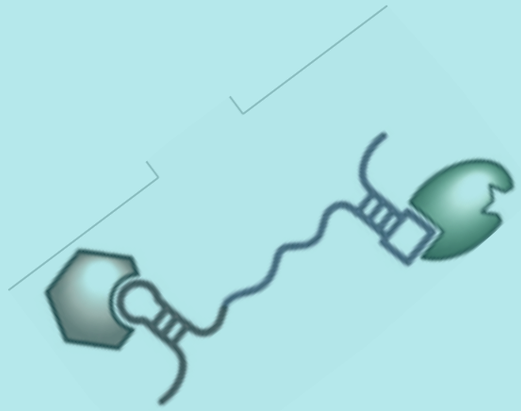
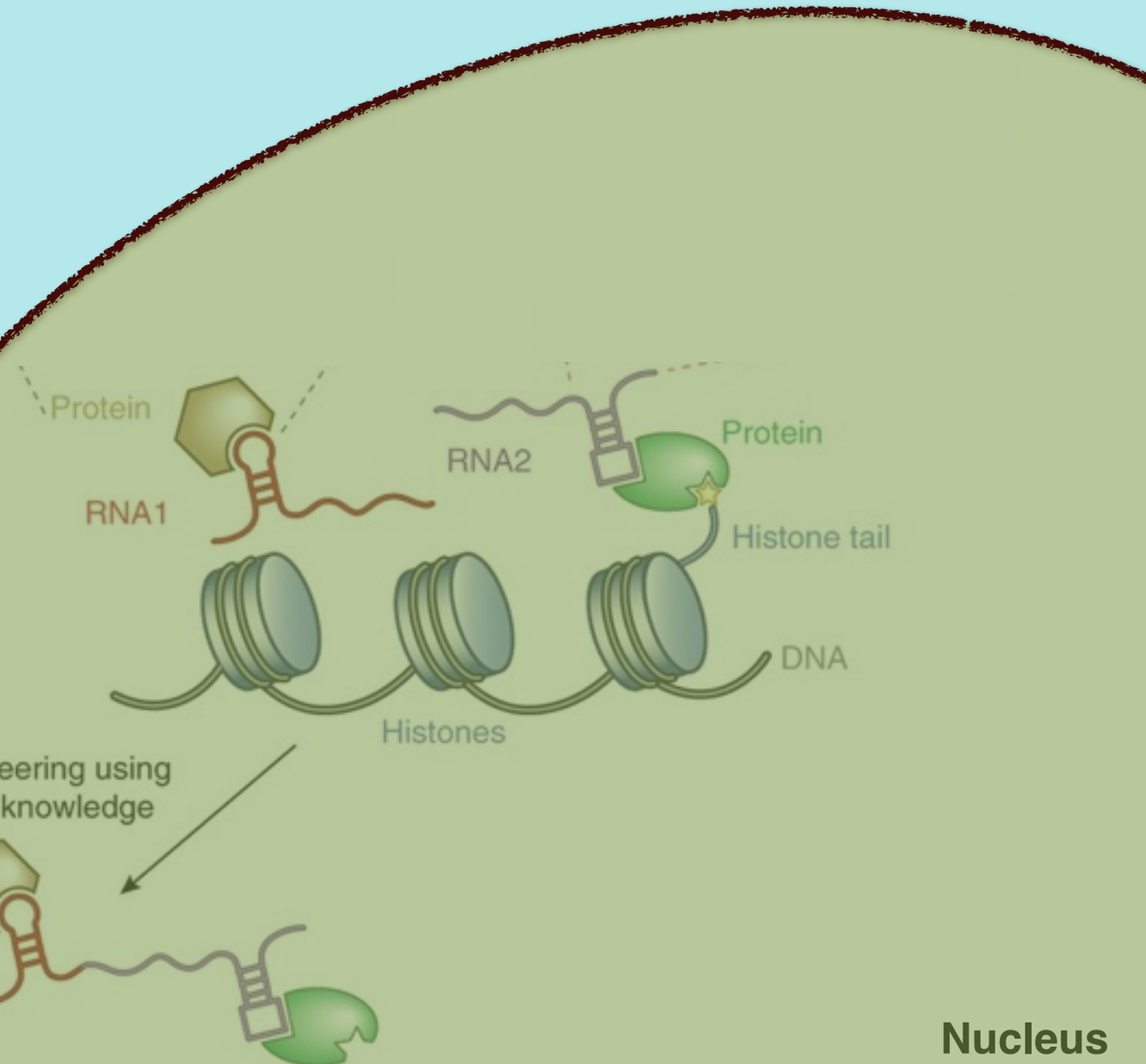
THE INTERACTOME STUDY

Andrea Cipriano

Inside a cell...

Cytoplasm





Nucleus

Molecular techniques for the study of the interaction between:

DNA-protein: **ChIP** (Chromatin immunoprecipitation)

*(Protein detection: **Western Blot**)*

RNA-Protein: **RNA pull down**

Protein-RNA: **RIP** (RNA immunoprecipitation)

Molecular techniques for the study of the interaction between:

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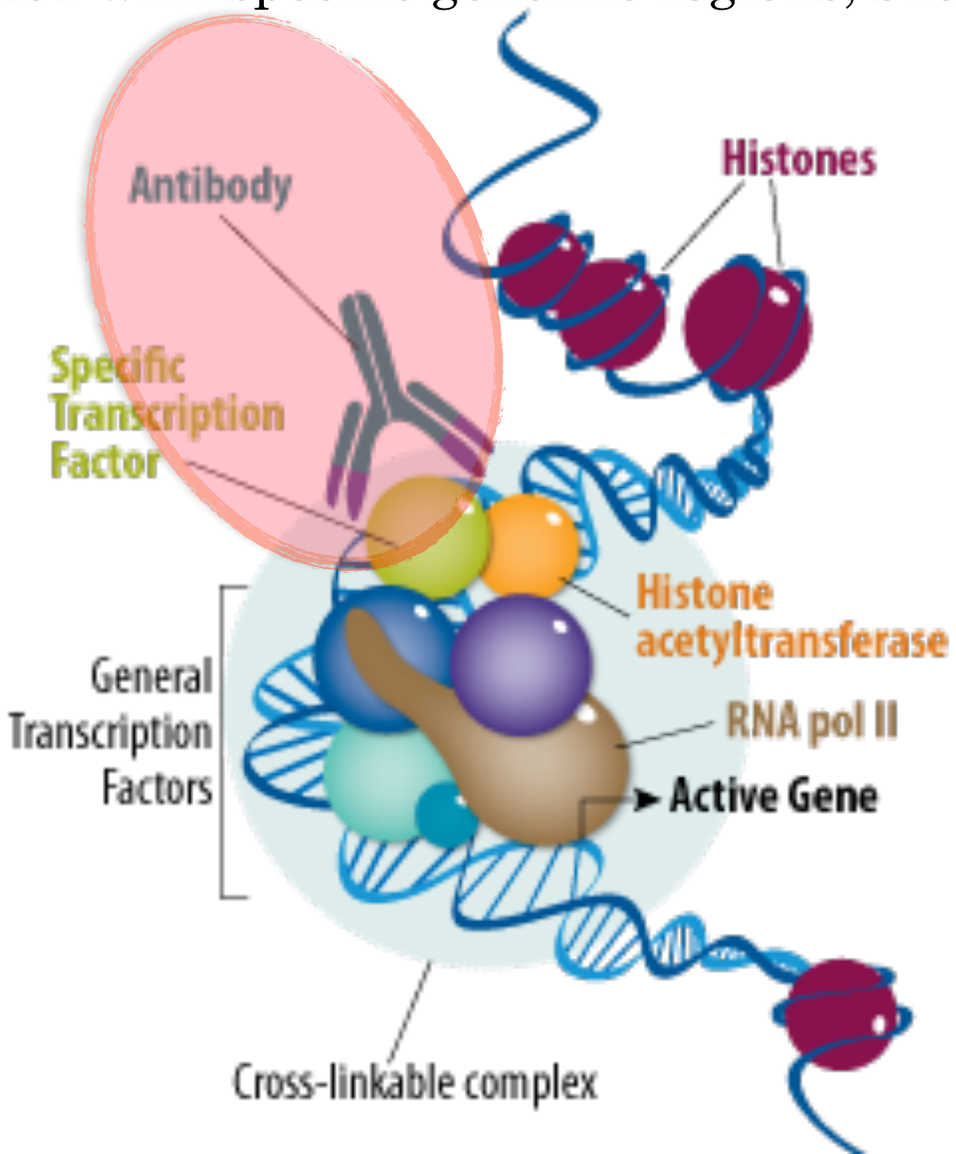
ChIP

(Chromatin Immunoprecipitation)

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. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factors on promoters.



ChIP

(Chromatin Immunoprecipitation)

What is an antibody?

An **antibody** also known as an **immunoglobulin** (Ig), is a large protein produced mainly by plasma cells (B linphocyte) and used by the immune system to identify and neutralize pathogens such as bacteria and viruses. The antibody recognizes a unique molecule of the pathogen, called **epitope (or antigene)**.

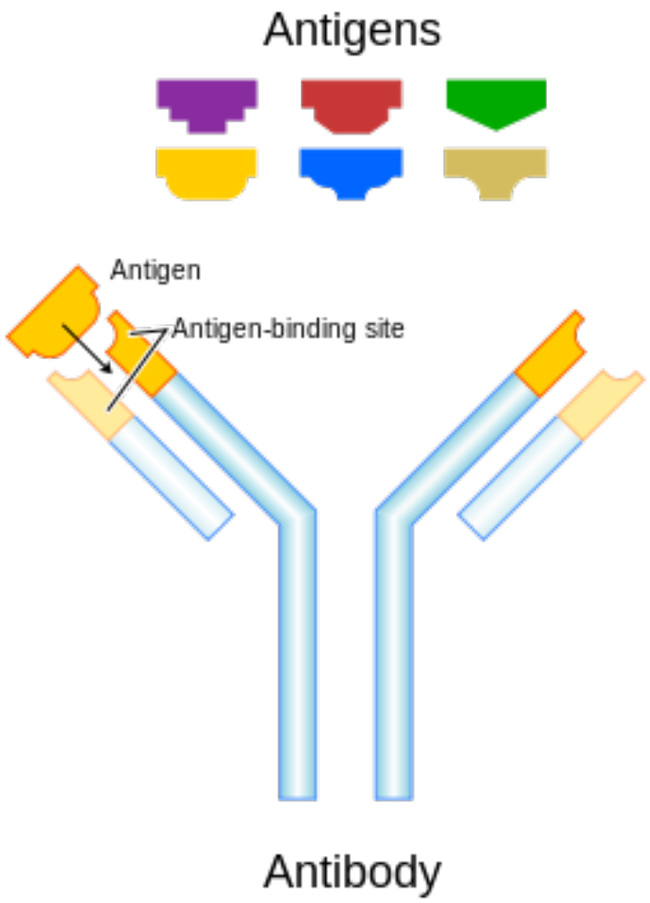
ChIP

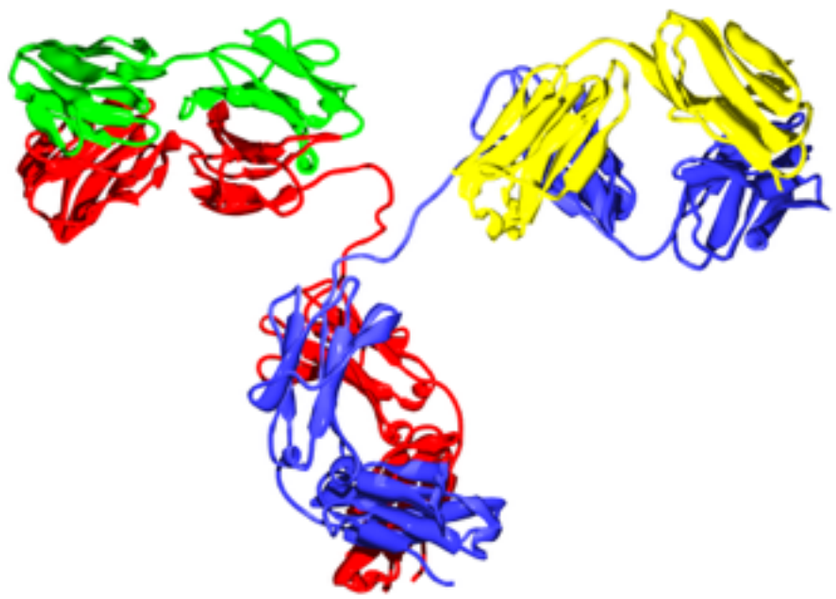
(Chromatin Immunoprecipitation)

How an antibody works?

