

Molecular techniques for the study of the interaction between macromolecules

Molecular techniques for the study of the interaction between:

Protein-protein:

Immunoprecipitation

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)

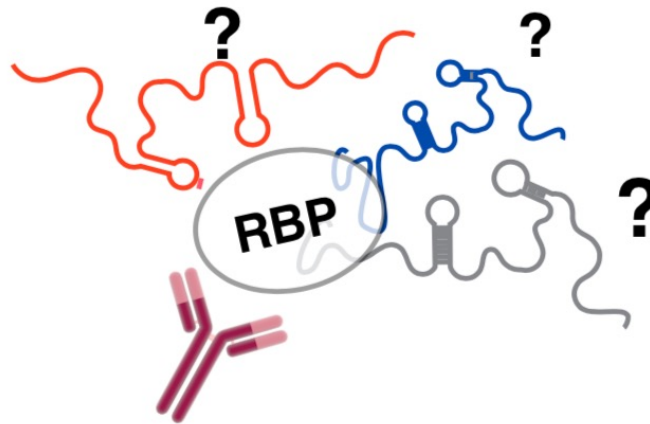
Protein-RNA (Protein centric):

RIP (RNA immunoprecipitation)

CLIP (Cross-linked immunoprecipitation)

RIP -RNA Immunoprecipitation-

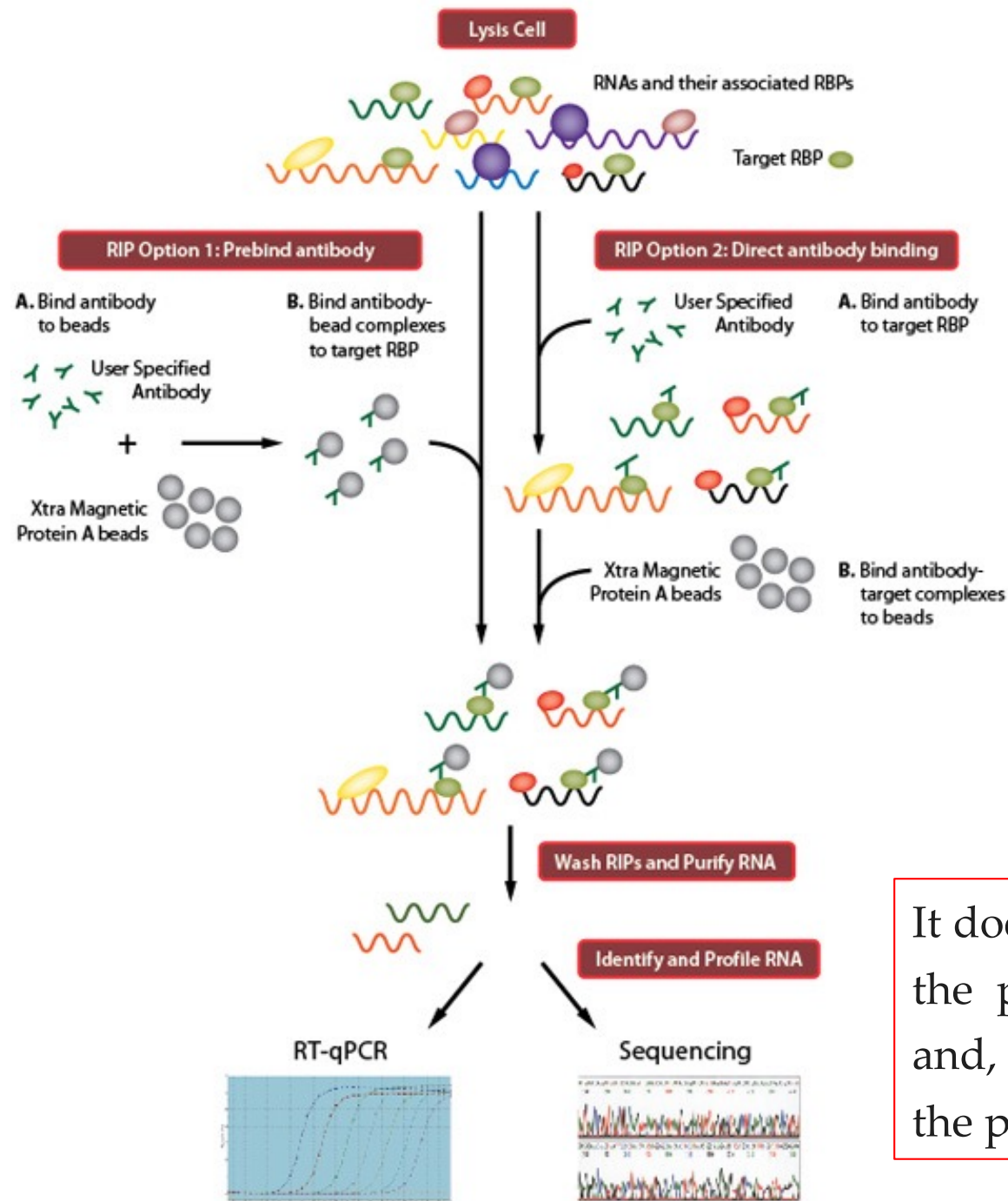
AIM: Identification of the RNAs bound to known proteins



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

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

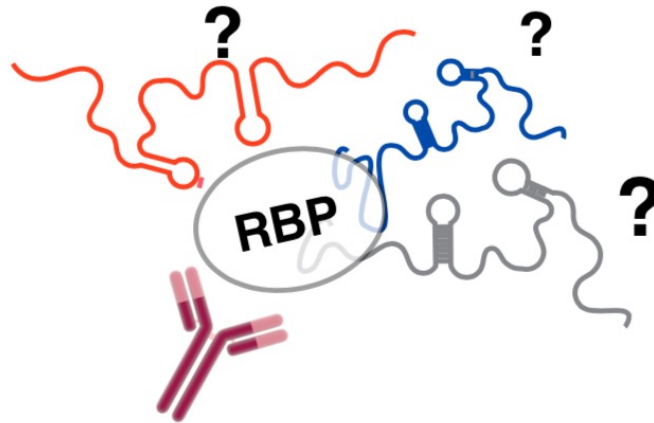
RIP -RNA Immunoprecipitation-



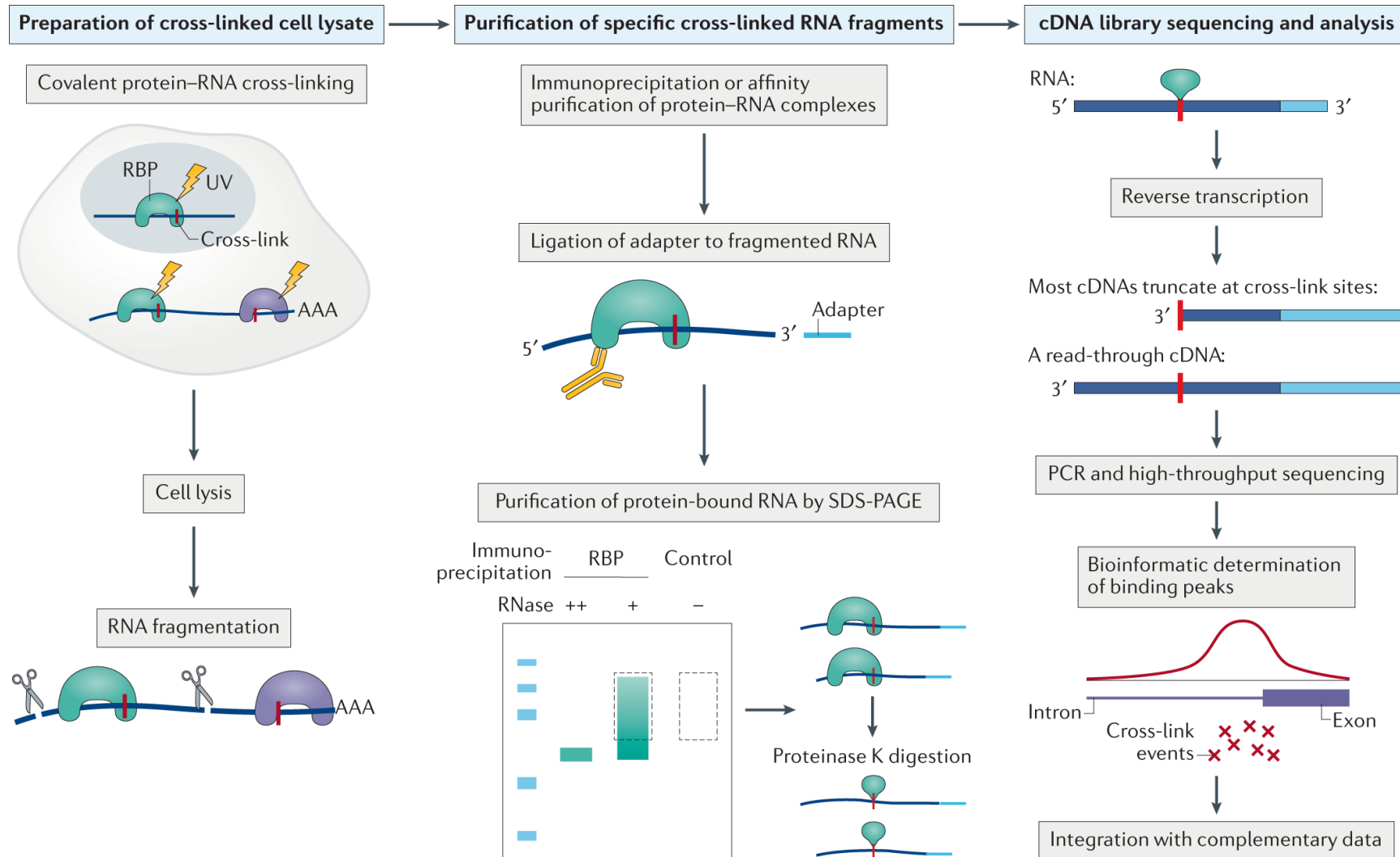
It does not determine whether the protein binding is direct and, more importantly, where the protein binds to RNA.

