Molecular techniques for the study of the interaction between……..

Protein-RNA: RIP (RNA immunoprecipitation)

Protein-RNA: CLIP (Cross-Linked RNA immunoprecipitation)

RNA-Protein and DNA: ChIRP

RNA-Protein: RNA pull down

The recognition and binding of certain RNAs by different RNA-binding proteins (RBPs) is essential to maintain the viability of any living cell. RBPs act on many kinds of RNA, such as rRNA, tRNA, siRNA, miRNA and lncRNA at different stages of the mRNA life-cycle from splicing, polyadenylation, various modifications and subcellular localization to translation.

The binding between an RNA and RBP involves the recognition of a specific sequence element and also often the identification of a specific secondary structure within the RNA molecule by the RBP. The RNA-binding domain (RBD) of an RBP recognizes a region of up to 5 or 6 nt (RRE: RNA Recognition Element), which determines its specificity and binding affinity to a particular RNA.

There are different techniques, which can be grouped in two approaches:

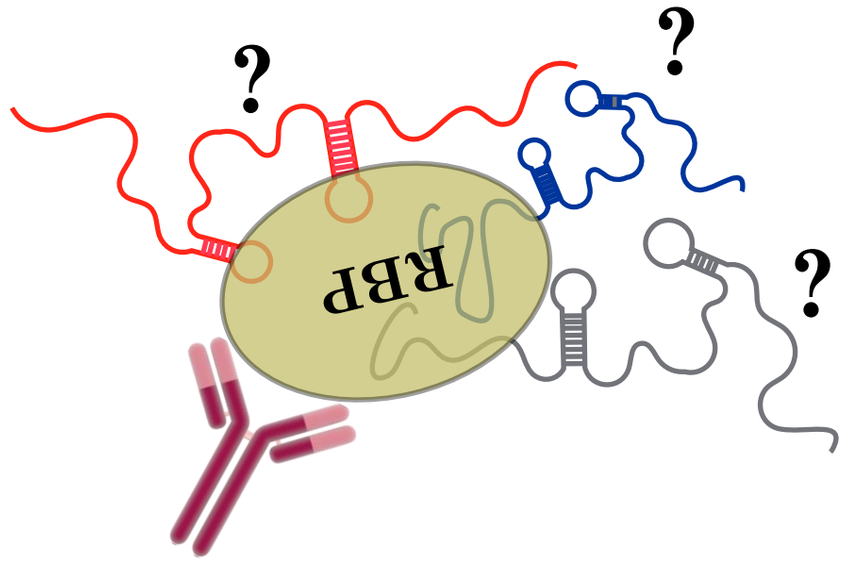
- **From RBP to the the bound RNA**
- **From the RNA to the RBPs**

The first method that used immunoprecipitation for the identification of protein-bound RNA transcripts is known as RNA immunoprecipitation chip (RIP-chip), which couples immunoprecipitation to microarray analysis.

RIP-Chip is performed without any treatment of the cells. After cell lysis, the RBP of interest and the bound RNAs are immunoprecipitated. After separation of the RBP and RNA, the purified RNA molecules are characterized by microarray analysis.

RIP
(RNA Immunoprecipitation)

AIM: Identification of the RNAs bound to a known Protein



RIP allows to study the physical association between individual proteins with specific nucleic acids such as mRNAs, ncRNAs and viral RNAs in vivo. It is based on the use of a specific antibody raised against the RBP of interest to pull down the RBP and target-RNA complexes. Any RNA that is associated with this protein complex will also be isolated and can be directly measured using down-stream applications including quantitative reverse transcription polymerase chain reaction (RT-PCR), microarrays analysis (RIP-chip) and "deep-sequencing" or second generation sequencing based platforms (RIP-seq).

The native RIP allows to reveal the identity of RNAs directly bound by the protein and their abundance in the immunoprecipitated sample, while cross-linked RIP leads to precisely map the direct and indirect binding site of the RBP of interest to the RNA molecule.