Each tip of the "Y" of an antibody contains a **paratope** (analogous to a lock) that is specific for one particular **epitope** (similarly analogous to a key), allowing these two structures to bind each other

Using this binding mechanism, an antibody is able to recognize and selectively bind a particular protein with a very high affinity.

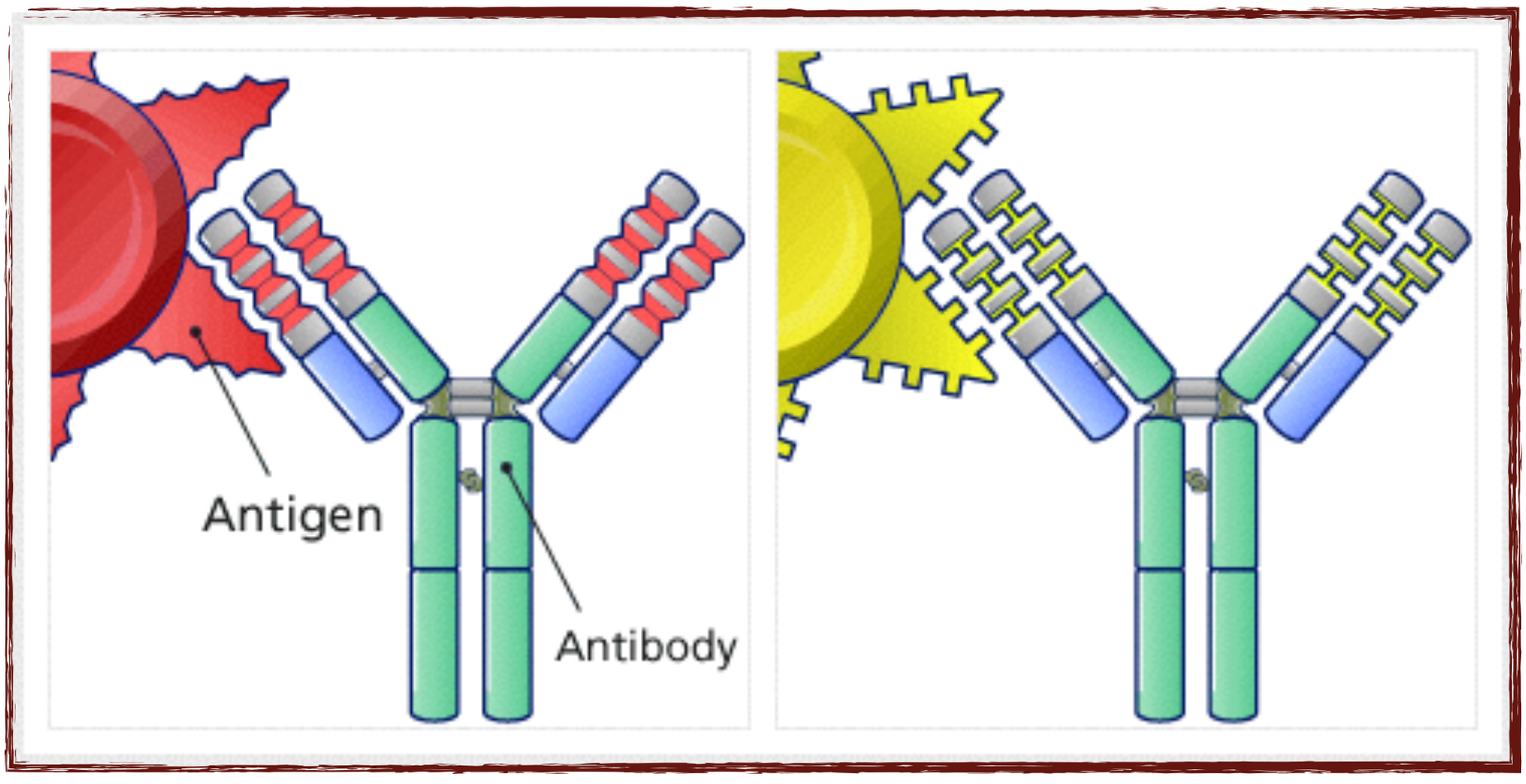




ChIP

(Chromatin Immunoprecipitation)

How an antibody works?



Antibody A **Antibody B**

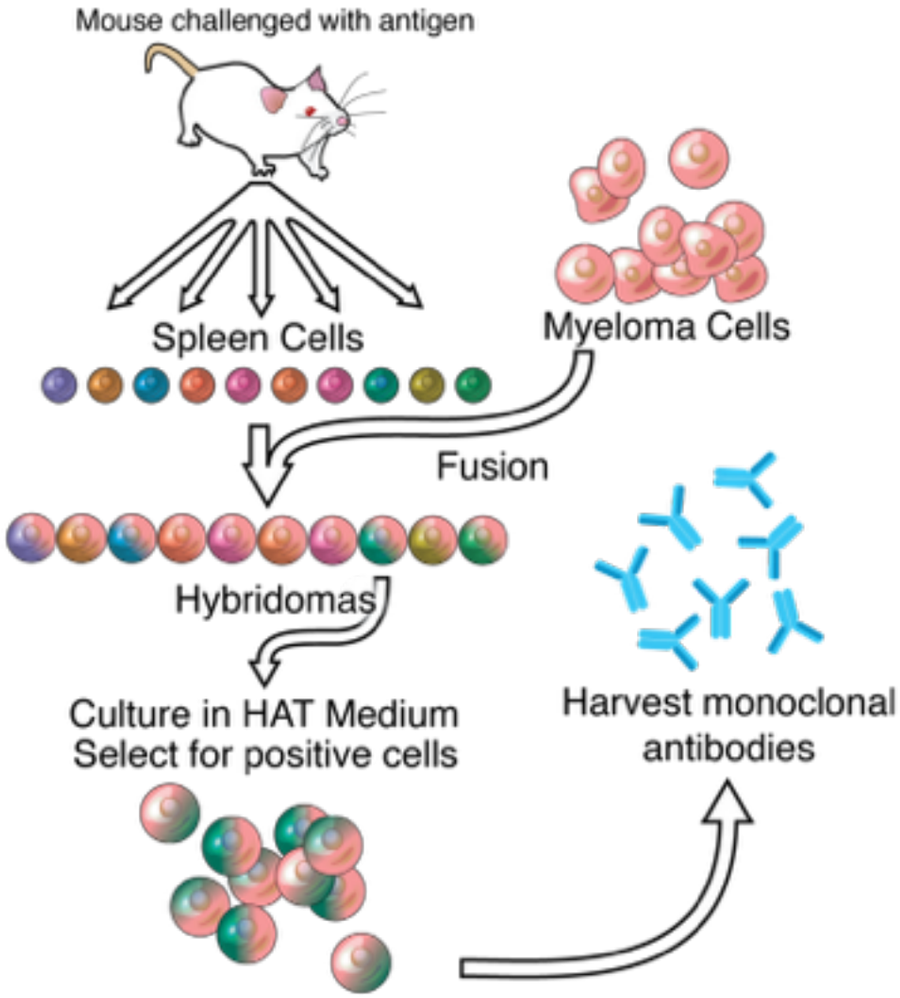
ChIP

(Chromatin Immunoprecipitation)

Polyclonal vs Monoclonal antibodies

Polyclonal antibodies are produced by injecting a protein into a mammal, such as a **mouse**, **rat**, **rabbit**, **goat**, **sheep**, or **horse.** These animal will produce different antibodies against the same protein
Blood isolated from these animals contains antibodies that are able to recognize **different epitopes of the same protein.**

Monoclonal antibodies are antibodies that are made by identical immune cells that are all clones of a unique parent cell, in contrast to polyclonal antibodies, which are made from several different immune cells. Monoclonal antibodies have monovalent affinity, in that they bind to the same epitope



ChIP

(Chromatin Immunoprecipitation)

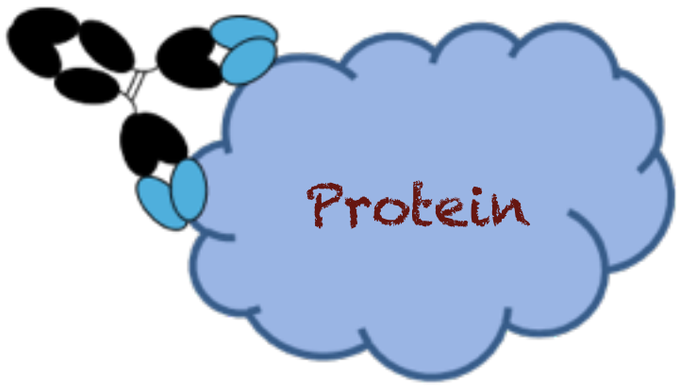
Monoclonal vs Polyclonal antibodies

Polyclonal antibody

Regognize several epitopes

More experimental variability

Monoclonal antibody



Regognize a single epitope

Less experimental variability

ChIP

(Chromatin Immunoprecipitation)

