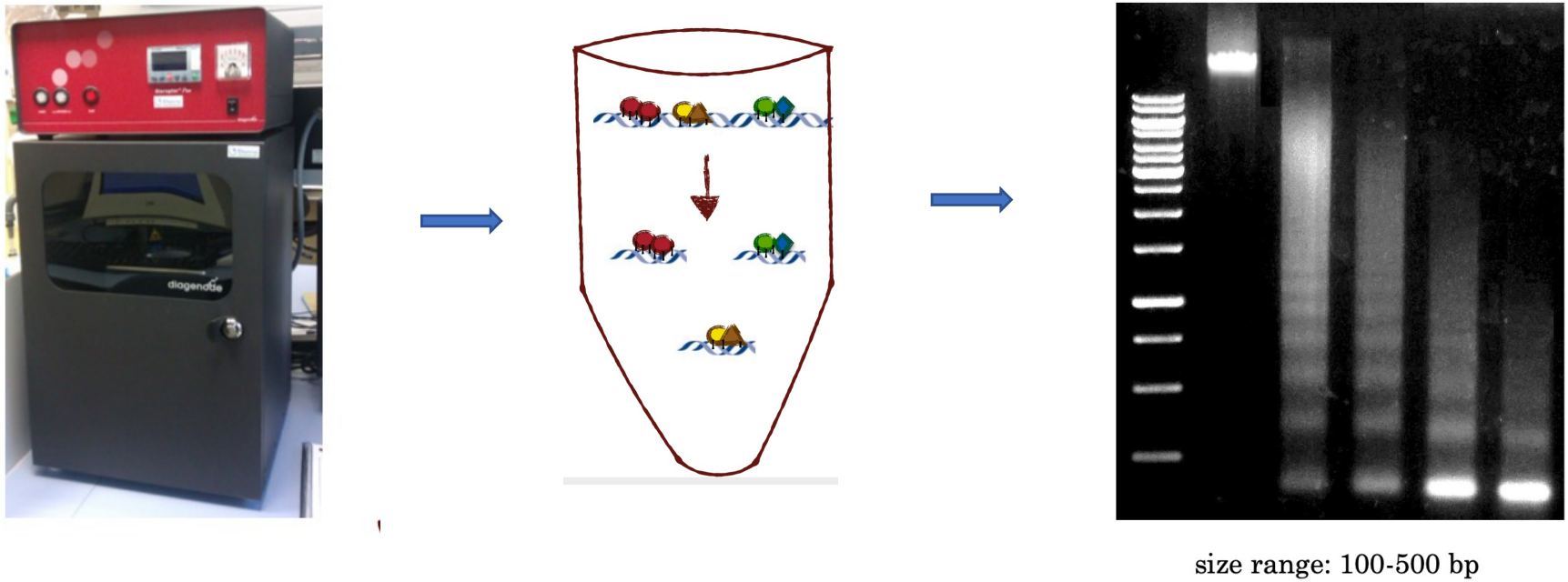
- The crosslinking is reversible (65°C reverse protein-DNA; 100°C reverse protein-protein)

- Reaction is stopped by providing an excess of primary amino groups (0.125M glycine)

Chromatin Immunoprecipitation (ChIP)

2. Chromatin Sonication

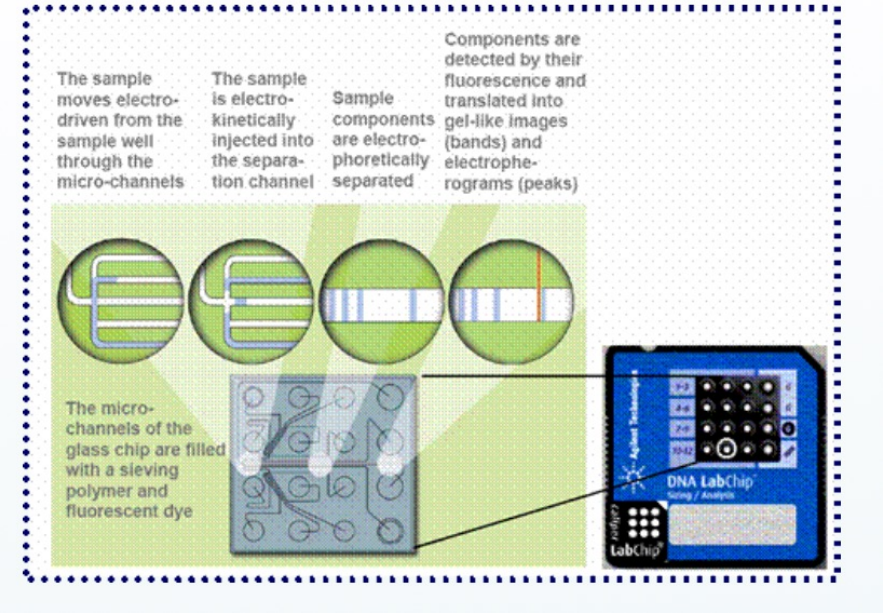
The DNA-protein complexes (chromatin-protein) are then sheared into ~500 bp DNA fragments by **sonication** or nuclease digestion.