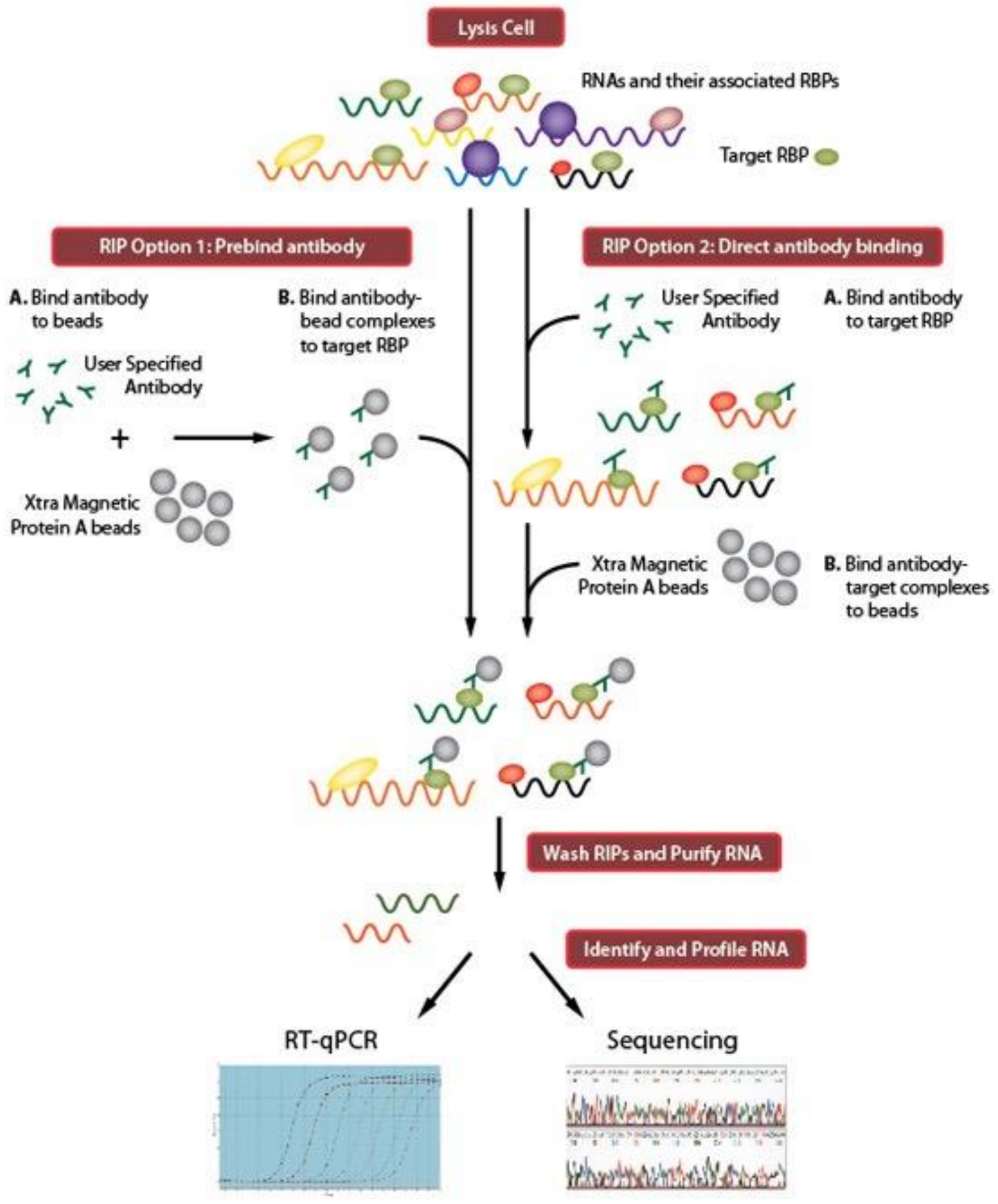
RIP

1. Lysis Cells and collect cell extract

2 Prebinding between AntiBody and Beads

3. Introduction of cellular extract (Binding step)

4. Wash and Purification of RNA-protein complexes



CLIP
(UV Cross-Linking and Immuno-Precipitation)

CLIP allows the characterization of the binding site of a given RBP because it contains an additional step in which the RNAs are partially degraded with Rnases. CLIP requires specific and high-affinity antibodies to recover RNA-protein complexes containing a given RBP by denaturing IP.

CLIP uses short wave UV irradiation at 254 nm to generate a covalent bond between RNA and proteins, in contrast to formaldehyde-based cross-linking typically used in DNA-ChIP.