WORK FLOW

1. Cell Crosslinking

2. Chromatin Sonication

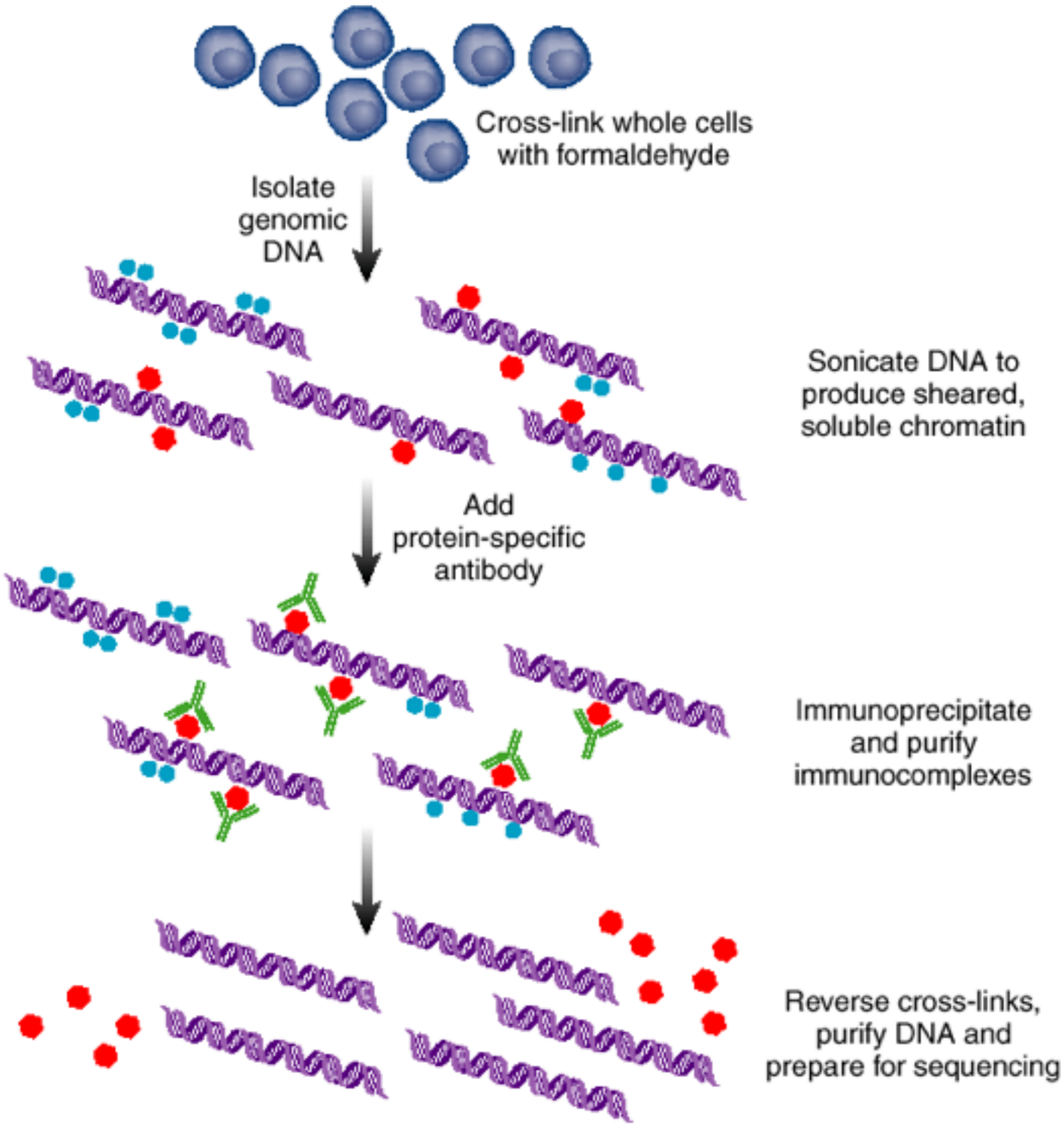
4. Antibody -Extract incubation

5. Binding between Beads and Antibody

6. Immunoprecipitation

7. Reverse crosslink and DNA purification

7. Analysis of DNA



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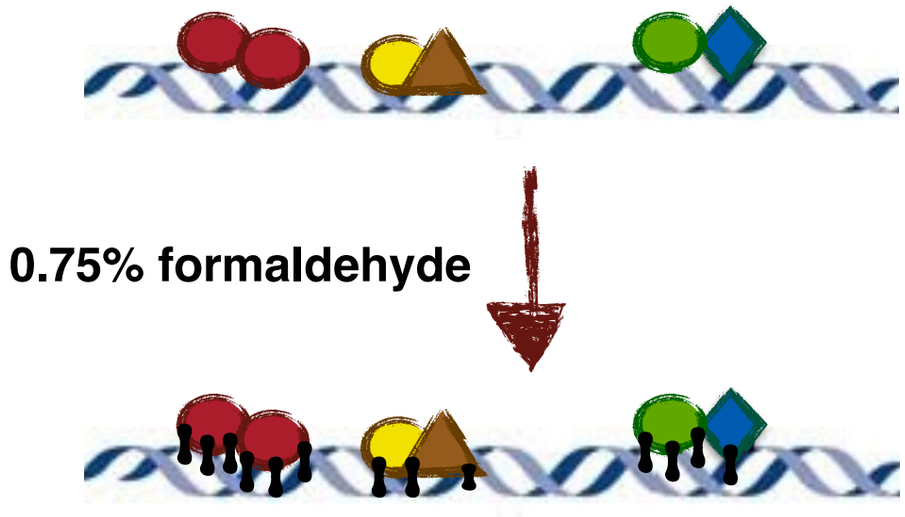
The cross-linking is an experimental procedure that convert in **covalent** all the the weak and non-covalent interactions between DNA - PROTEINS and PROTEIN-PROTEIN

Crosslinking strategies

UV-Crosslinking

Formaldehyde

Glutaraldehyde



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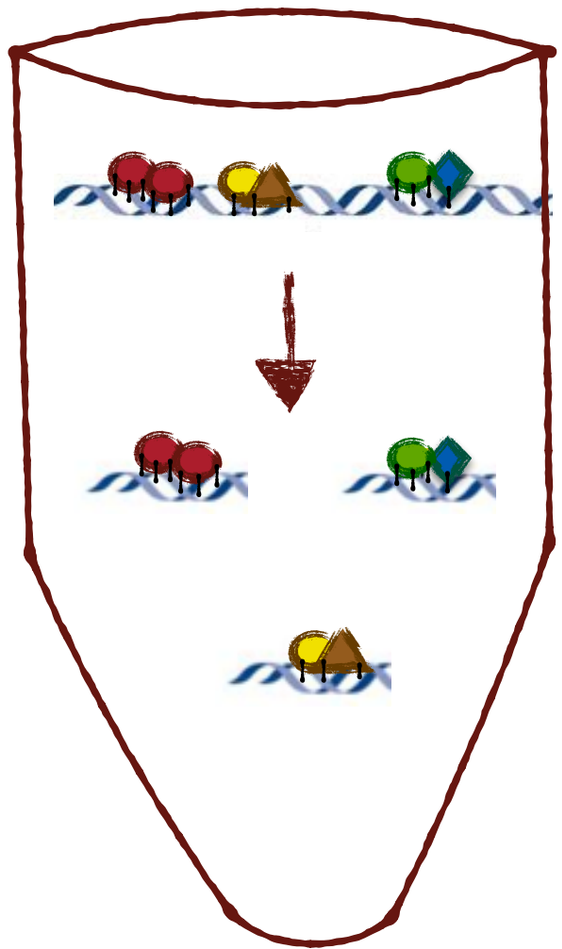
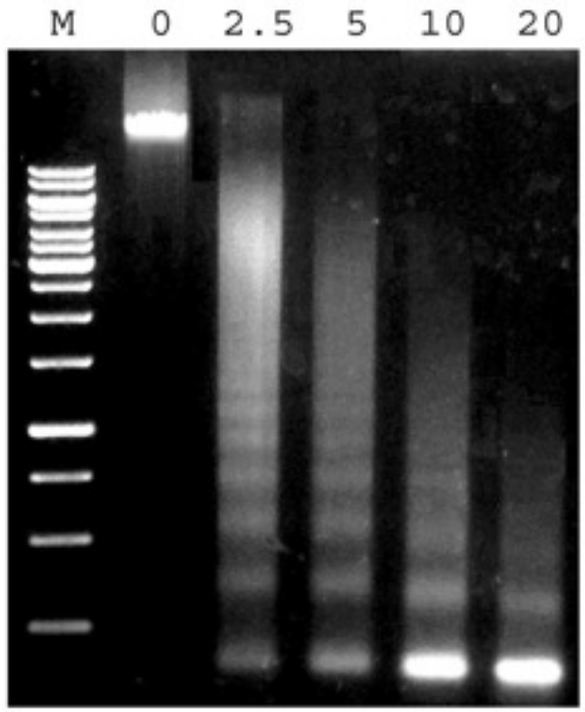
The DNA-protein complexes (chromatin-protein) are then sheared into ~500 bp DNA fragments by **sonication** or (nuclease digestion).





size range: 100-500 bp

ChIP

(Chromatin Immunoprecipitation)

1. Cell Crosslinking

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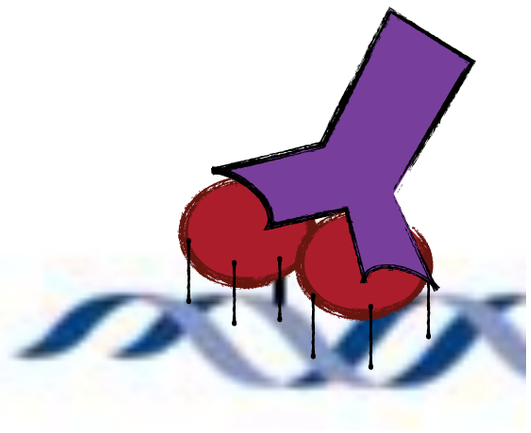
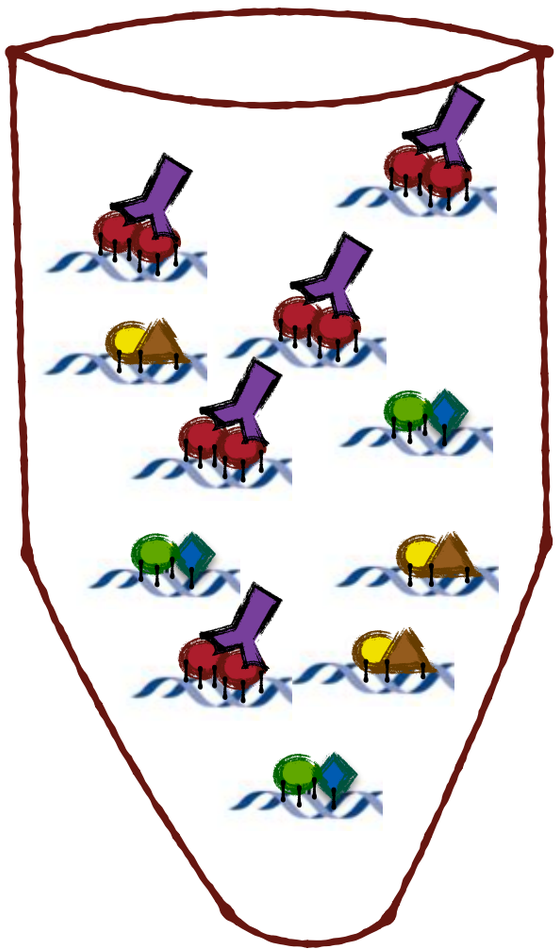
The antibody specific for the protein of interest, is incubated together with the extract, this step allows the formation of strongly interaction between the antibody and the protein of interest





Incubation at 4°
with gentle agitation for 1 hr.