Crosslinking and Immunoprecipitation (CLIP)

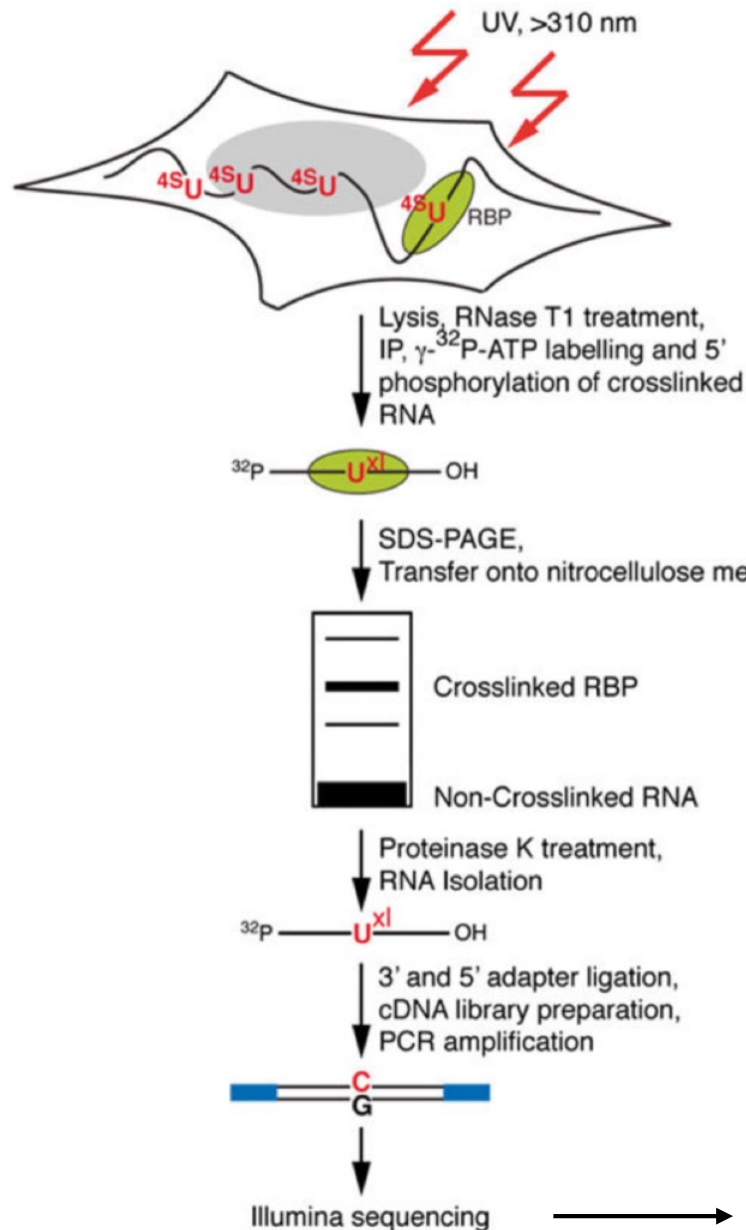
AIM: To identify the positions of protein–RNA interactions with high resolution and specificity



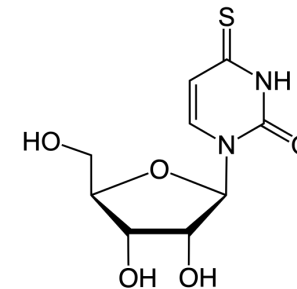
Crosslinking and Immunoprecipitation (CLIP)



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



4-thiouridine (4^SU)



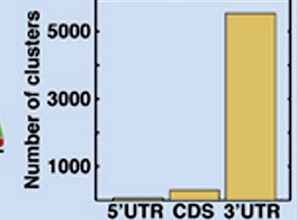
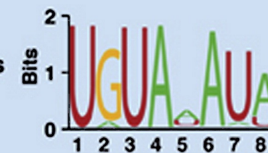
Recover crosslinked RNA,
deep sequence,
align to genome/mRNAs,
cluster sequence reads

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UUUUUAGAUUACUUUUUACAUGUAAAUAAUGUACAUUUAAUGUCAC
-----ATTACTTTTCAACTGTAAACAATGTACATTT-----
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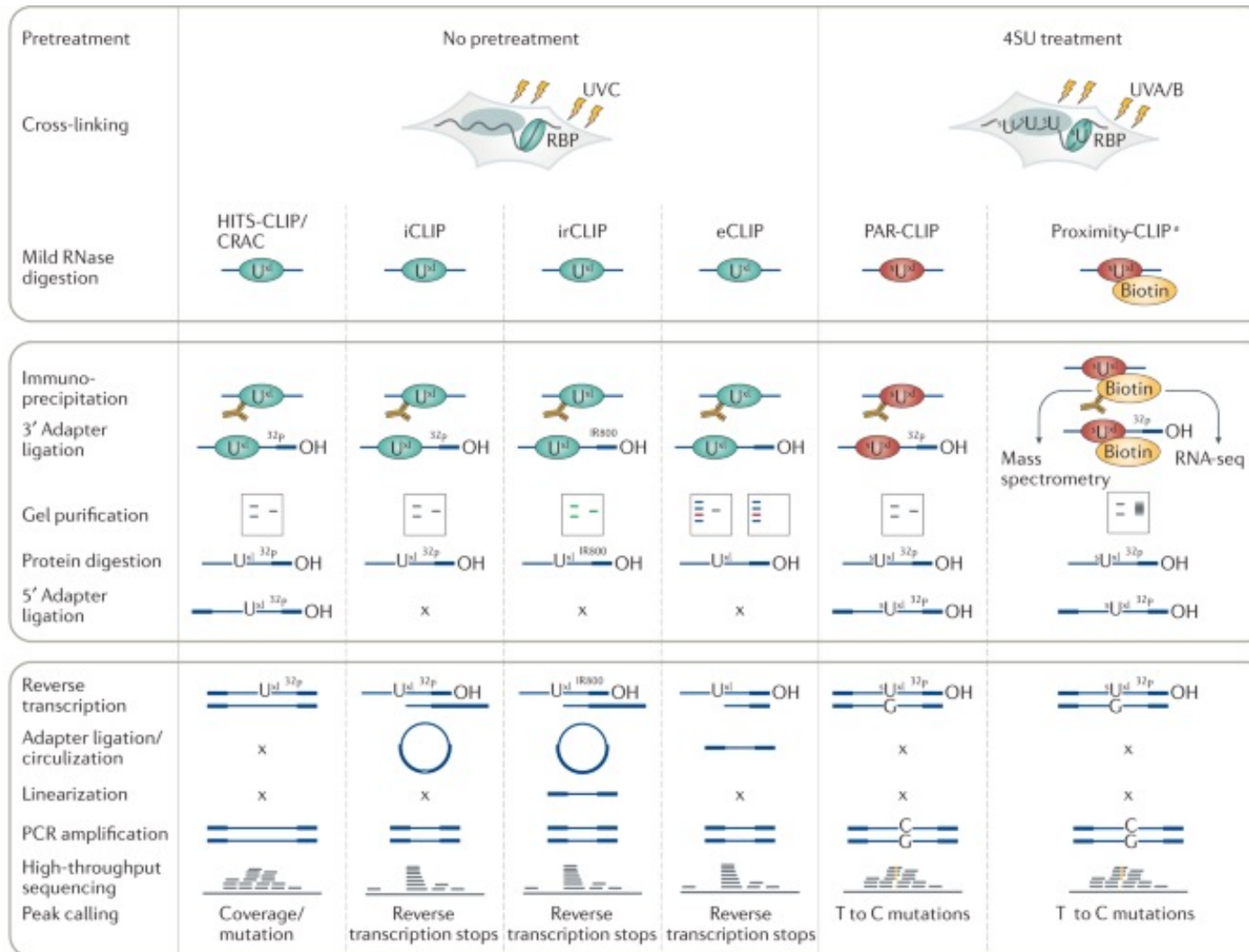
Binding sites