1. UV crosslink Cells or Tissue

2. Partial RNA digestion

3. Immunoprecipitate RBP, add P to RNA 5' end

4. Ligate the 5' RNA adapter

5. Dephosphorylate RNA 3' end

6. Purify RBP-RNA on SDS-PAGE

7. Digest the RBP

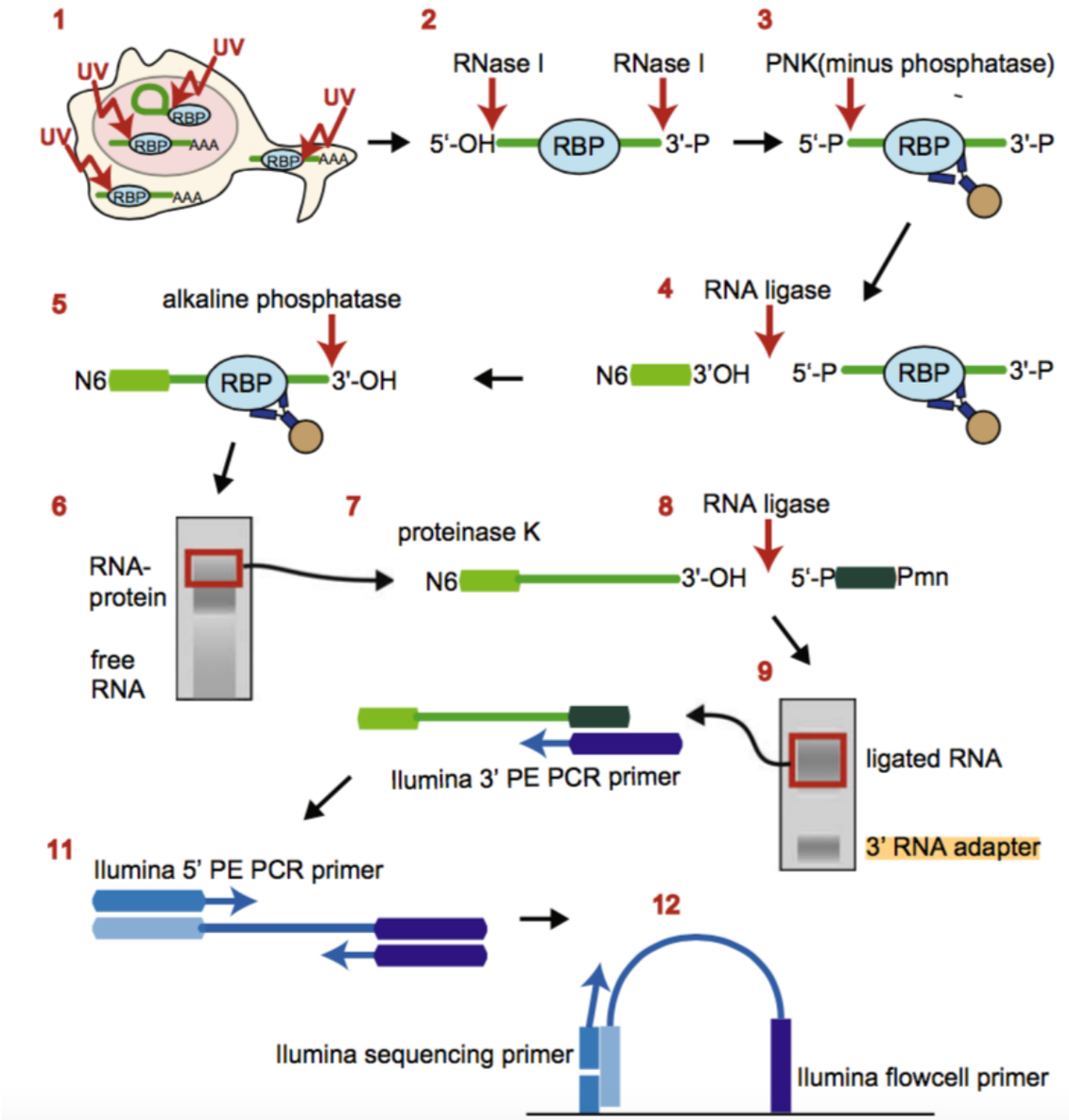
8. Ligate the 3' RNA adapter

9. Purify RNA on urea-TBE gel

10. Reverse transcription

11. PCR

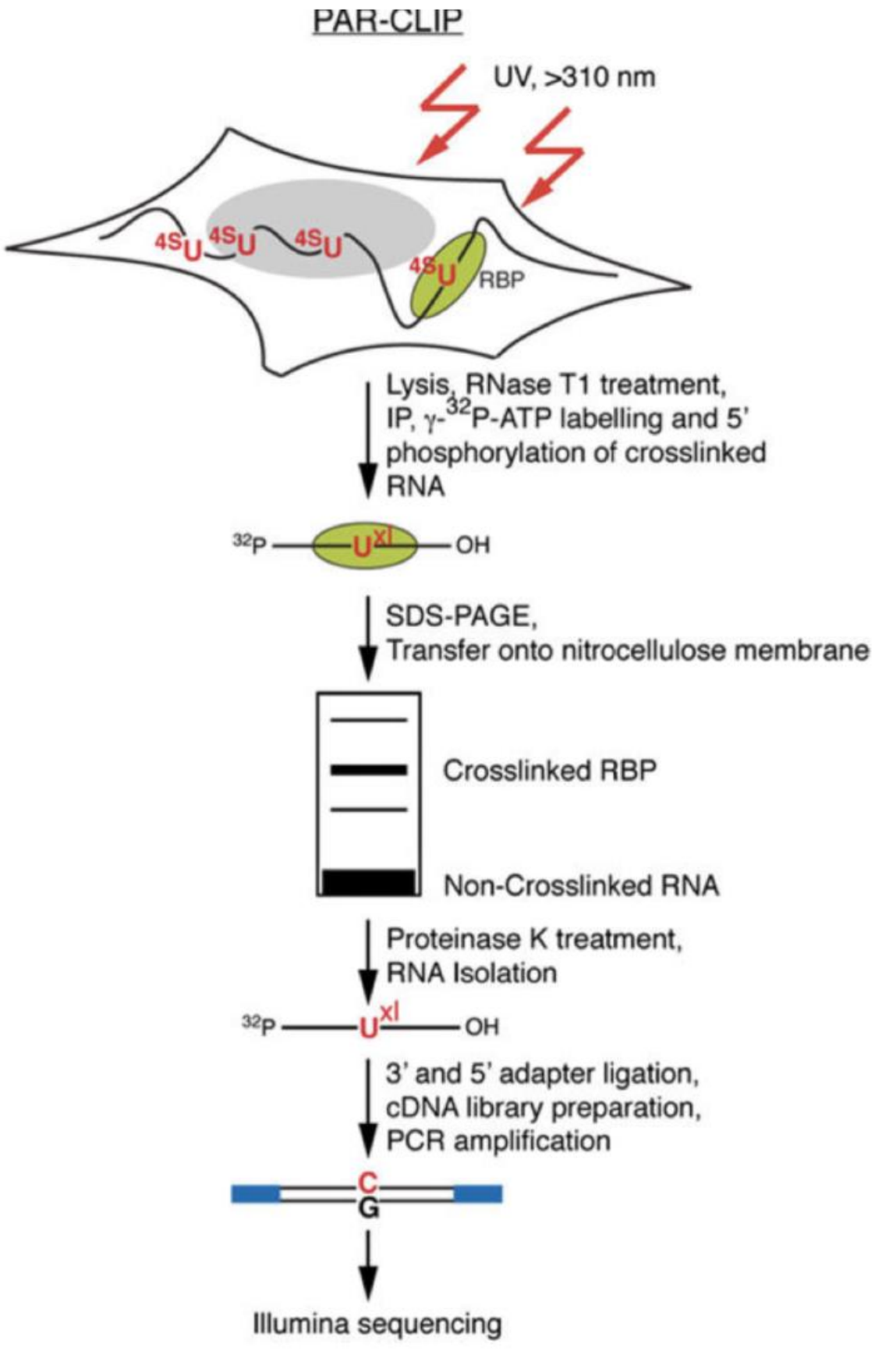
12. Illumina paired-end sequencing



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

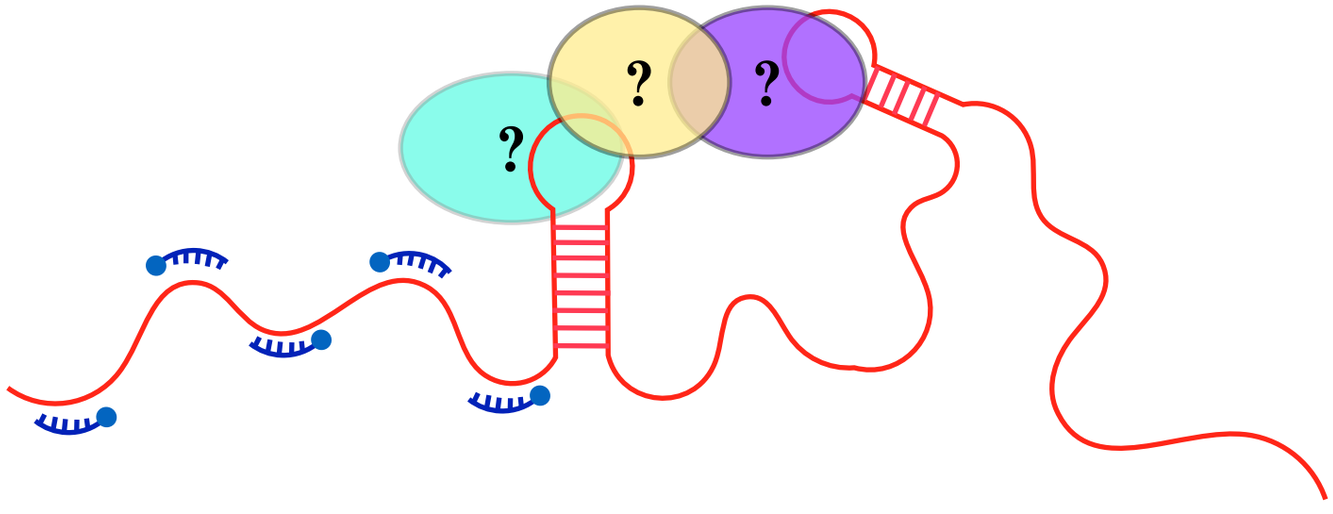
PAR-CLIP improves the cross-linking compared with standard UV cross-linking by using a photoactivatable nucleoside (e.g. 4-thiouridine), which is incorporated into nascent transcripts. This incorporation leads to more cross-linked sites between the RNA and the protein because of the higher photoreactivity of such nucleosides