ChIP

(Chromatin Immunoprecipitation)

1. Cell Crosslinking

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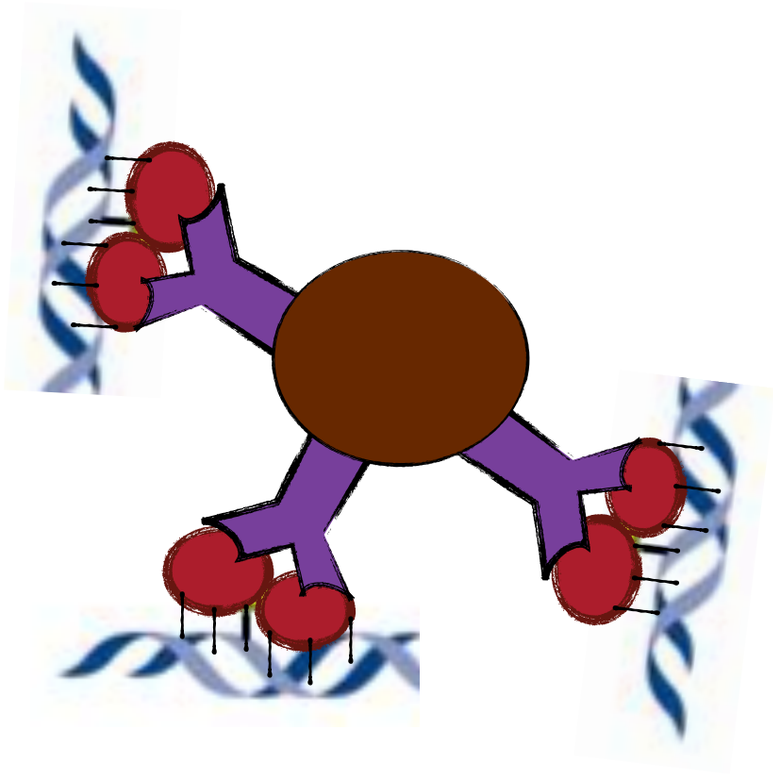
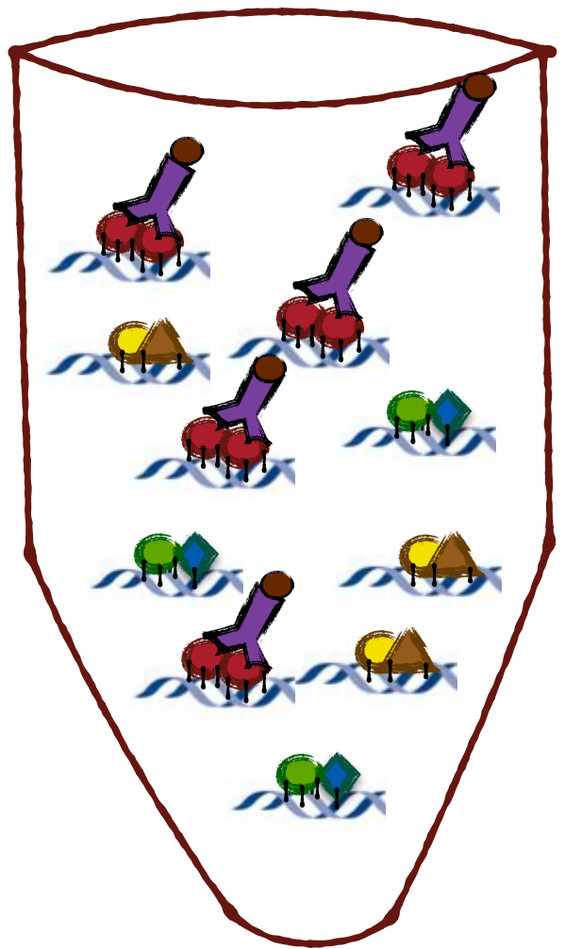
7. Analysis of DNA

Agarose or Magnetic beads (that are able to bind the antibody) are added to the extract.

This step is fundamental for the precipitation of the complex **Bead-Antibody-Protein-DNA**





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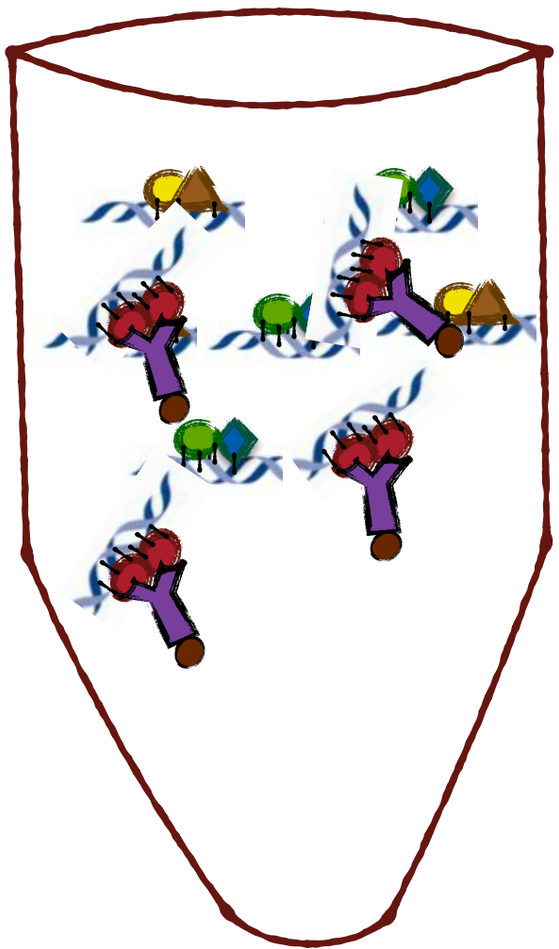
7. Analysis of DNA

The immuniprecipitation step allows the purification of the complexes that are bound to the antibody

Centrigugation (Agarose Beads)
Using a Magnetic Rec (Magnetic Beads)



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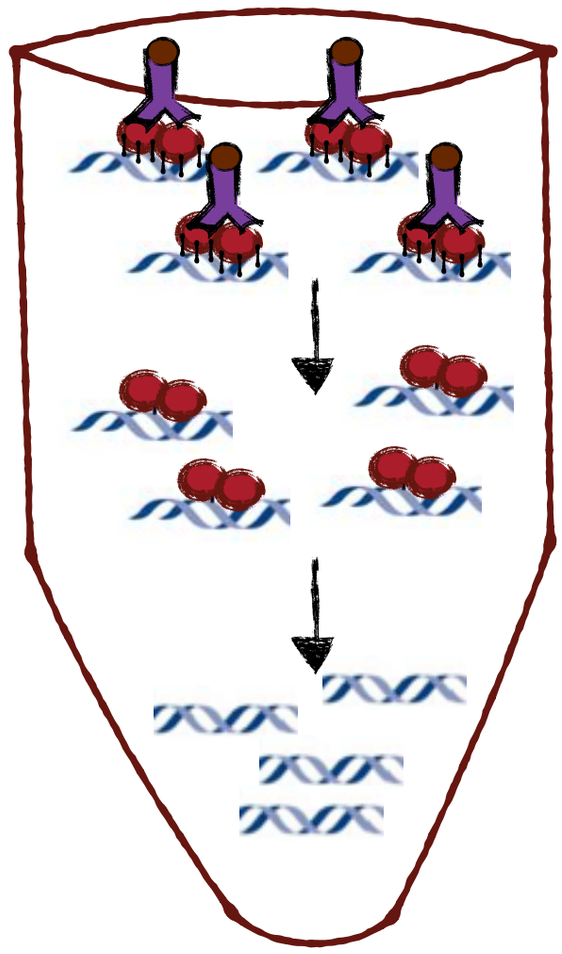
7. Analysis of DNA

The cross-linking with formaldehyde is able to be removed through the incubation of the extract at High temperature,

this step allows the detach of the protein from the DNA that is subsequently purified and analyzed



incubation at 70 °C for 5 minutes

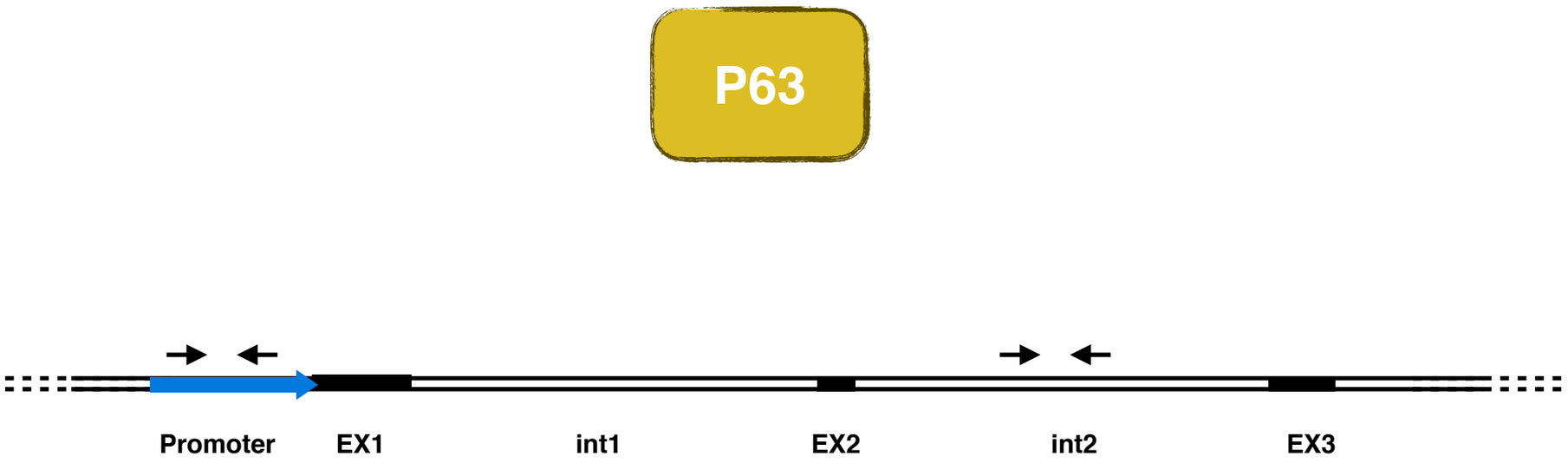
ChIP

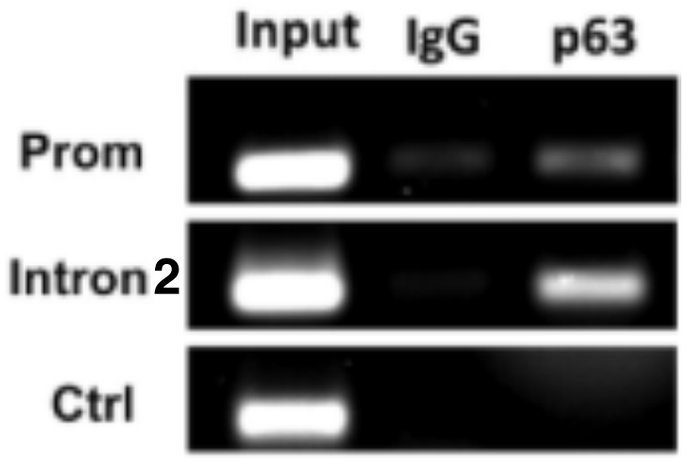
(Chromatin Isolation by Rna Purification)

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 a





ChIP on chip

Microarray technology allows the generation of high resolution genome-wide maps of protein/protein modifications. DNA purified from the immunoprecipitated chromatin and input are labeled with fluorescent dyes using ligation mediated PCR.

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

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*Protein detection: **Western Blot***

RNA-Protein: **RNA pull down**

Protein-RNA: **RIP** (RNA Immunoprecipitation)

Western Blot

AIM: Detection and quantification of a the protein

The **western blot** (sometimes called the protein immunoblot) is a widely used analytic technique performed to **detect** and **quantify** specific **protein** in a sample of tissue homogenate or extract.

It is based on the usage of an **electrophoresis** to separate denatured proteins by their length.

The proteins are then transferred on a membrane (typically nitrocellulose), where they are stained with **antibodies** specific for the target protein.