Determine
recognition motifs
and binding
preferences



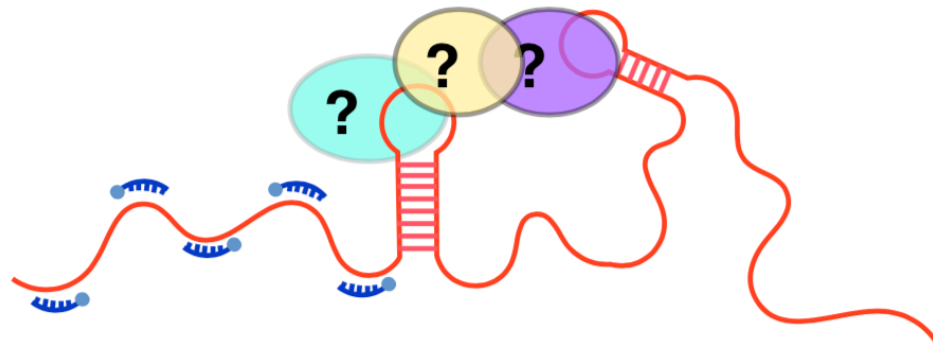
CLIP methodologies

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

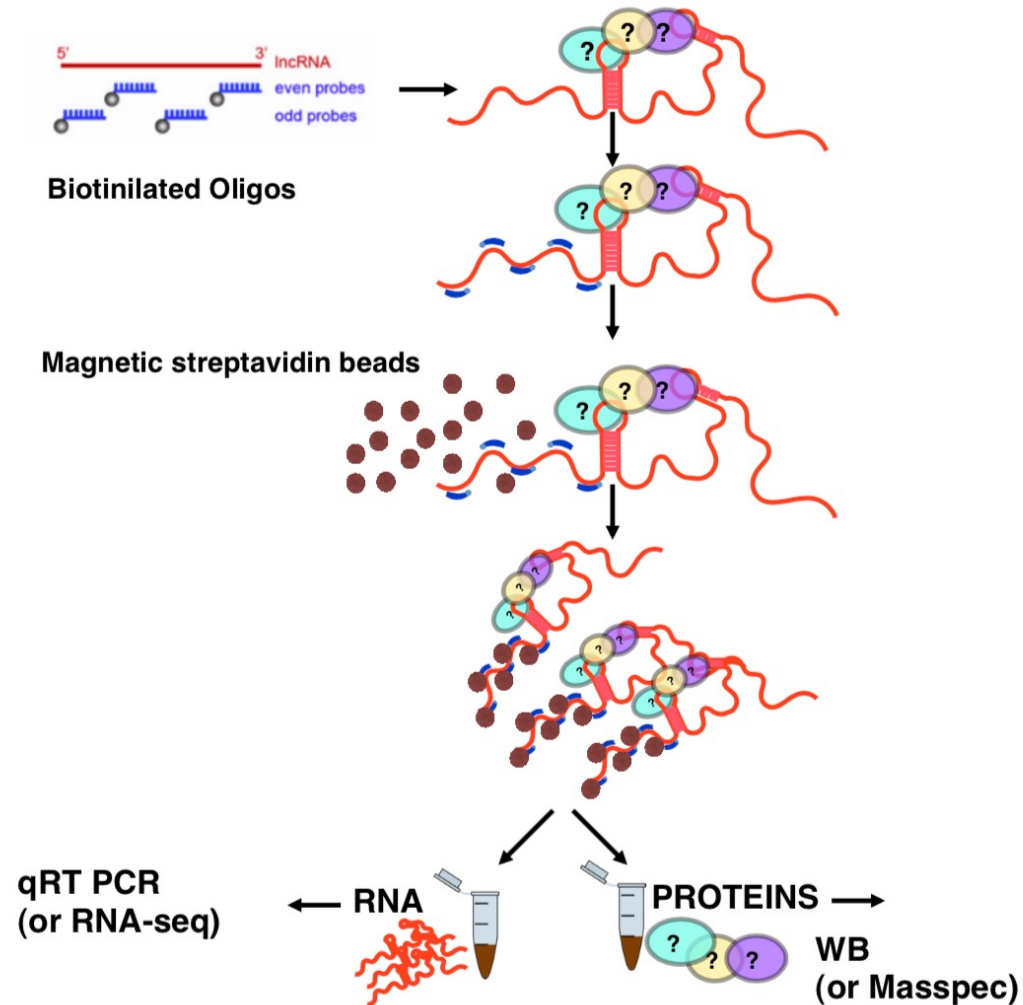
2 Collect cell extract

3. Binding step

4. Introduction of Streptavidin-magnetic beads

5. Pull down

6. Protein and RNA analysis



Exogenous RNA pull down

1 Label RNA using
T4 RNA Ligase

2 Capture labeled RNA
with streptavidin
magnetic beads

3 Bind proteins to RNA

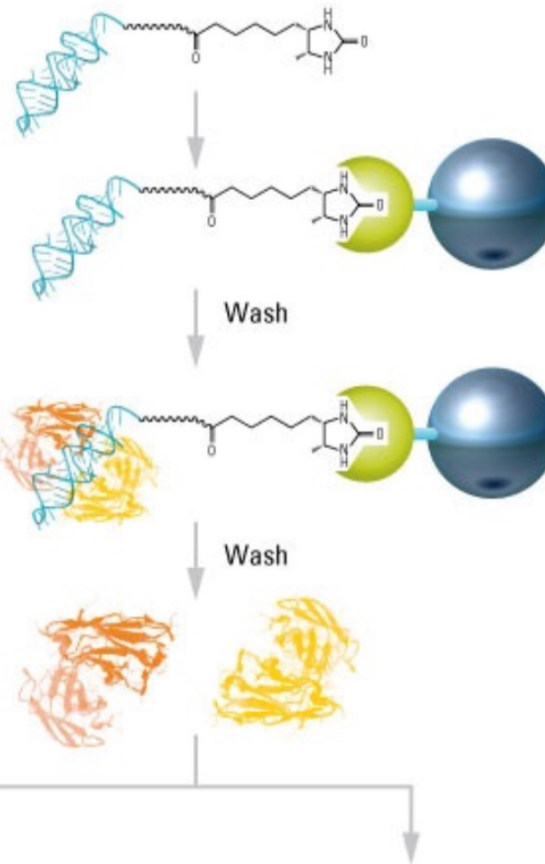
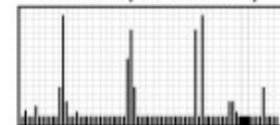
4 Elute

5 Detect

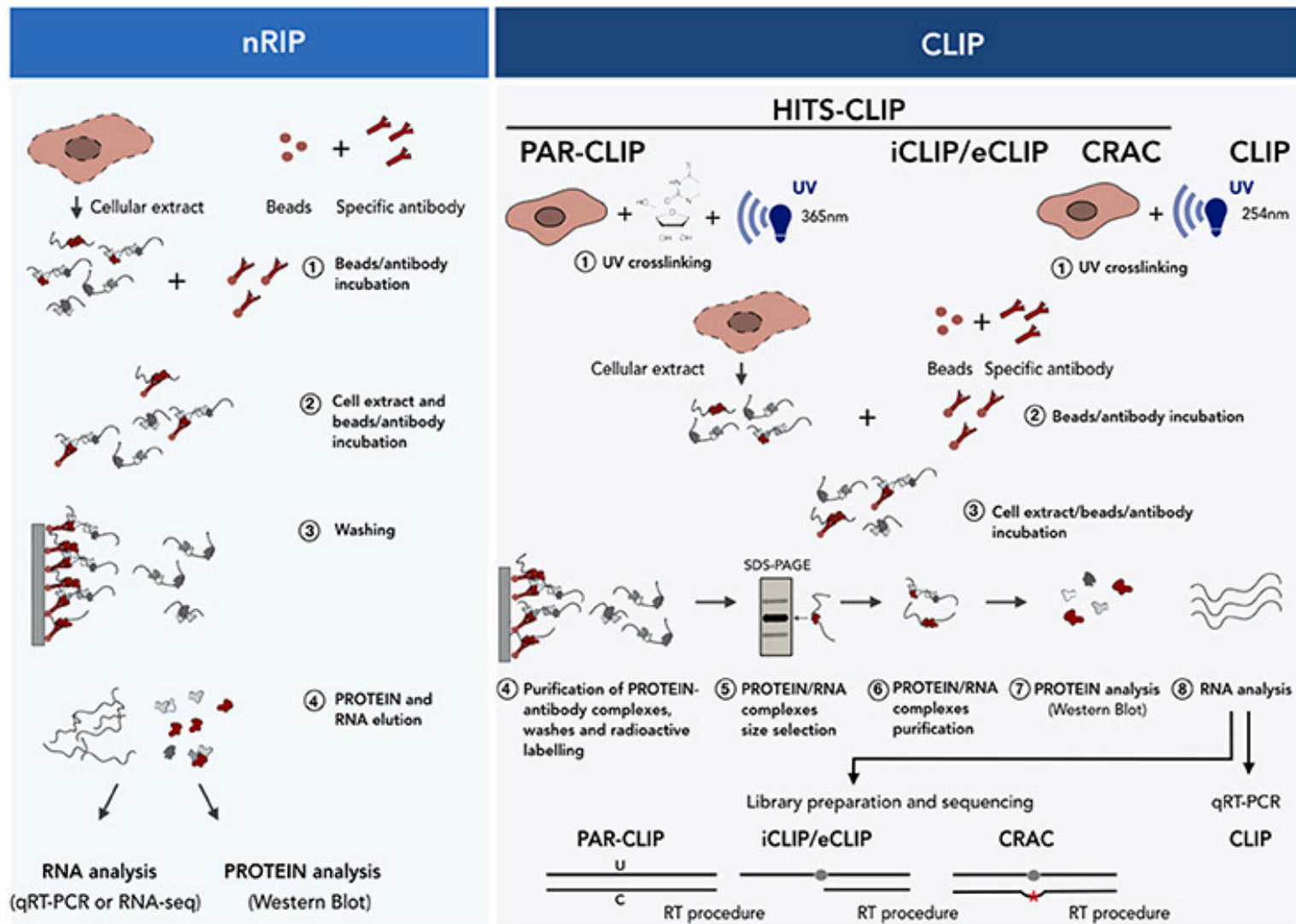
Western blotting



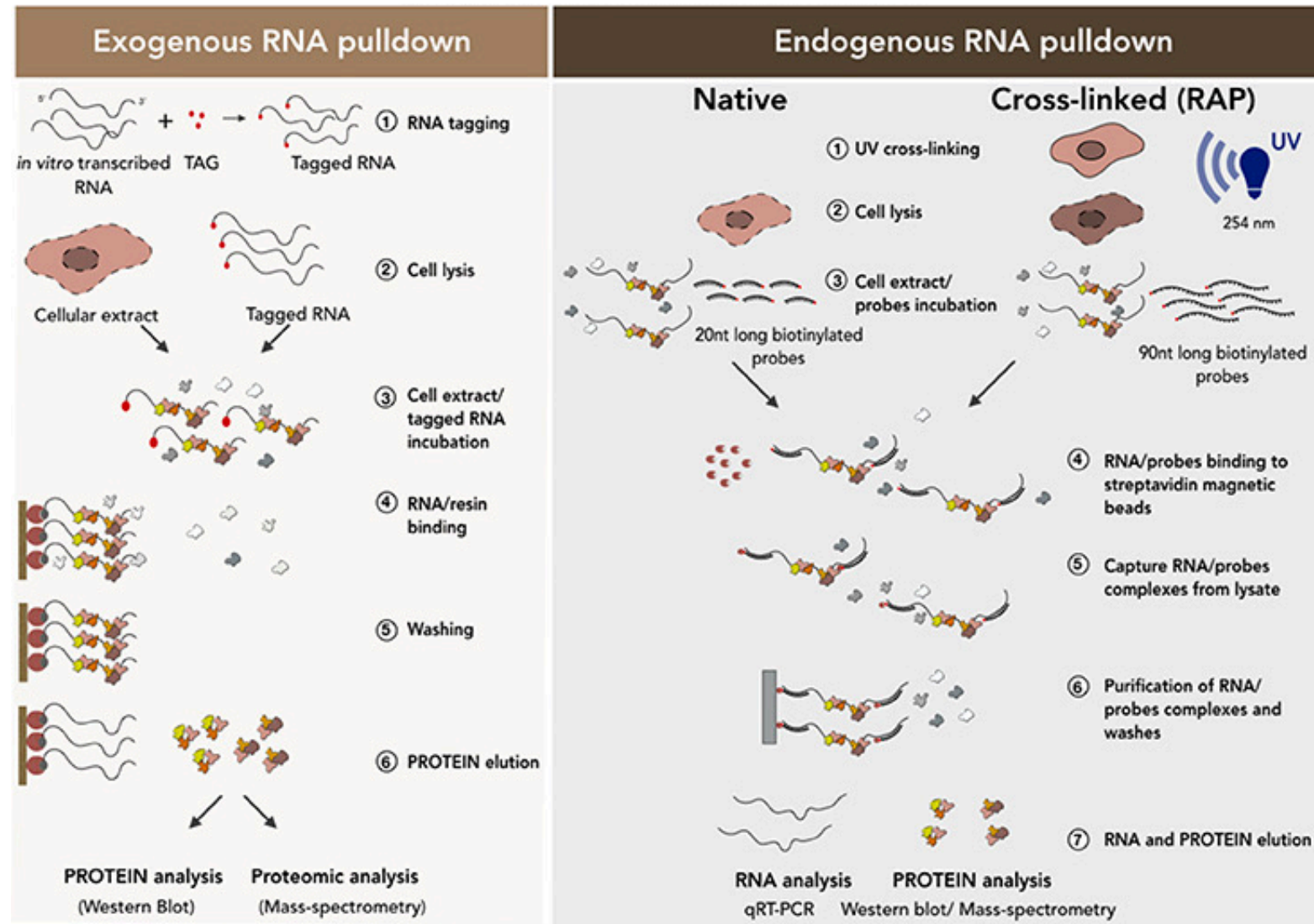
Mass spectrometry



Methods for the identification of RNAs that interact with specific proteins



Methods for the identification of lncRNAs interactors



Chromatin Immunoprecipitation (ChIP)

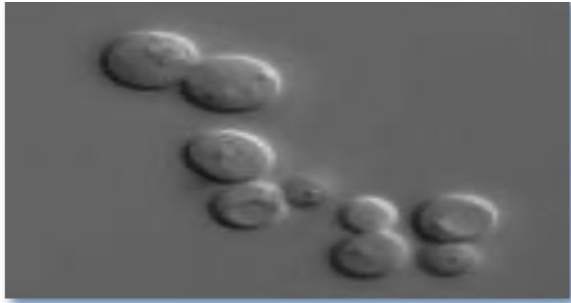
AIM: Identification of the genomic loci bound to a DNA binding protein.