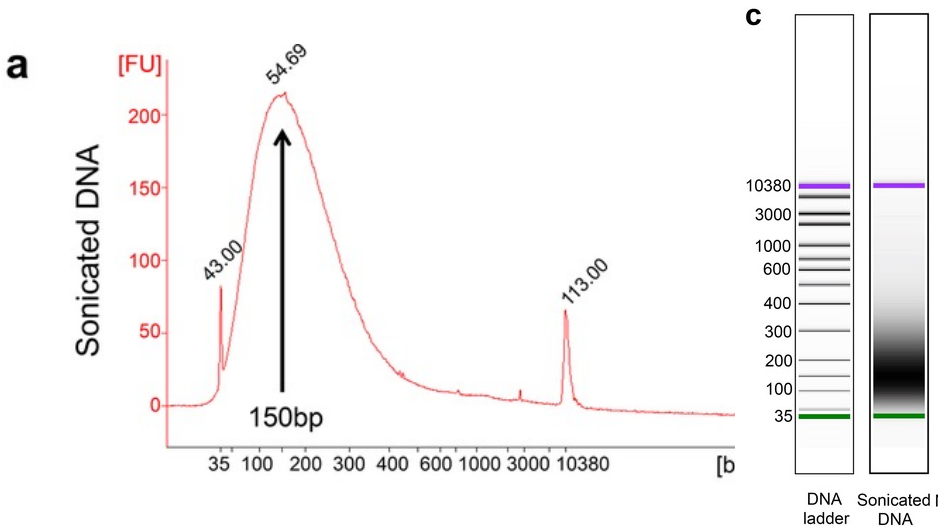


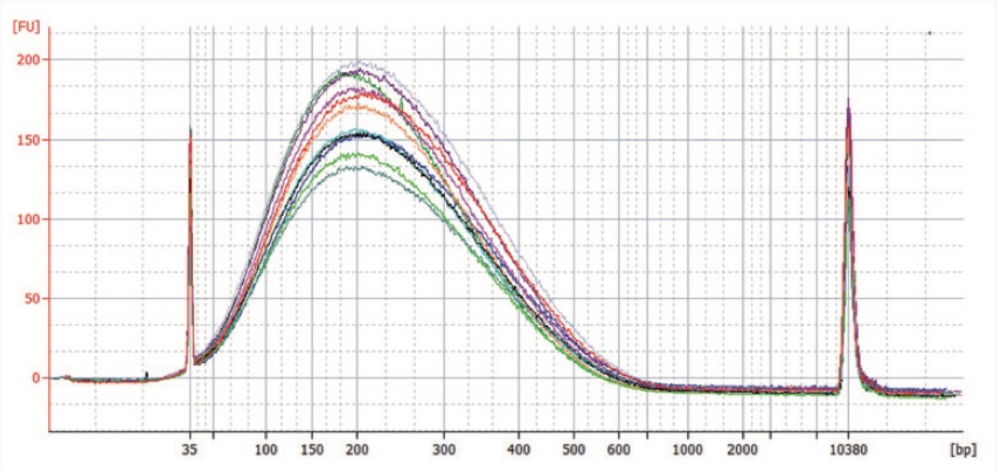
size range: 100-500 bp

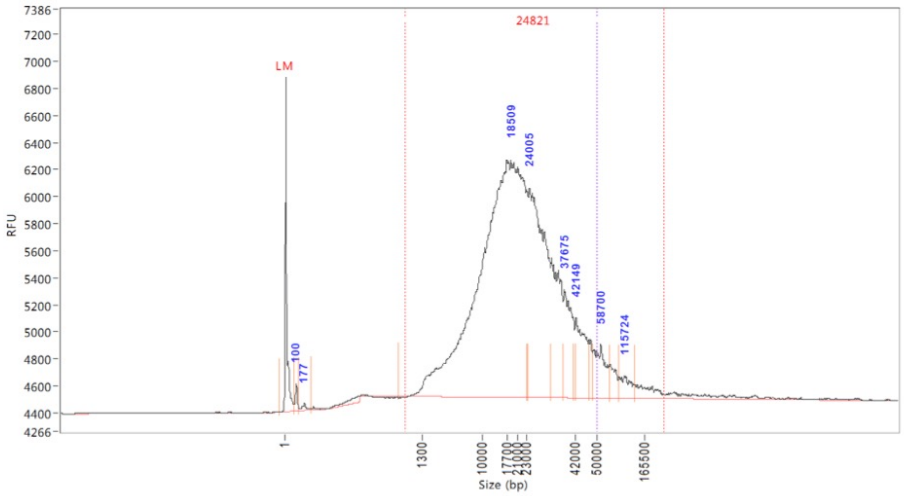
Chromatin Immunoprecipitation (ChIP)

2. Chromatin Analysis (Bioanalyzer)





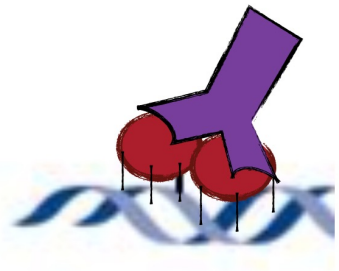