Western Blot

WORK FLOW

1. Gel electrophoresis

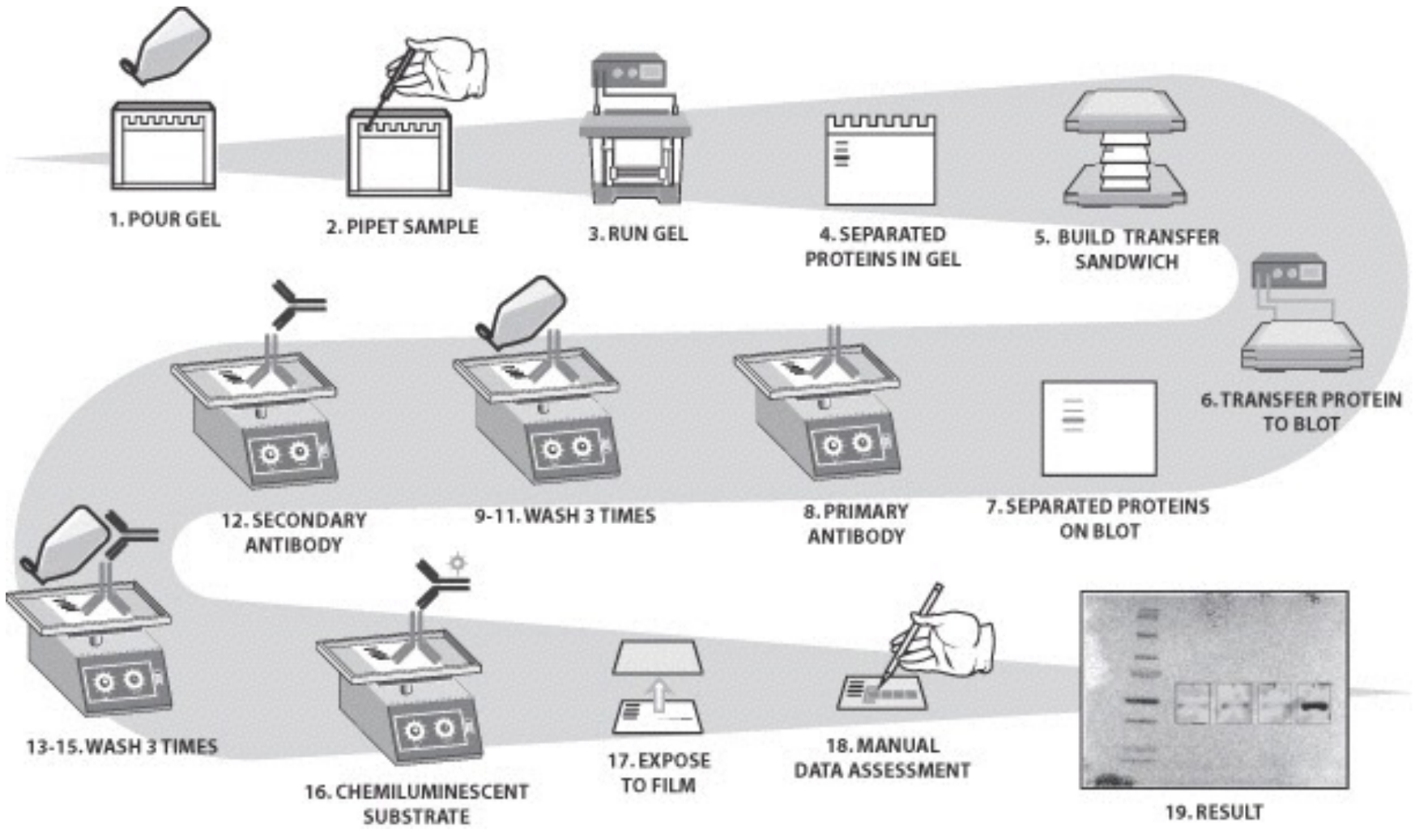
2 Transfer Membrane (Blot)

3. Saturation or Blocking

4. Bindind with primary antibody

5.Binding with secondary antibody

6. Protein detection



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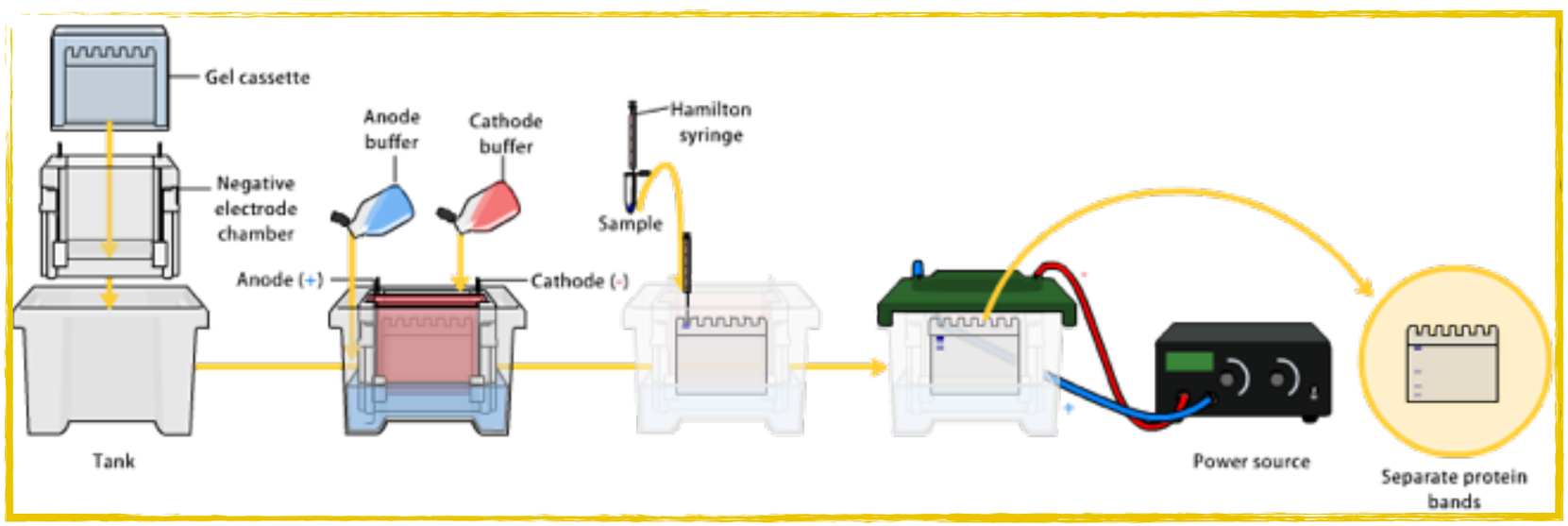
During the gel electrophoresis the proteins have to be separated according to they **molecular weight**:

- Remove Secondary and tertiary structures
- The proteins need to have the same negative charge

SDS (sodium dodecyl sulphate)

$CH_3(CH_2)_{10}CH_2OSO_3^-Na^+$



Western Blot

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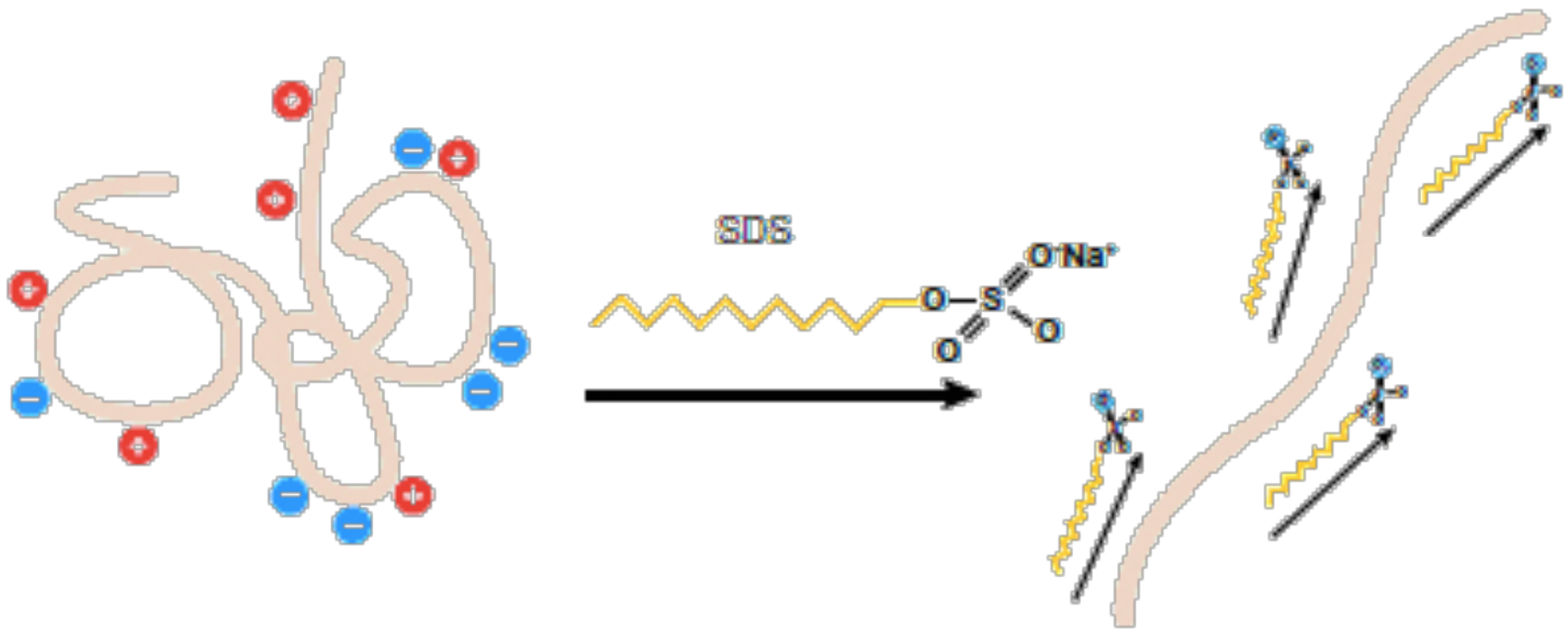
SDS (sodium dodecyl sulphate)

$CH_3(CH_2)_{10}CH_2OSO_3^-Na^+$

The SDS maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups (DTT).

Sampled proteins become covered by the SDS, negatively charged, and move to the positive electrode through the polyacrylamide gel. Smaller proteins migrate faster through the gel and the **proteins are thus separated according to size**