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.

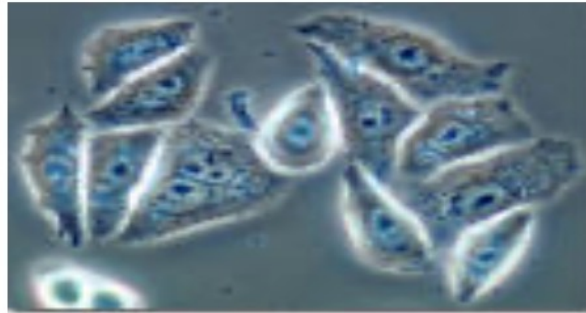


Chromatin Immunoprecipitation (ChIP)

Chromatin from most sources is a suitable substrate for ChIP:



yeast



cell cultures



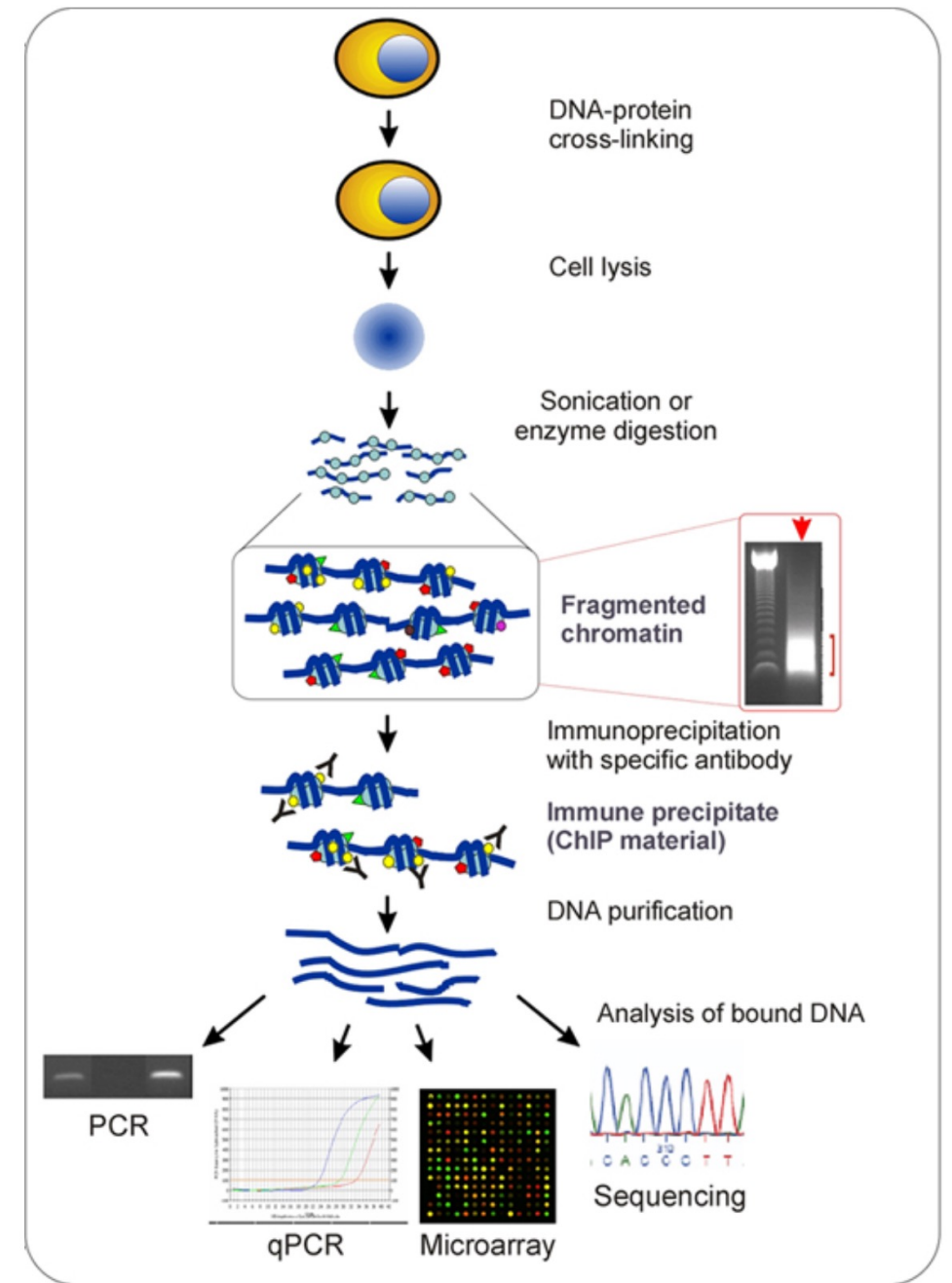
tissues

- 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 μ g 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)

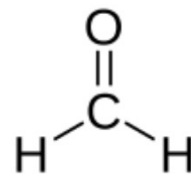
1. Cell Crosslinking

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

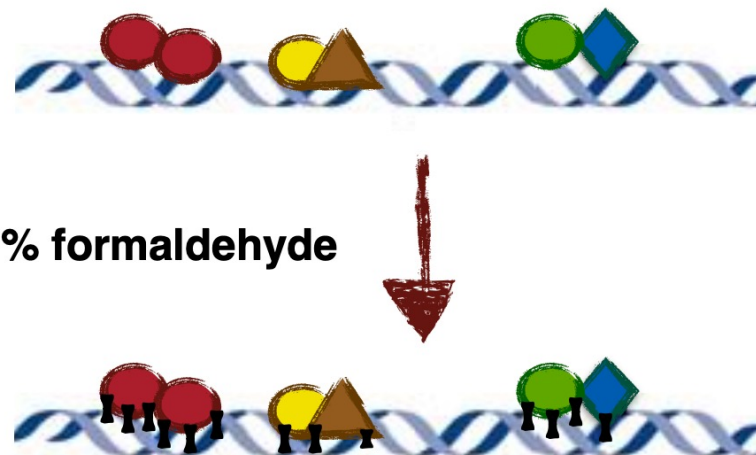
Crosslinking strategies:

Formaldehyde

Glutaraldehyde



1% formaldehyde



Chromatin Immunoprecipitation (ChIP)

1. Cell Crosslinking

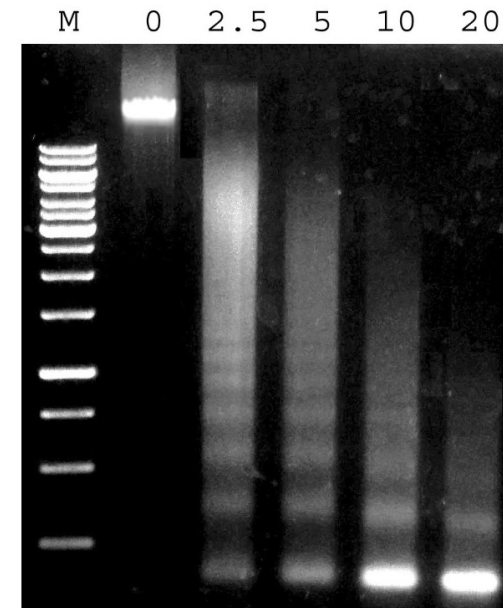
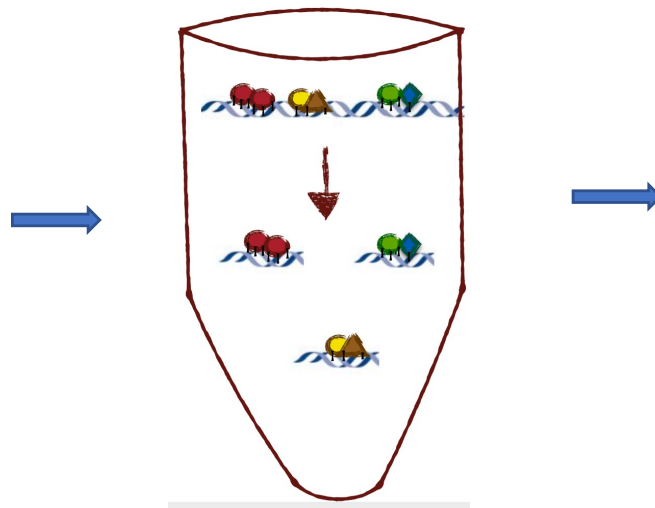
Formaldehyde Cross-linking