Outline of the PAR-CLIP methodology. PAR-CLIP begins with incorporation of photoactivatable thioribonucleosides into nascent transcripts followed by cross-linking with long-wavelength >310 nm UV. Cross-linked RNA–RBP complexes are isolated by IP and further purified by SDS-PAGE. After recovery from the purified radioactive band, the RNA is carried through a small RNA cDNA library preparation protocol for sequencing. Reverse transcription of cross-linked RNA with incorporated photoactivatable thioribonucleosides, followed by PCR amplification, leads to a characteristic mutation (T-to-C when using 4SU 4-thiouridine and G-to-A when using 6SG 6-thioguanine) that is used to identify the RNA recognition elements



RNA pull down

AIM: Identification of the protein interactors of an RNA



Long non-coding RNA (lncRNA) are sequences of more than 200 nt without reading frame, belonging to the regulatory non-coding RNA's family. Although their biological functions remain largely unknown, the number of these lncRNAs has steadily increased and it is now estimated that humans may have more than 10,000 such transcripts. Some of these are known to be involved in important regulatory pathways of gene expression which take place at the transcriptional level, but also at different steps of RNA co- and post-transcriptional maturation.

- **Nuclear and Cytoplasmic RNAs**
- **Exogenous (in vitro) vs Endogenous (in vivo) RNA pull down**
- **The amount of cellular extract depends by the abundance of the RNA**