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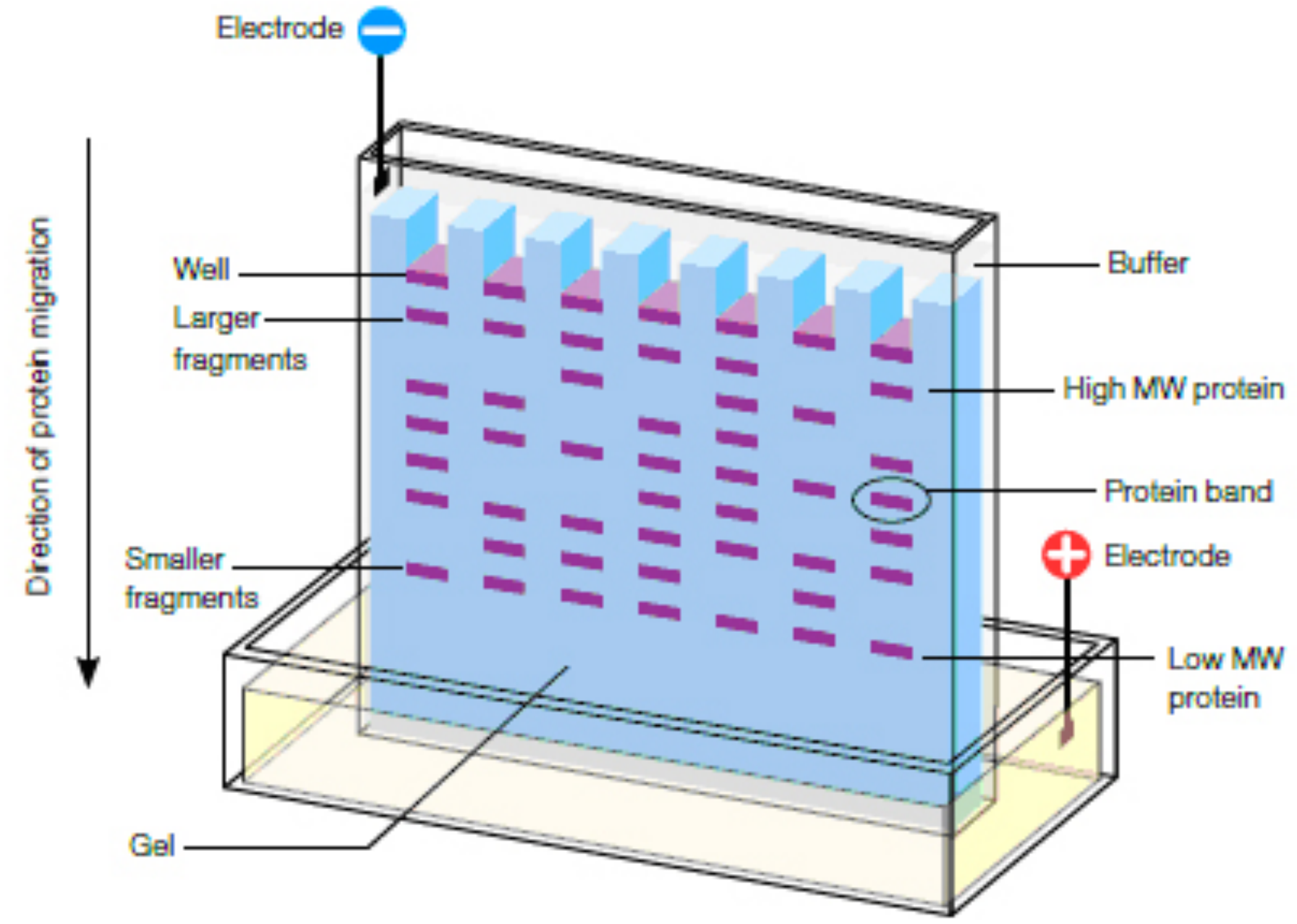

Western Blot

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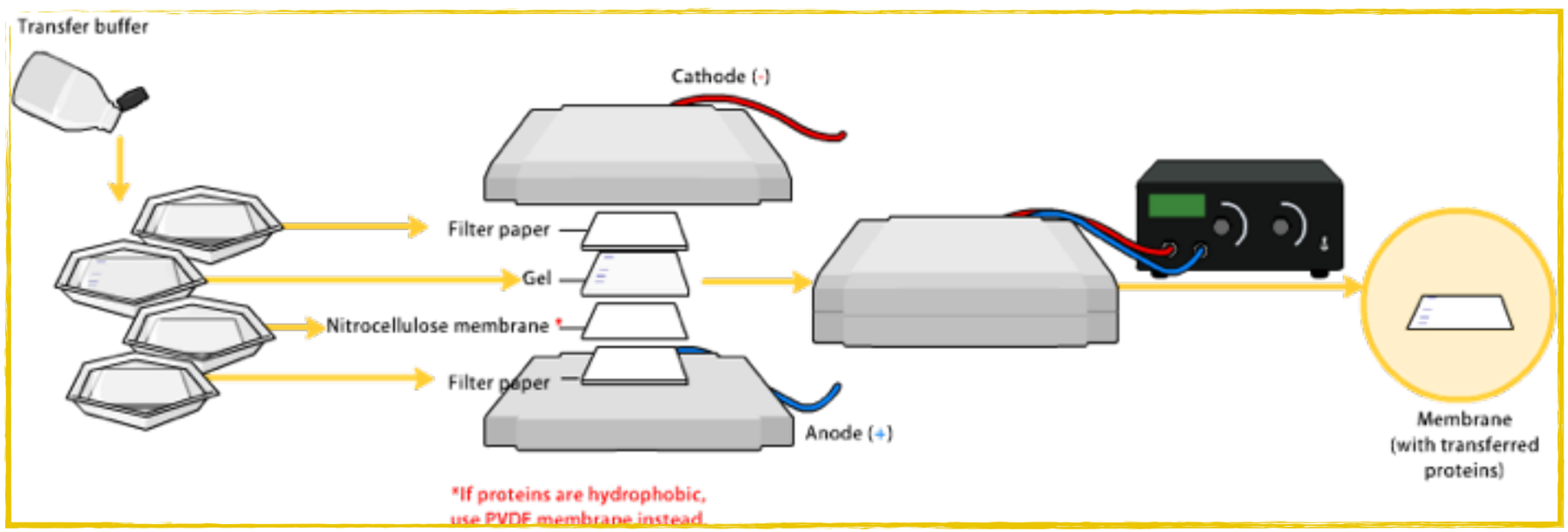
6. Protein detection

In order to make the proteins accessible to antibody detection, they are transferred from the gel to the **nitrocellulose membrane.**

The primary method for transferring the proteins is called **electroblotting** and uses an electric current to pull proteins from the gel to the nitrocellulose membrane.



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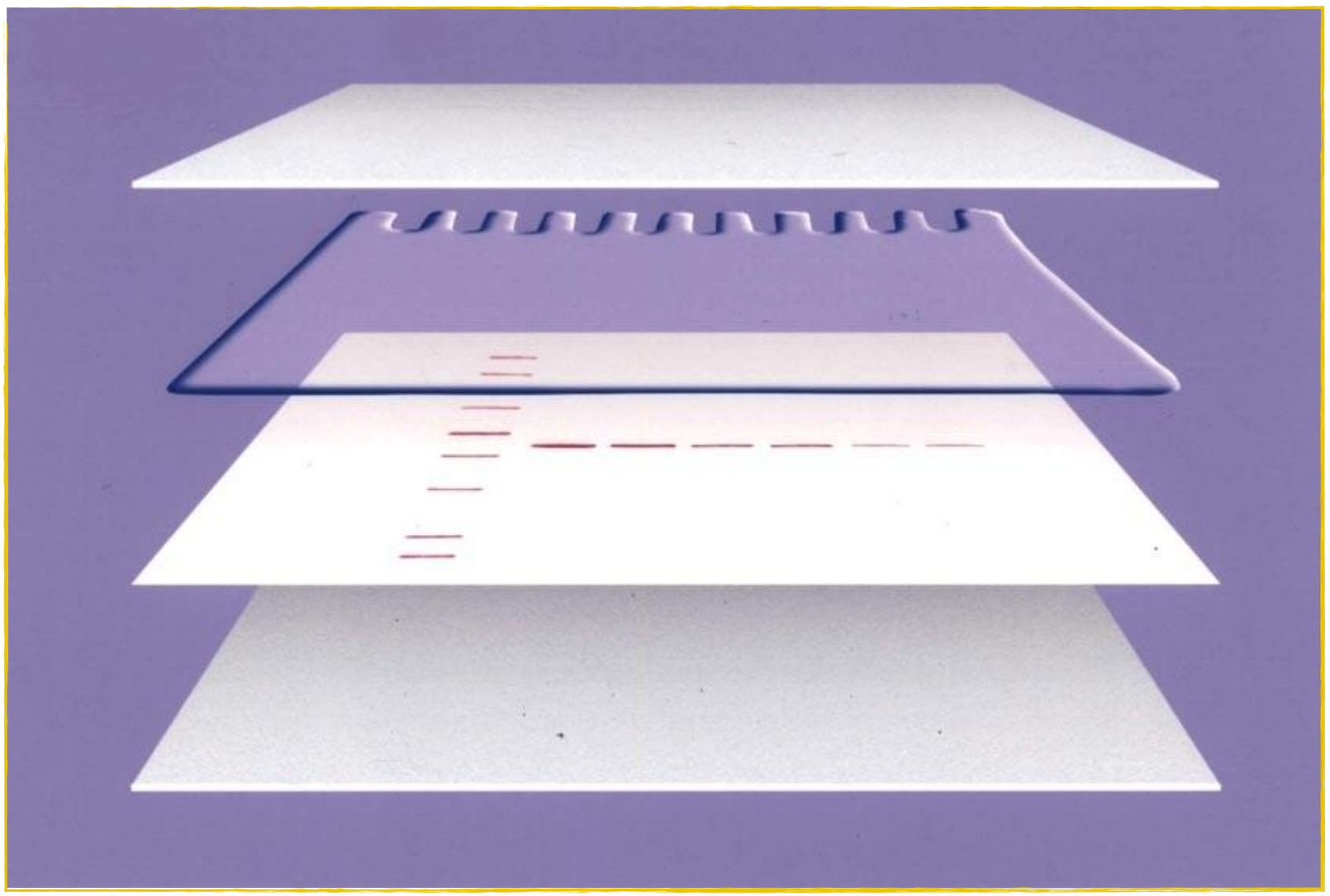

Western Blot

WORK FLOW

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5.Binding with secondary antibody

6. Protein detection

Blocking

This steps must be performed to prevent the aspecific interactions between the membrane and the antibody used for detection of the target protein.

Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein (3-5% Bovine serum albumin (BSA) or 10% of milk in Tris-Buffered Saline (TBS) or I-Block, with a minute percentage (0.1%) of detergent such as Tween or Triton)

The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane.

This reduces **background leading to clearer results**, and eliminates false positives.

Western Blot

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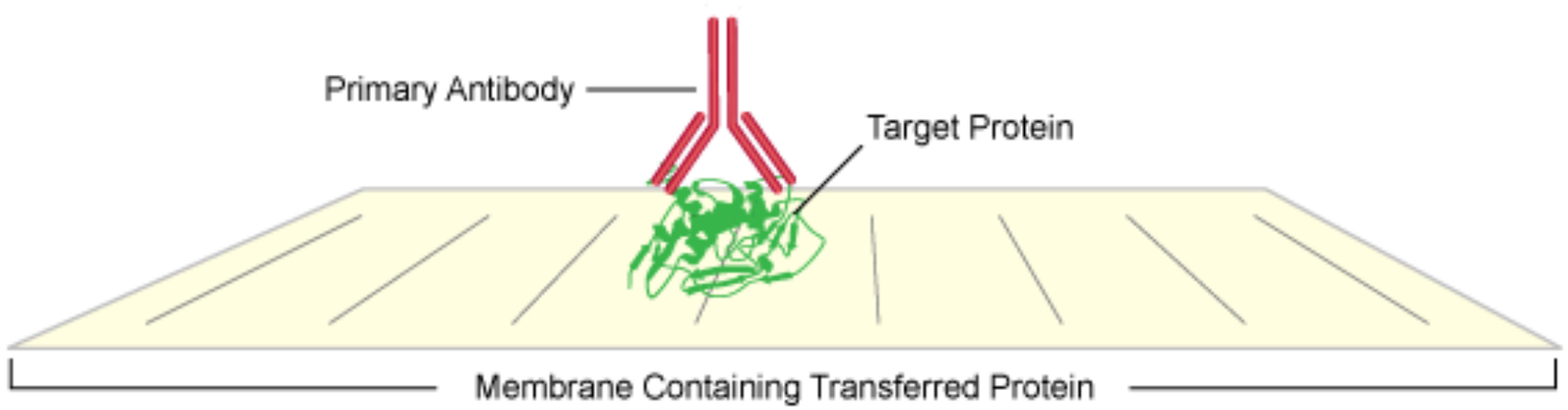

Western Blot

WORK FLOW

1. Gel electrophoresis

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4. Bindind with primary antibody