- 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

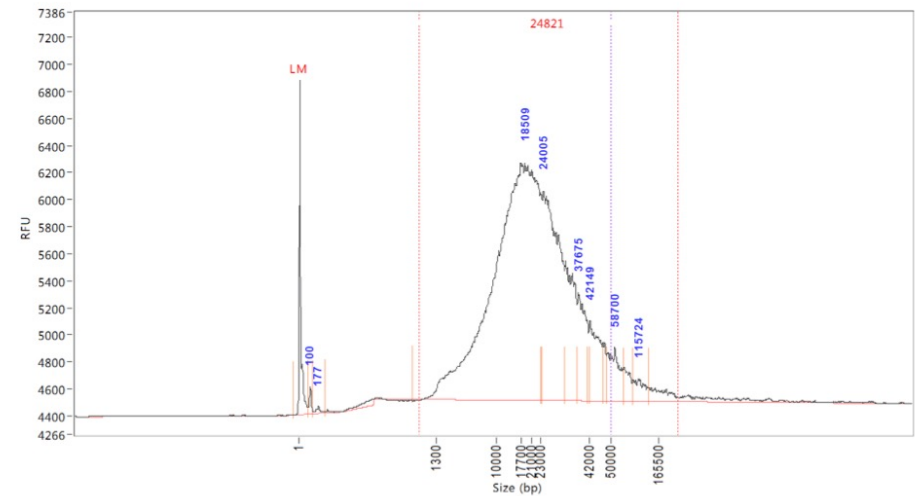
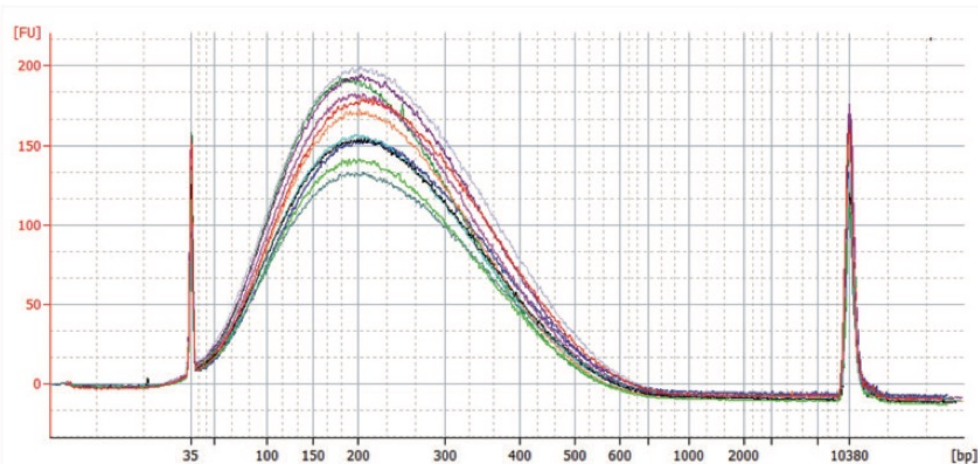
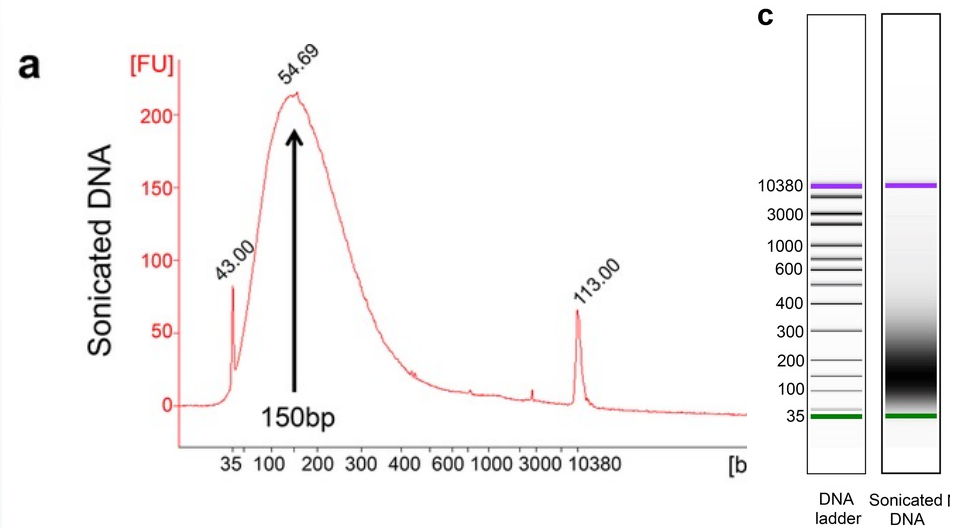
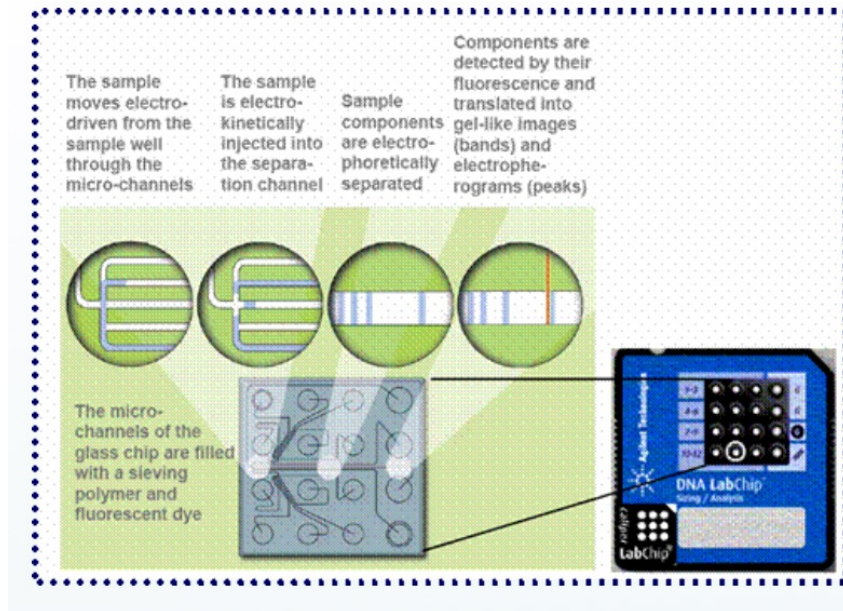
The DNA-protein complexes (chromatin-protein) are then sheared into ~500 bp DNA fragments by **sonication** or nuclease digestion.



size range: 100-500 bp

Chromatin Immunoprecipitation (ChIP)

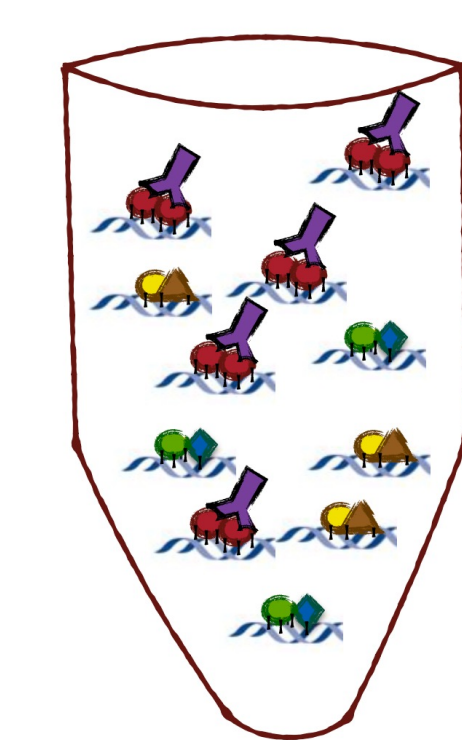
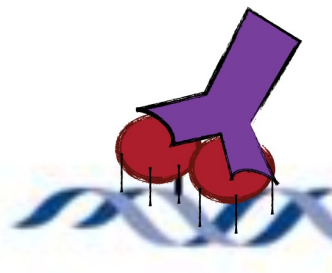
2. Chromatin Analysis (Bioanalyzer)



Chromatin Immunoprecipitation (ChIP)

3. Antibody -Extract incubation

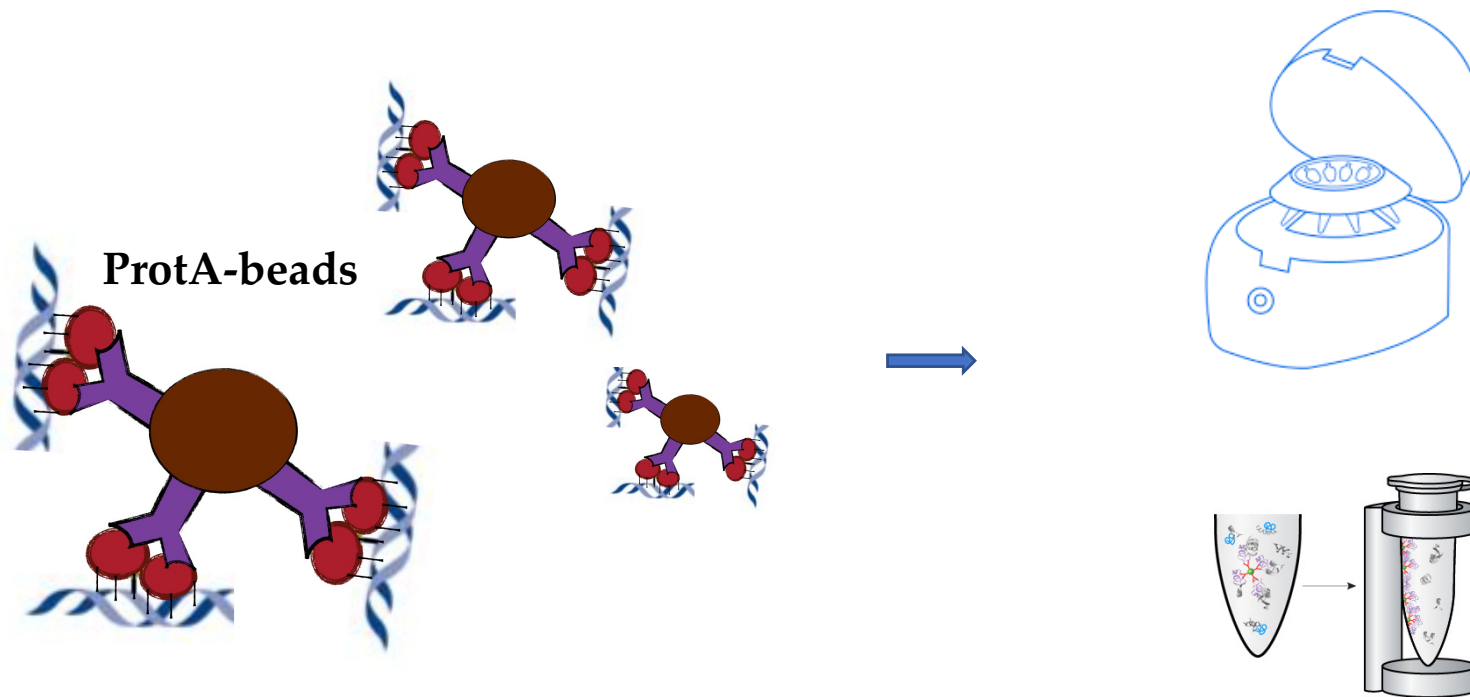
The antibody specific for the protein of interest, is incubated together with the extract, this step allows the formation of strongly interaction between the antibody and the protein of interest



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**

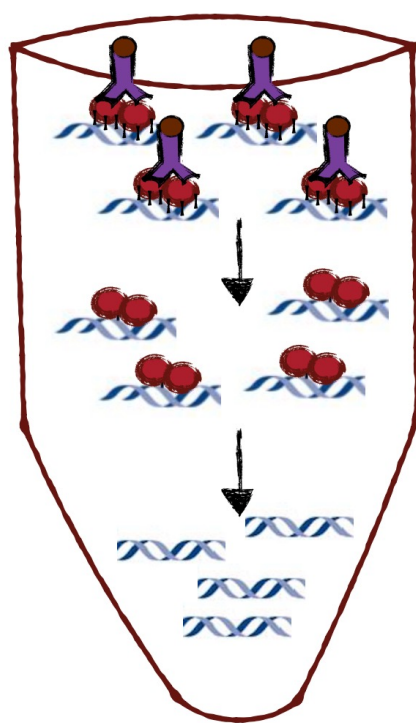


Centrifugation or magnetic recovery step allows the purification of the complexes that are bound to the antibody

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.