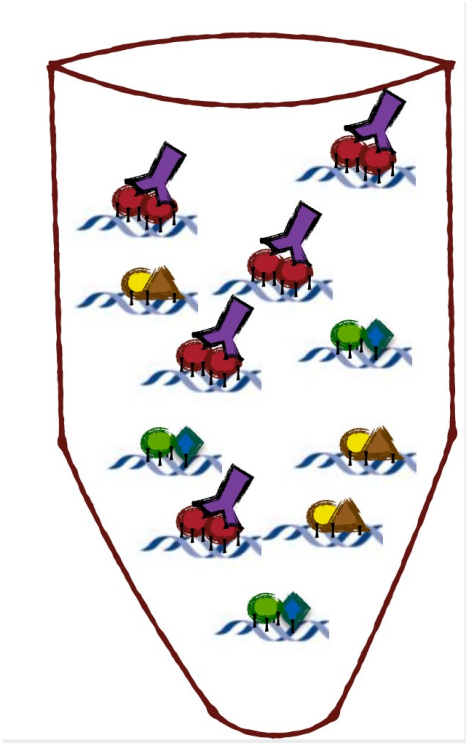


Chromatin Immunoprecipitation (ChIP)

3. Antibody -Extract incubation

The antibody specific for the protein of interest, is incubated together with the extract, this step allows the formation of strongly interaction between the antibody and the protein of interest