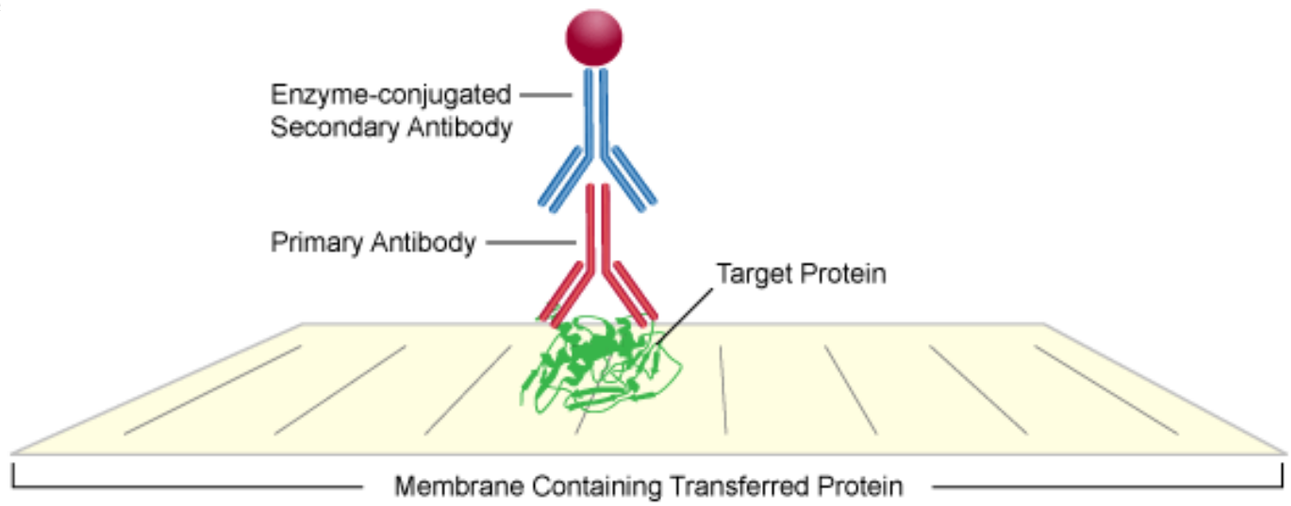
5.Binding with secondary antibody

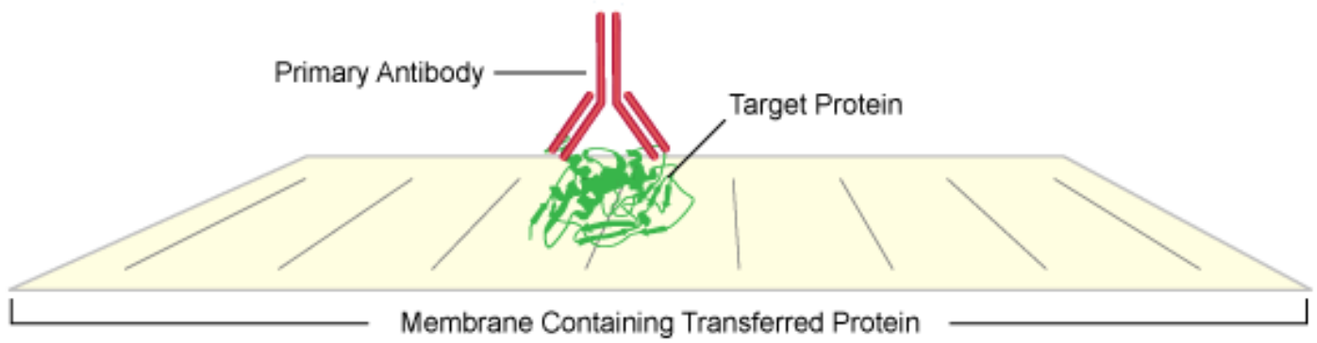
6. Protein detection

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a **species-specific** portion of the primary antibody.

SECONDARY ANTIBODIES

a- mouse
a- goat
a- rabbit
a- sheep
a- horse





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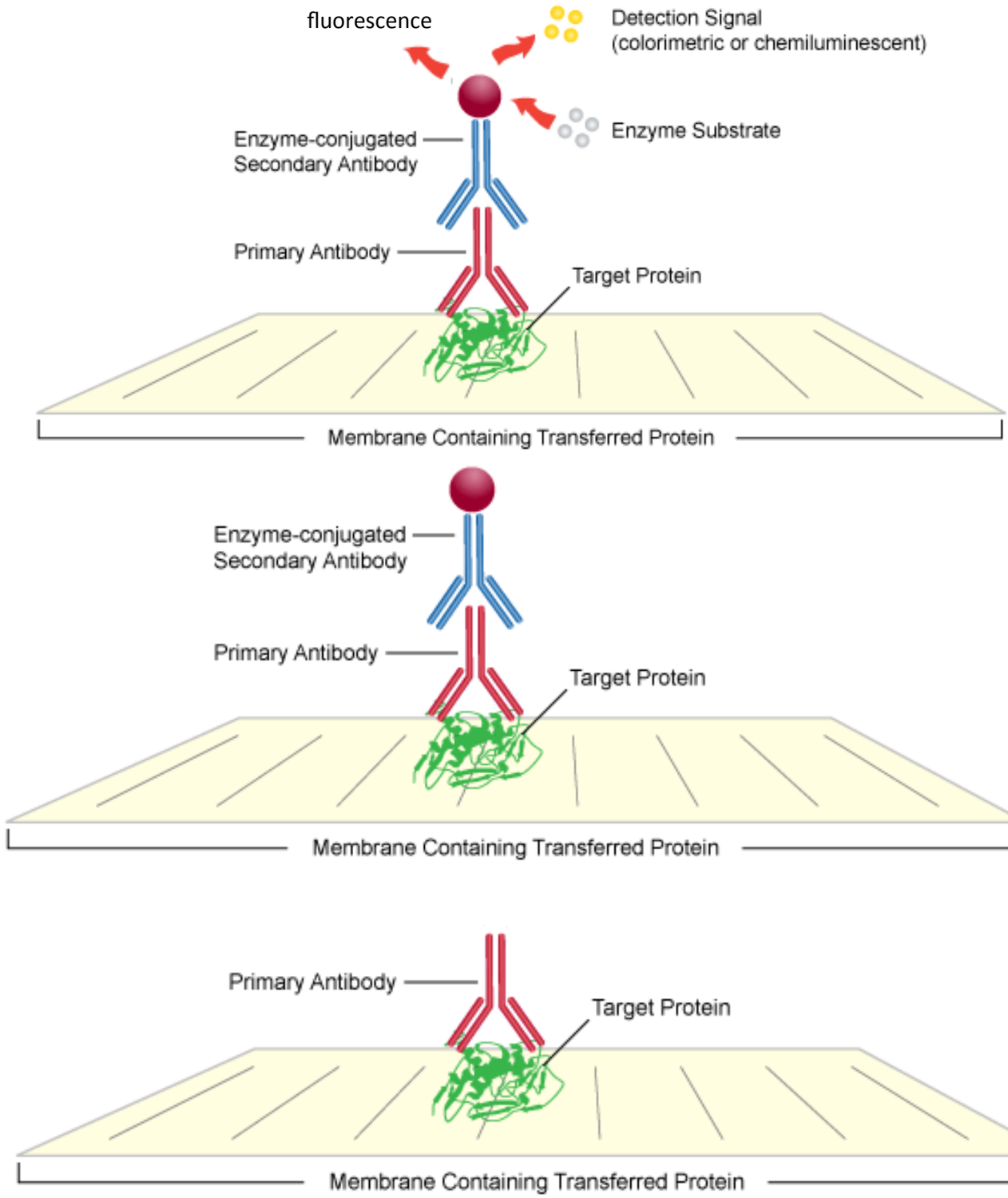

Western Blot

WORK FLOW

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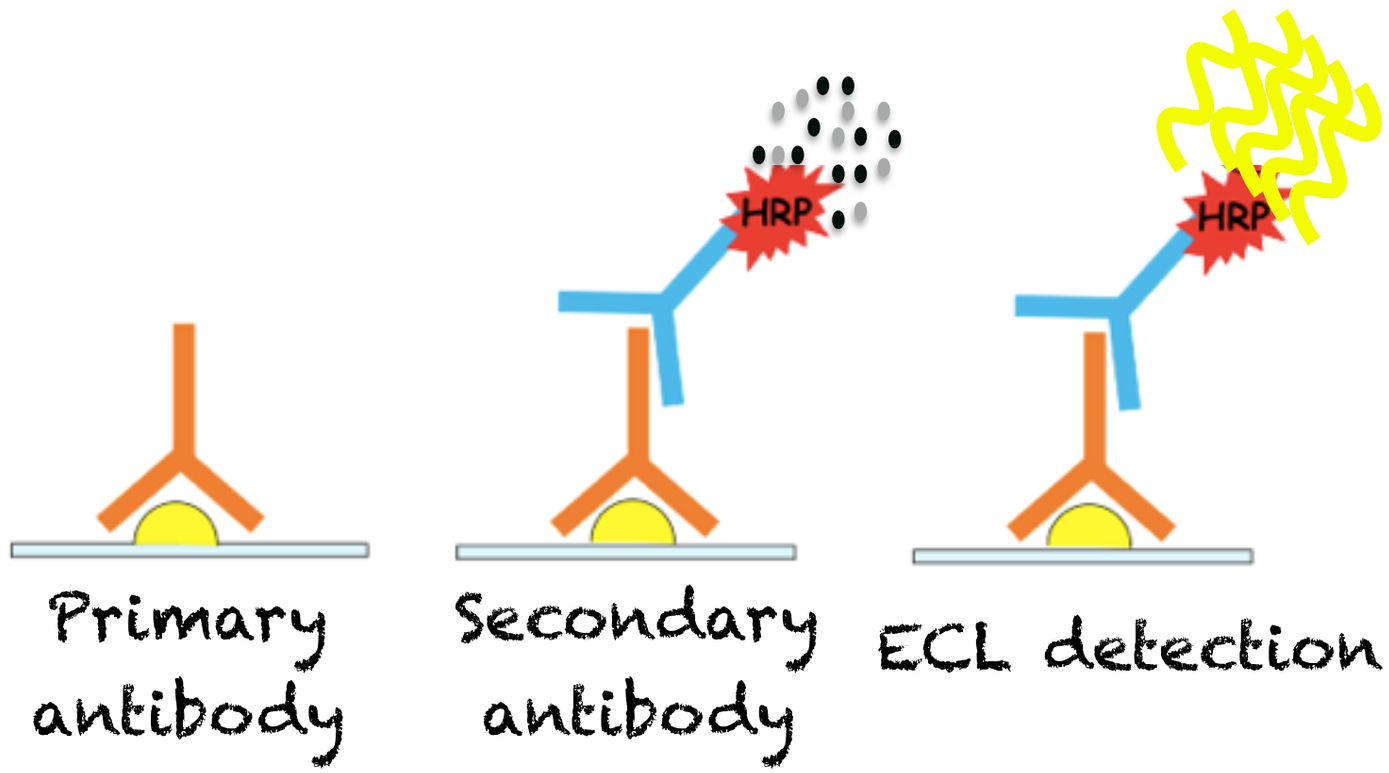
6. Protein detection