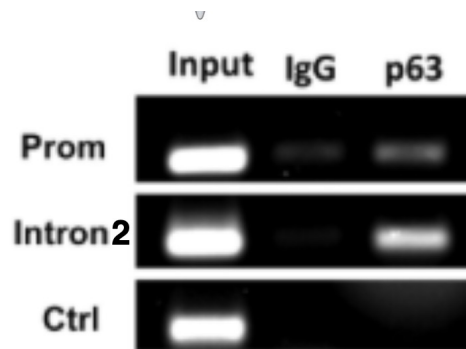
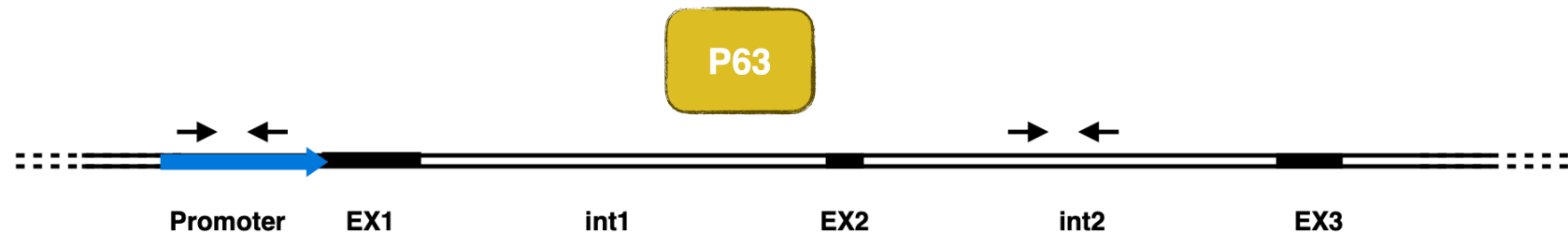


Chromatin Immunoprecipitation (ChIP)

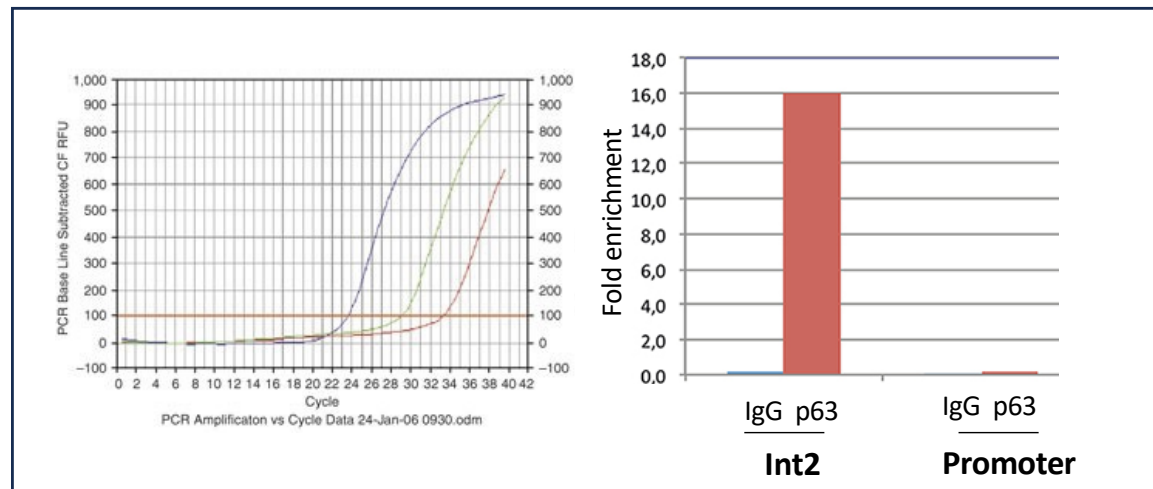
6. DNA analysis

ChIP (PCR)

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



Dismissed !

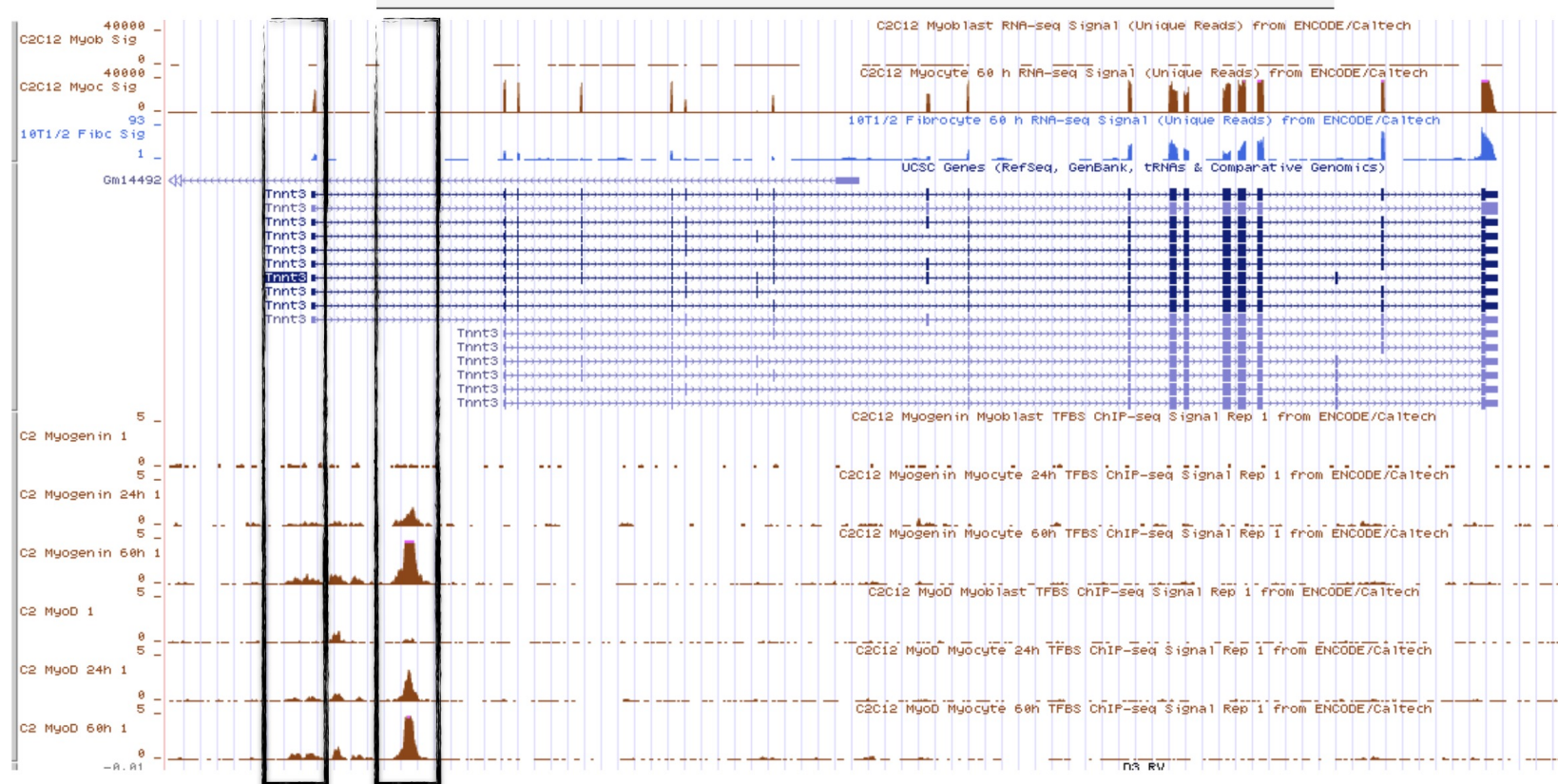


Chromatin Immunoprecipitation (ChIP)

6. DNA analysis

ChIP Seq

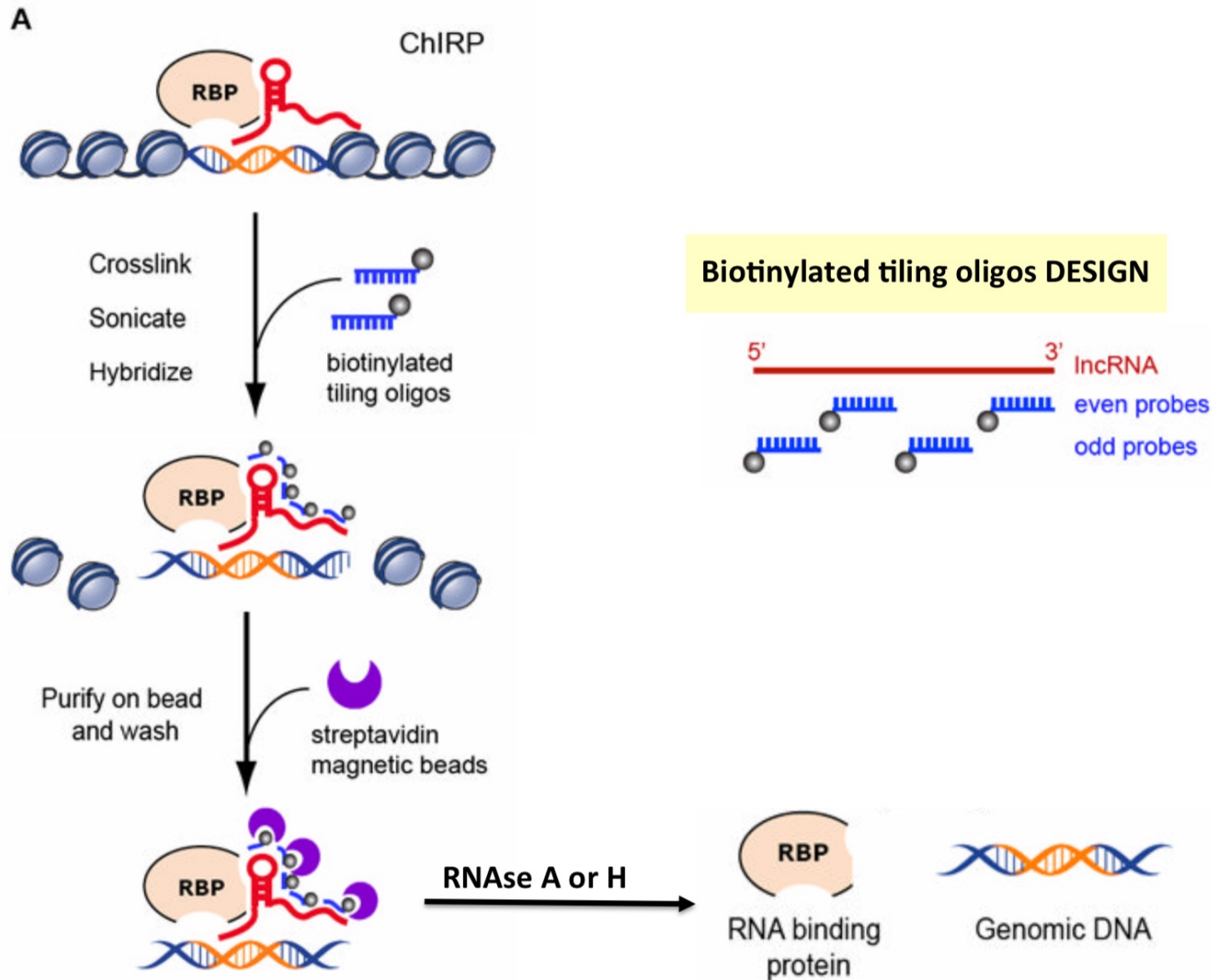
Direct sequencing of the DNA isolated, generates genome wide profiles. ChIP-seq combines ChIP and direct sequencing technology for genome-wide analysis of antigen distribution. Immunoprecipitated DNA is sequenced and mapped to the genome