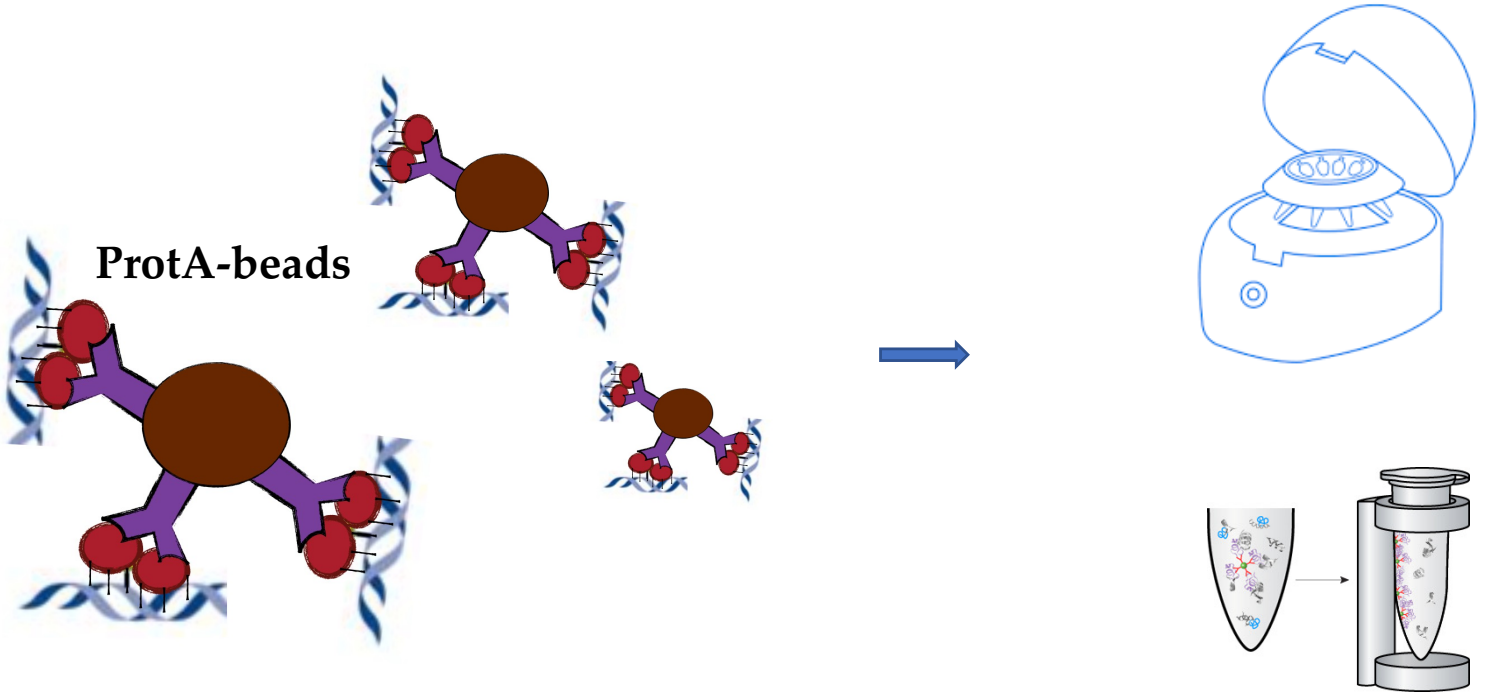




Chromatin Immunoprecipitation (ChIP)

4. Binding between Beads and Antibody

Agarose, Sepharose or Magnetic beads (Protein A or Protein G conjugated) are added to the extract. This step is fundamental for the precipitation of the complex **Bead-Antibody-Protein-DNA**

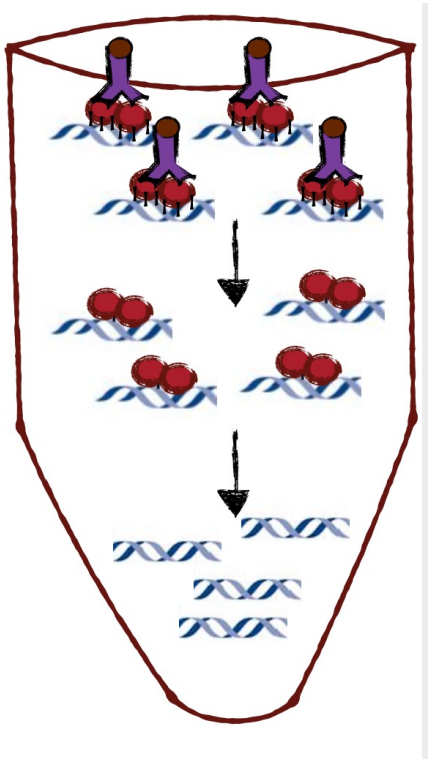


Centrifugation or magnetic recovery step allows the purification of the complexes that are bound to the antibody

Chromatin Immunoprecipitation (ChIP)

5. Reverse crosslink and DNA purification

The cross-linking with formaldehyde is removed through the incubation of the extract at High temperature (70° C for 5 minutes). This step allows the detach of the protein from the DNA that is subsequently purified by Proteinase K digestion and phenol extraction.