Endogenous RNA pull down

1. Probe Design (biotinylated oligos)

2 Collect cell extract

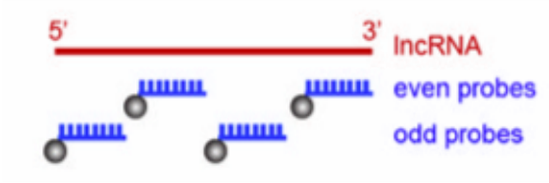
3. Binding step

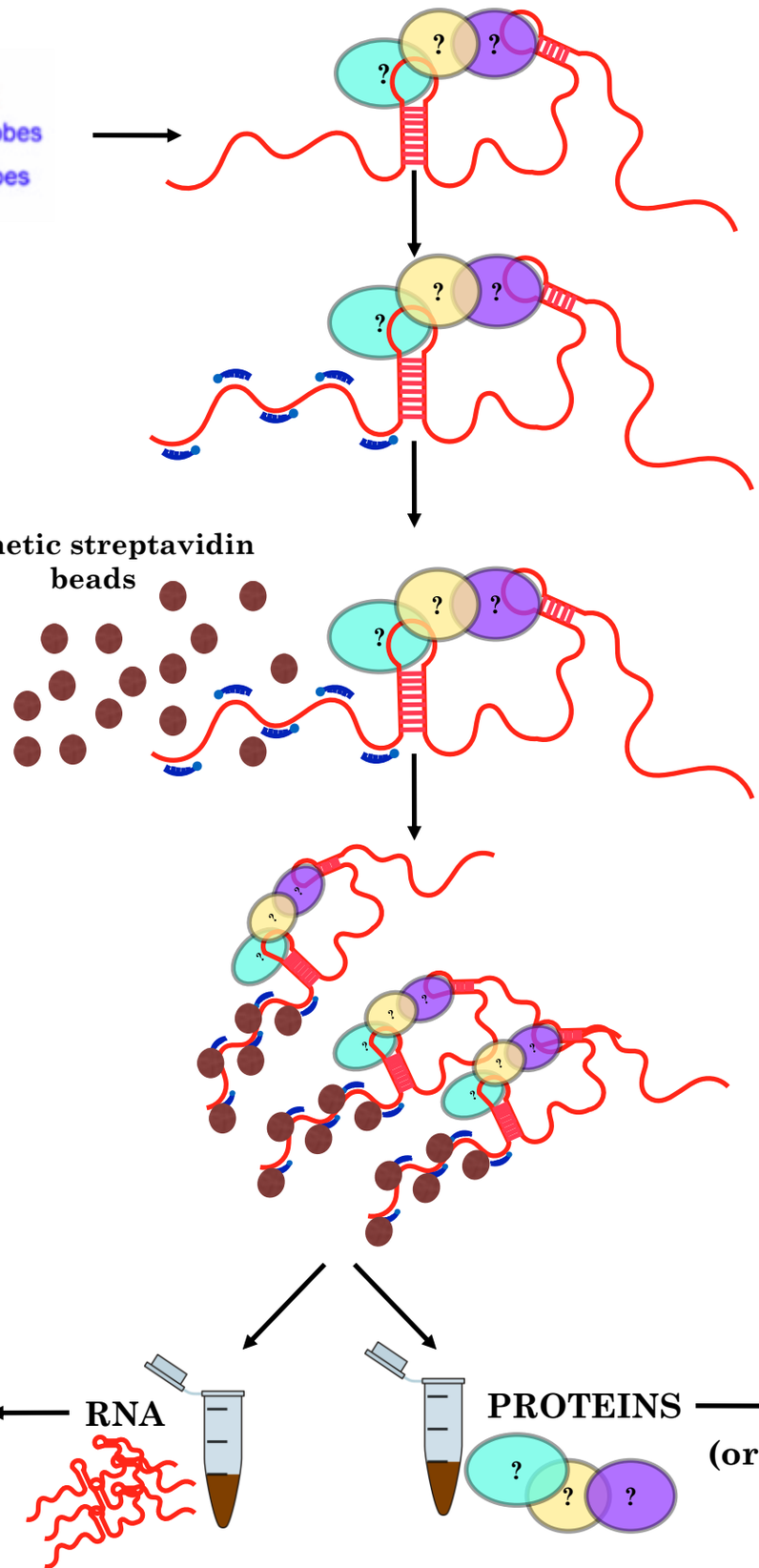
4. Introduction of Streptavidin-magnetic beads