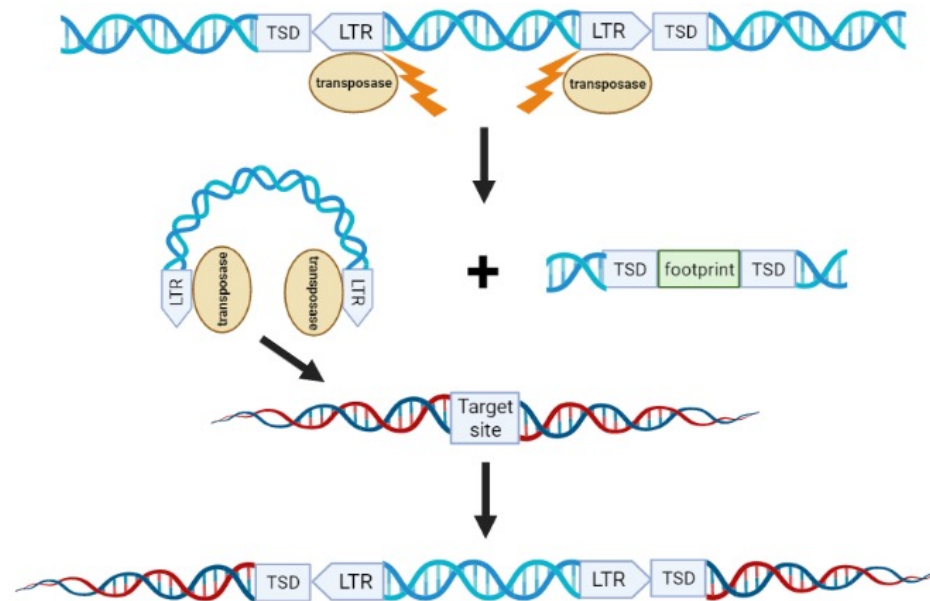
Chromatin Isolation by RNA Purification (ChIRP)

- 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)



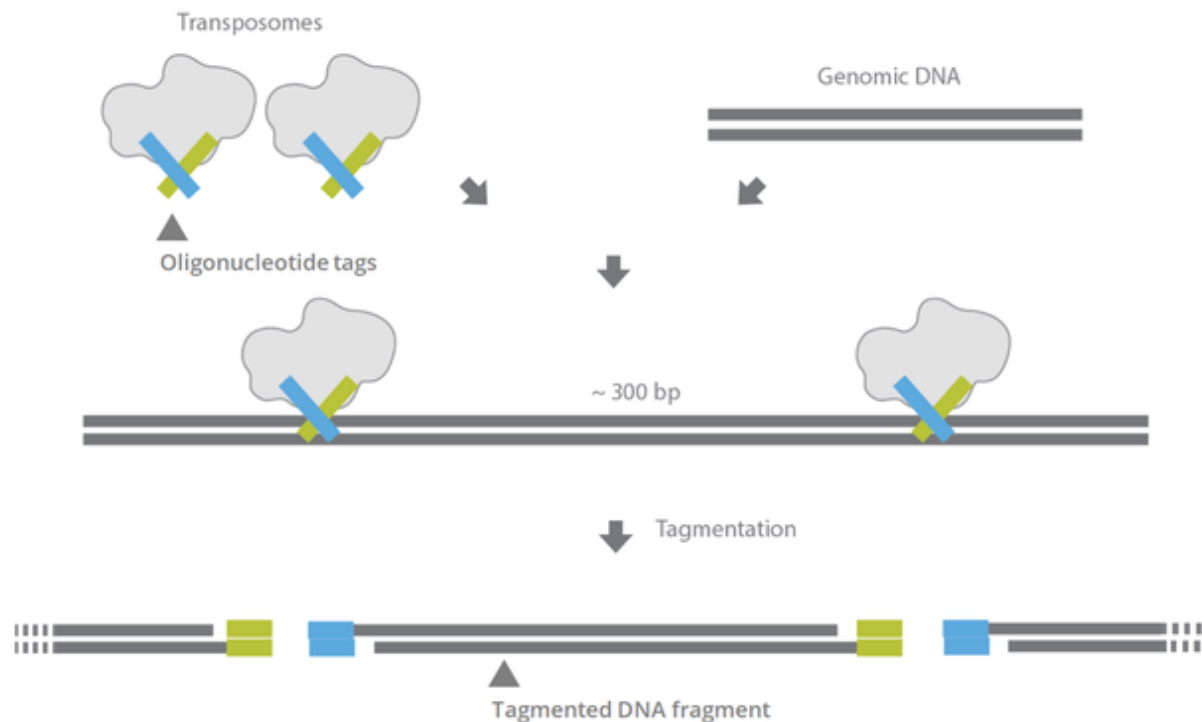
ATAC-seq for chromatin-accessibility studies



DNA transposons are most commonly used in the laboratory for genome manipulation. When transposons are used in the lab, the transposase gene is provided *in trans* so that a gene of interest can be inserted between the transposon's LTRs. The integration on the genome depends on the chromatin accessibility.

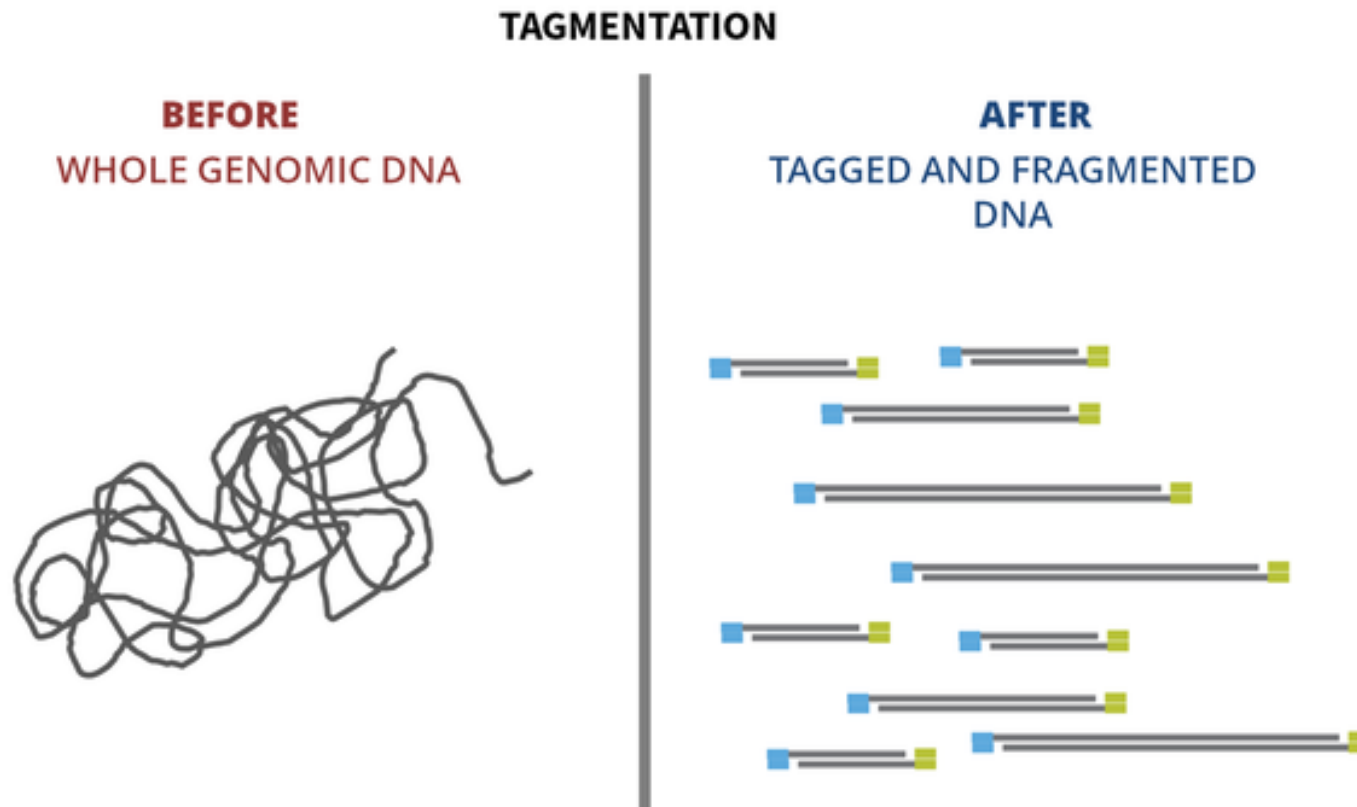
ATAC-seq for chromatin-accessibility studies

Tagmentation is transposase-based methods to prepare high-throughput sequencing libraries, in which a hyperactive transposase is used to simultaneously fragment target DNA and append universal adapter sequences.