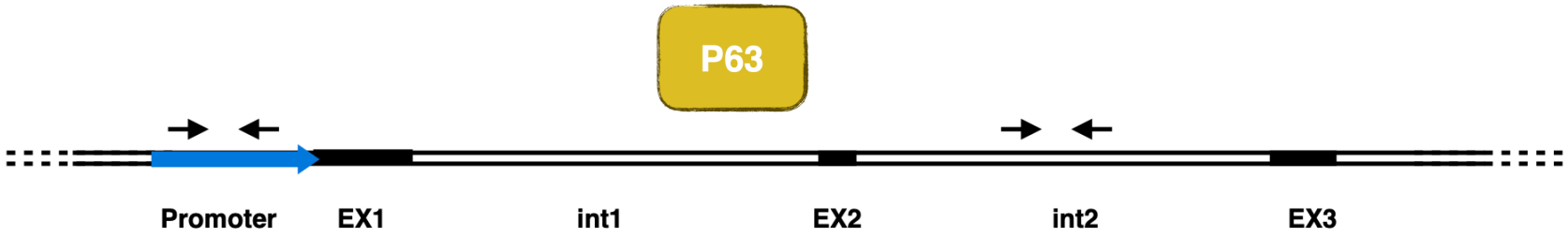


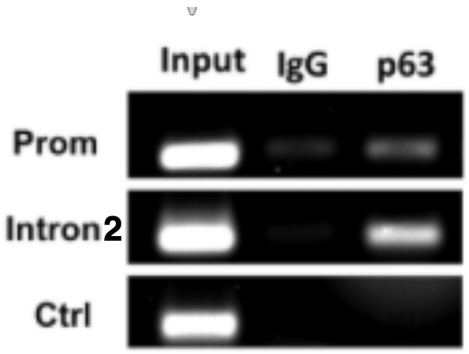
Chromatin Immunoprecipitation (ChIP)

6. DNA analysis

ChIP (PCR)

The isolated DNA can be quantified by PCR using specific probes. This allows the analysis of a specific region in multiple samples.