5. Pull down

6. Protein and RNA analysis

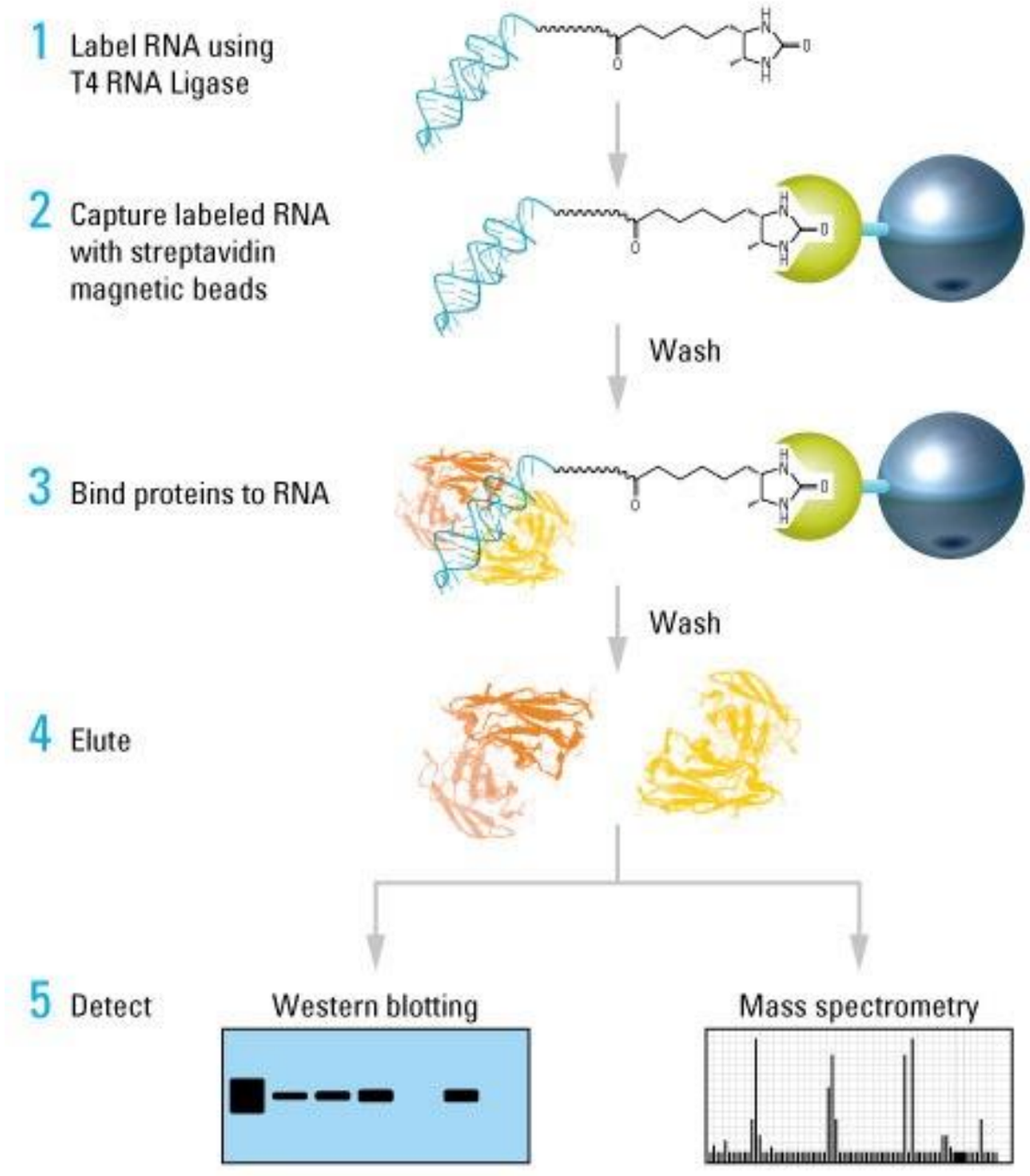
Biotinilated Oligos
residuo di **biotina legato ad un UTP** in posizione 5'





Exogenous RNA pull down

In the exogenous RNA pull-down the transcript of interest is tagged (i.e., biotinylation) by in vitro transcription. The co-purified proteins are collected and analyzed by Western Blot or Mass-spectrometry analyses.



Chromatin Isolation by RNA Purification (ChIRP)

ChIRP is a novel and rapid technique to map genomic binding sites of lncRNAs. The method takes advantage of the specificity of anti-sense tiling oligonucleotides to allow the enumeration of lncRNA-bound genomic sites.

Flow chart of the ChIRP procedure. Chromatin is crosslinked to lncRNA:protein adducts in vivo. Biotinylated tiling probes are hybridized to target lncRNA, and chromatin complexes are purified using magnetic streptavidin beads, followed by stringent washes. Elute lncRNA bound DNA and/or proteins with a cocktail of Rnase A and H. A putative lncRNA binding sequence is schematized in orange.