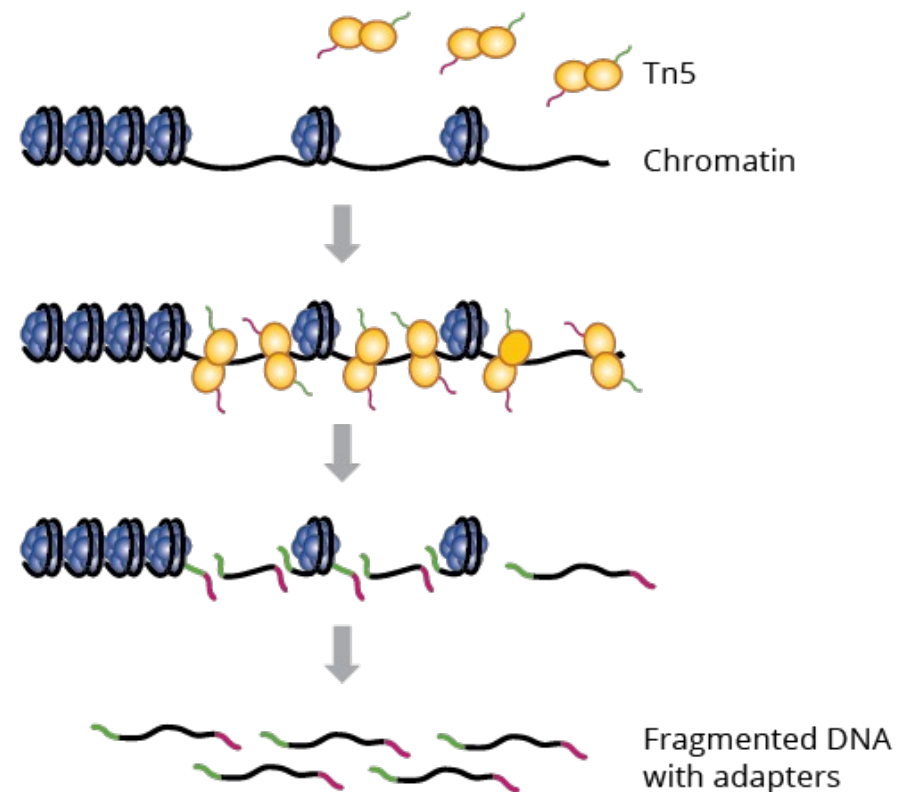
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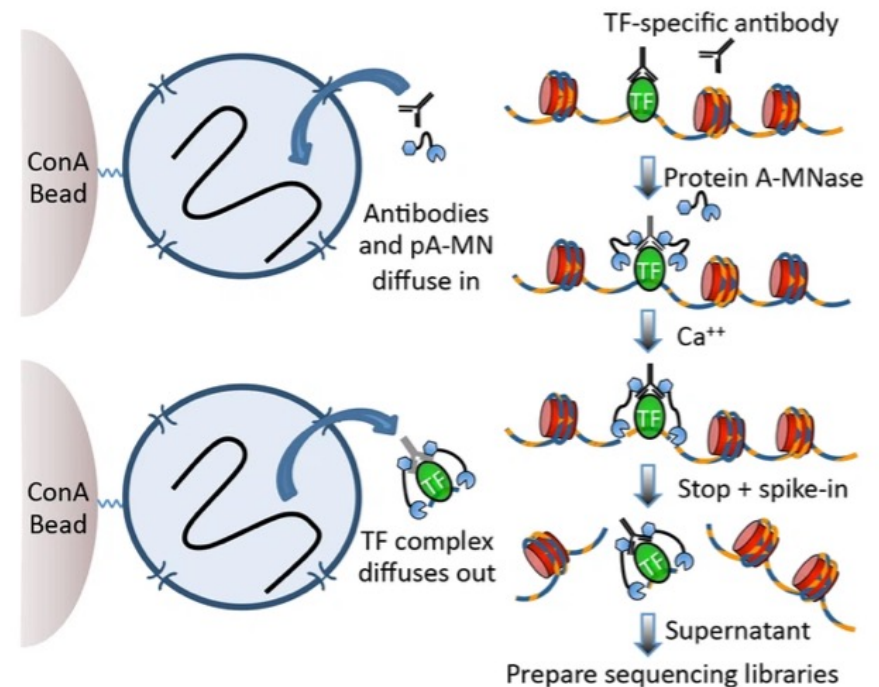
ATAC-seq for chromatin-accessibility studies

Assay for transposase-accessible chromatin sequencing (ATAC-Seq) employs a hyperactive form of Tn5 transposase to identify regions of open chromatin, which are important for global epigenetic control of gene expression. Tn5 simultaneously cleaves and adds adapters to nucleosome-free regions of DNA, priming them for sequencing.



Cut & Run: Cleavage Under Targets and Release Using Nuclease

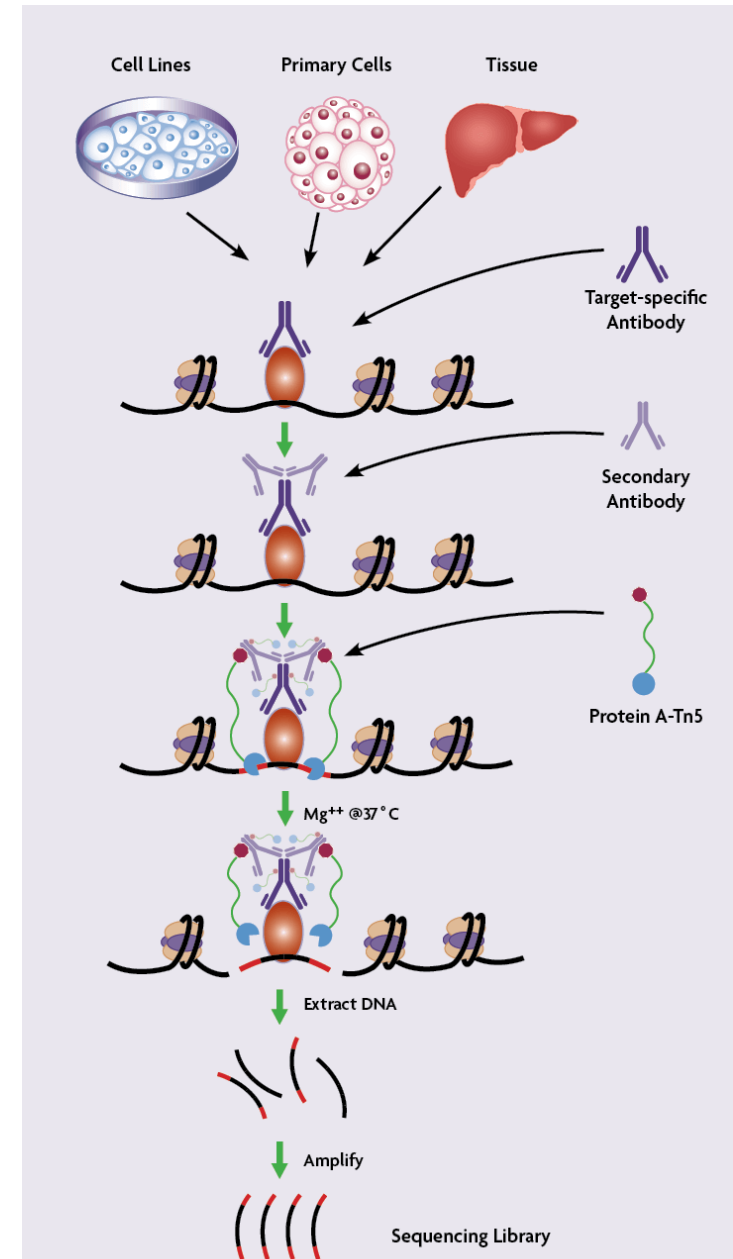
Unfixed nuclei/cells are immobilized on Concanavalin A*-coated magnetic beads, successively incubated with antibodies and protein A-MNase (pA-MN) followed by minimal washing steps, mixed with Ca^{++} on ice to initiate the cleavage reaction then stopped seconds-to-minutes later by chelation, and centrifuged to recover the supernatant containing the released TF-DNA complexes. DNA is then extracted from the supernatant and used directly for sequencing library preparation.



*Concanavalin A binds to glycoproteins on the surfaces of cells and nuclei

Cut & Tag: Cleavage Under Targets and Tagmentation

Cells are first permeabilized and incubated with an antibody immobilized on Concanavalin A-coated magnetic beads to facilitate the subsequent washing steps. Next, the cells are incubated with a primary antibody specific for the target protein of interest followed by incubation with a secondary antibody. The cells are then incubated with assembled transposomes, which consist of protein A fused to the Tn5 transposase enzyme that is conjugated to NGS adapters. After the incubation, unbound transposome is washed away using stringent conditions. Tn5 is an Mg^{2+} -dependent enzyme so Mg^{2+} is added to activate the reaction, which results in the chromatin being cut close to the protein binding site and simultaneous addition of the NGS adapter DNA sequences. This leads to chromatin cleavage and library preparation in one single step.



Advantages of Cut & Tag

CUT&Tag is Compatible with Low Cell Numbers

In the publication that first described the CUT&Tag method, the authors went down as low as 60 cells to analyze H3K27me3 profiles across the genome. CUT&Tag is compatible with low cell numbers because pA-Tn5 cleaves DNA directly at the binding site of the antibody and does not require chromatin preparation and sonication steps that can lead to sample loss.

The ability to work with small numbers of cells is an advantage for researchers working on specific cell types, such as rare cell populations.

CUT&Tag Does Not Require Fixation or Sonication

CUT&Tag is performed on native cells or nuclei, avoiding the need for fixation, chromatin preparation, and sonication steps of standard ChIP workflows. Sonication can be quite challenging to set up and requires specialized equipment. Moreover, over-fixation and over-sonication can destroy protein epitopes preventing them from being immunoprecipitated. Some antibodies work better under native conditions.

CUT&Tag is Fast

Relative to ChIP, which is a multi-step process, CUT&Tag is much faster. Cells are immobilized on magnetic beads and the entire protocol happens in a single tube. The tagmentation step involves chromatin shearing and sequencing library adapter insertion at the same time, which is amenable to high-throughput experiments.

Cut & Tag vs. Cut & Run vs. ChIP

	CUT&Tag	CUT&RUN	ChIP-Seq
Performed Under Native Conditions?	Yes	Yes	No
Chromatin Fragmentation Method	Tn5-based tagmentation	MNase digestion	Sonication
Cell Number Requirements	5,000-500,000 cells	500,000 cells	1-10 million cells
Sequencing Depth Required *	2 million reads **	8 million reads	20-50 million reads
Integrated Library Preparation?	Yes, uses tagmentation	No, separate library prep required	No, separate library prep required
Compatible Targets	Primarily histone modifications, some transcription factors and co-factors	Wide range of histone modifications, transcription factors, and co-factors	Wide range of histone modifications, transcription factors, and co-factors
Workflow Length	1-2 days	1-2 days	2-3 days