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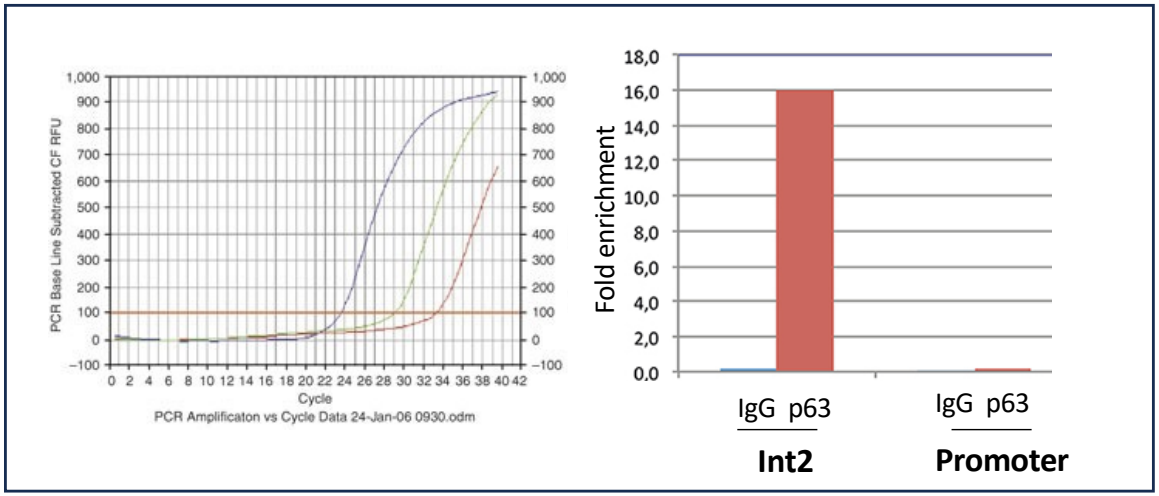
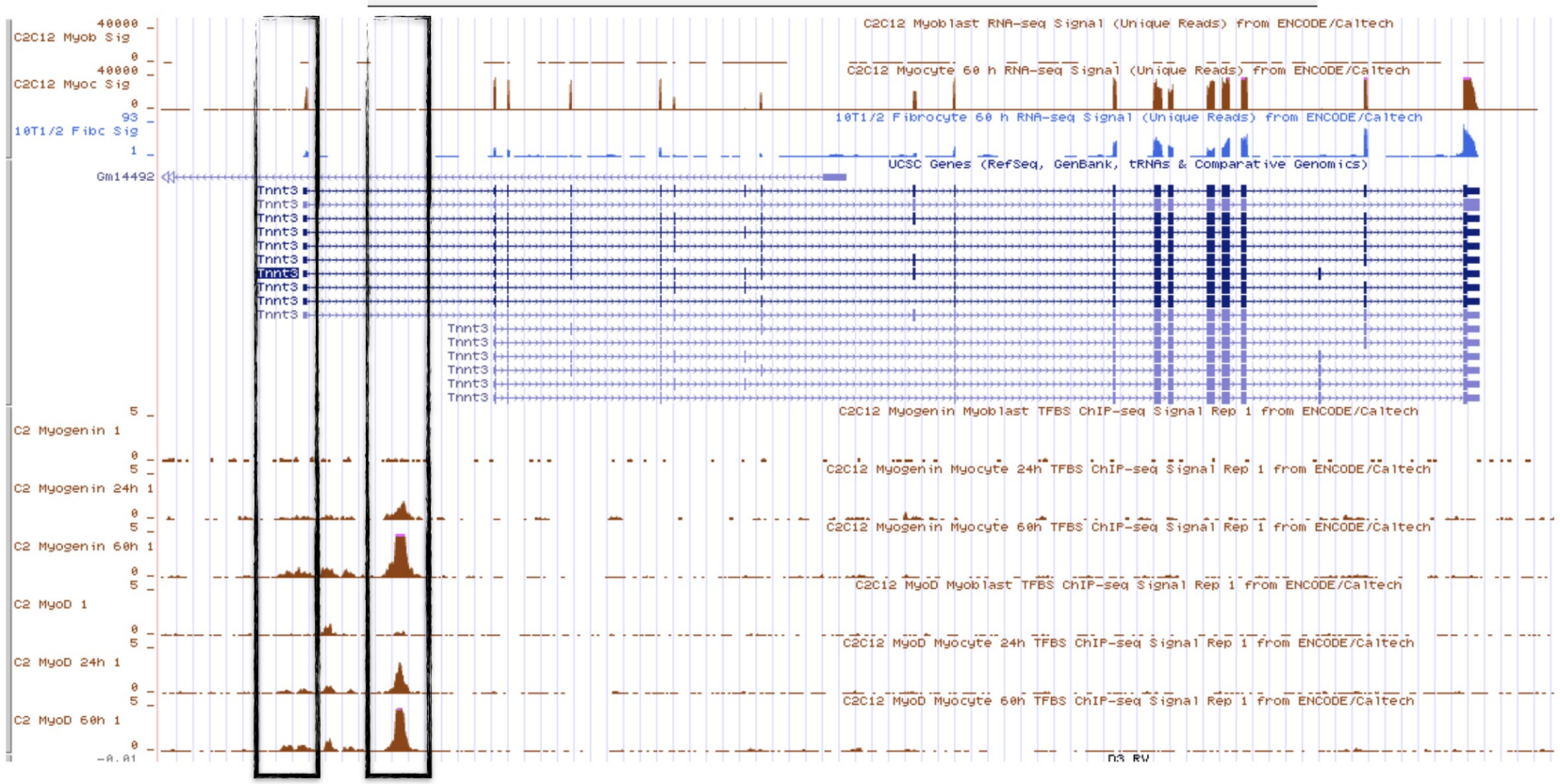


Chromatin Immunoprecipitation (ChIP)

6. DNA analysis

ChIP Seq

Direct sequencing of the DNA isolated, generates genome wide profiles. ChIP-seq combines ChIP and direct sequencing technology for genome-wide analysis of antigen distribution. Immunoprecipitated DNA is sequenced and mapped to the genome



Chromatin Isolation by RNA Purification (ChIRP)

- allows to map *in vivo* the **RNA occupancy** (genome-wide) at high resolution.
- has enabled the first genome-wide views of **lncRNA occupancy** on the human genome.

Chromatin Isolation by RNA Purification (ChIRP)