ECL



Western Blot

WORK FLOW

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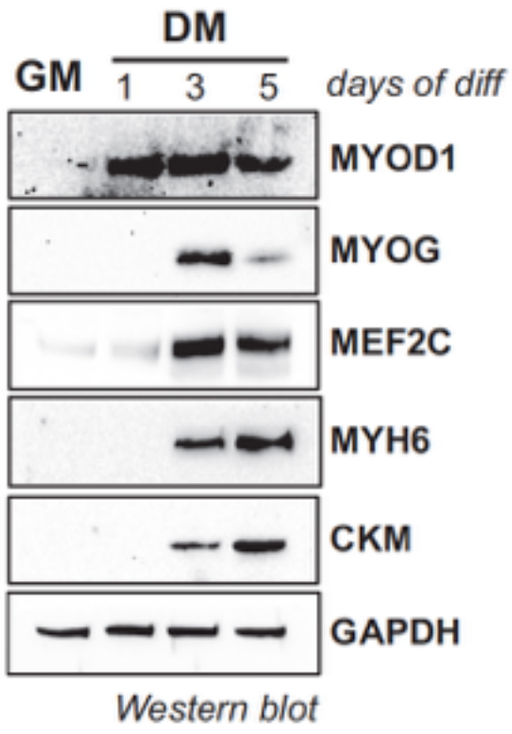
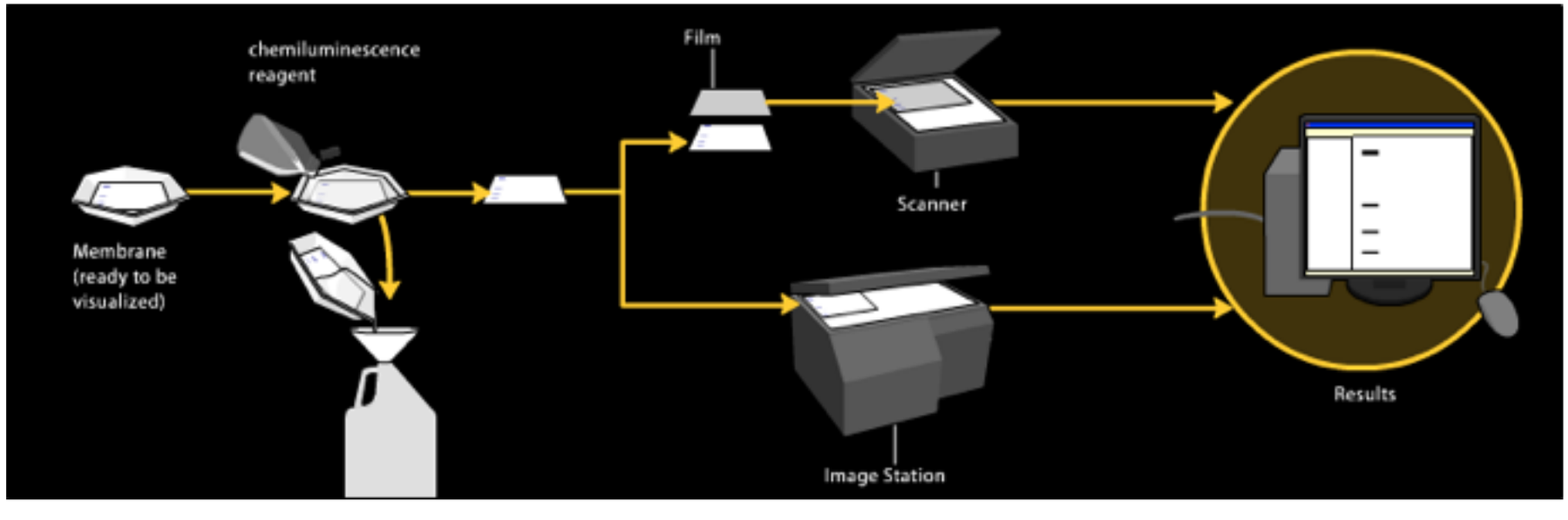
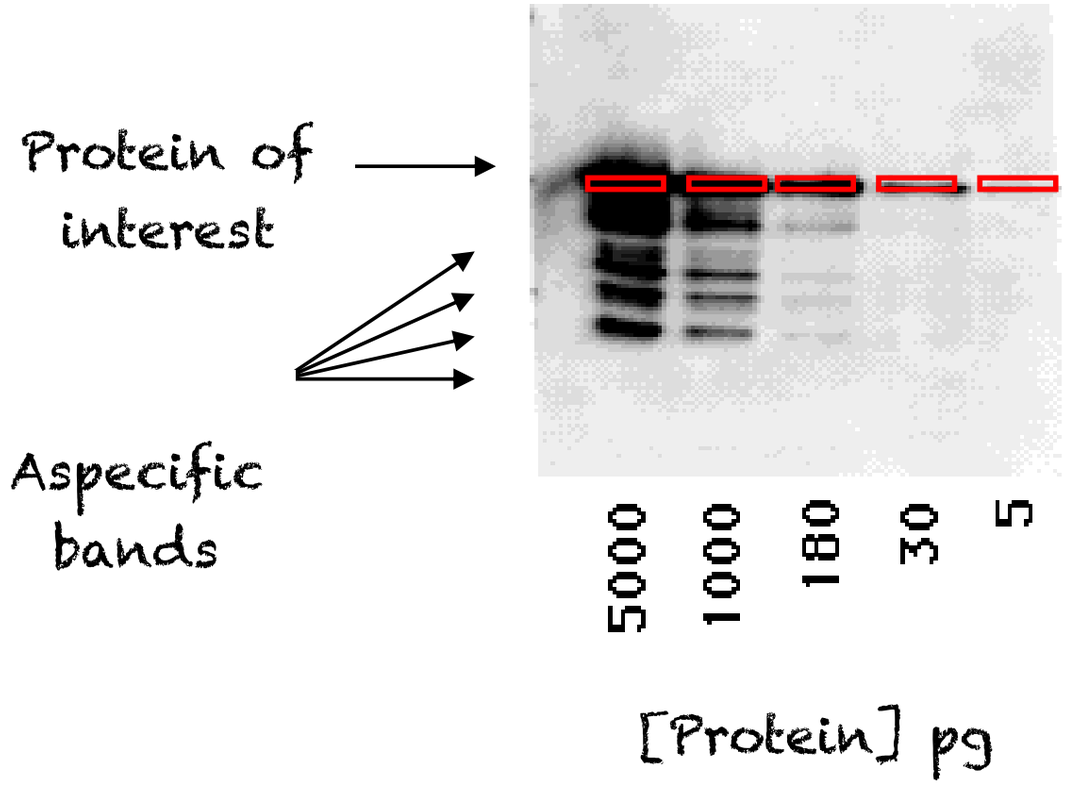


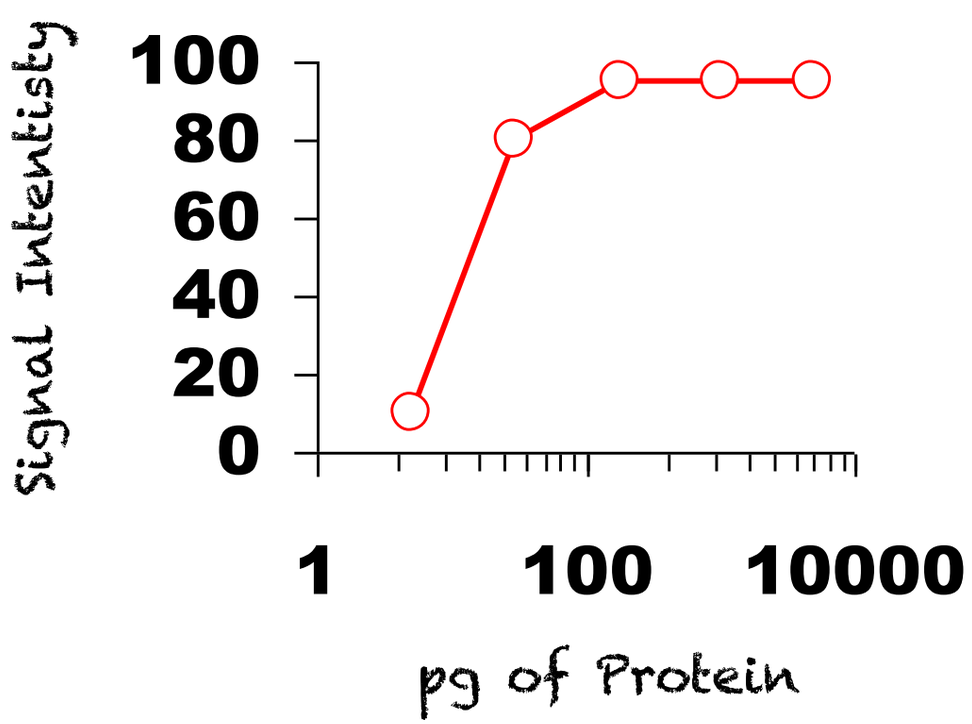
Adapted from Ballarino et al, 2015



Western Blot

How is possible to Quantify a protein?





Molecular techniques for the study of the interaction between:

DNA-protein: **ChIP** (Chromatin immunoprecipitation)

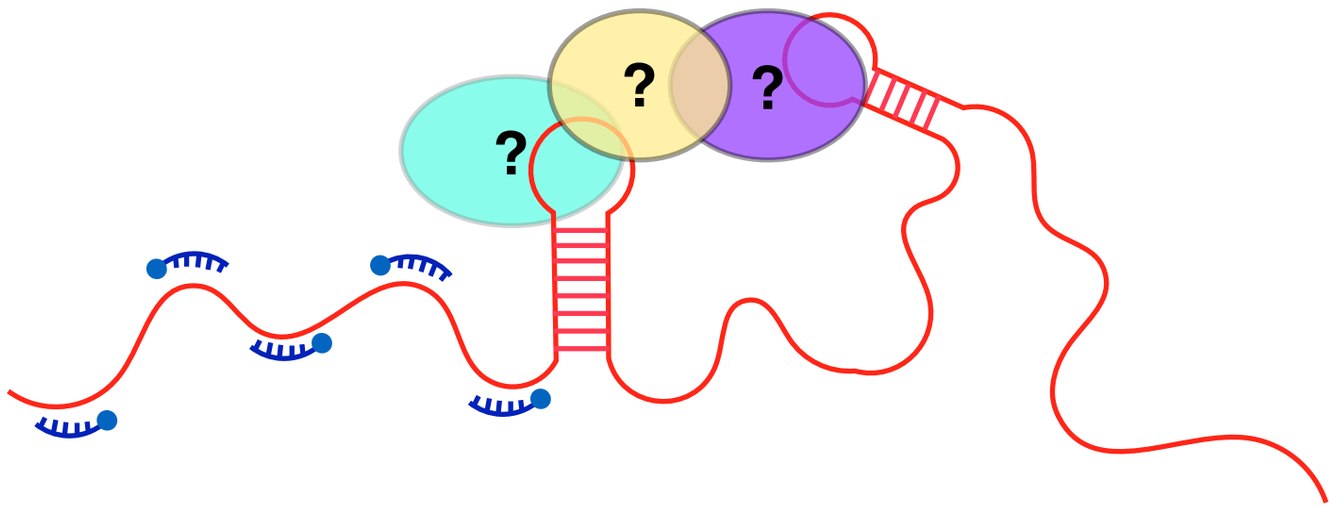
Protein detection: Western Blot

RNA-Protein: **RNA pull down**

Protein-RNA: RIP (RNA immunoprecipitation)

RNA pull down

AIM: Identification of the protein interactors of an RNA.



- Nuclear and Cytoplasmic RNAs

- Exogenous (*in vitro*)
- Endogenous (*in vivo*) RNA pull down

Endogenous RNA pull down

WORK FLOW

1. Probe Design

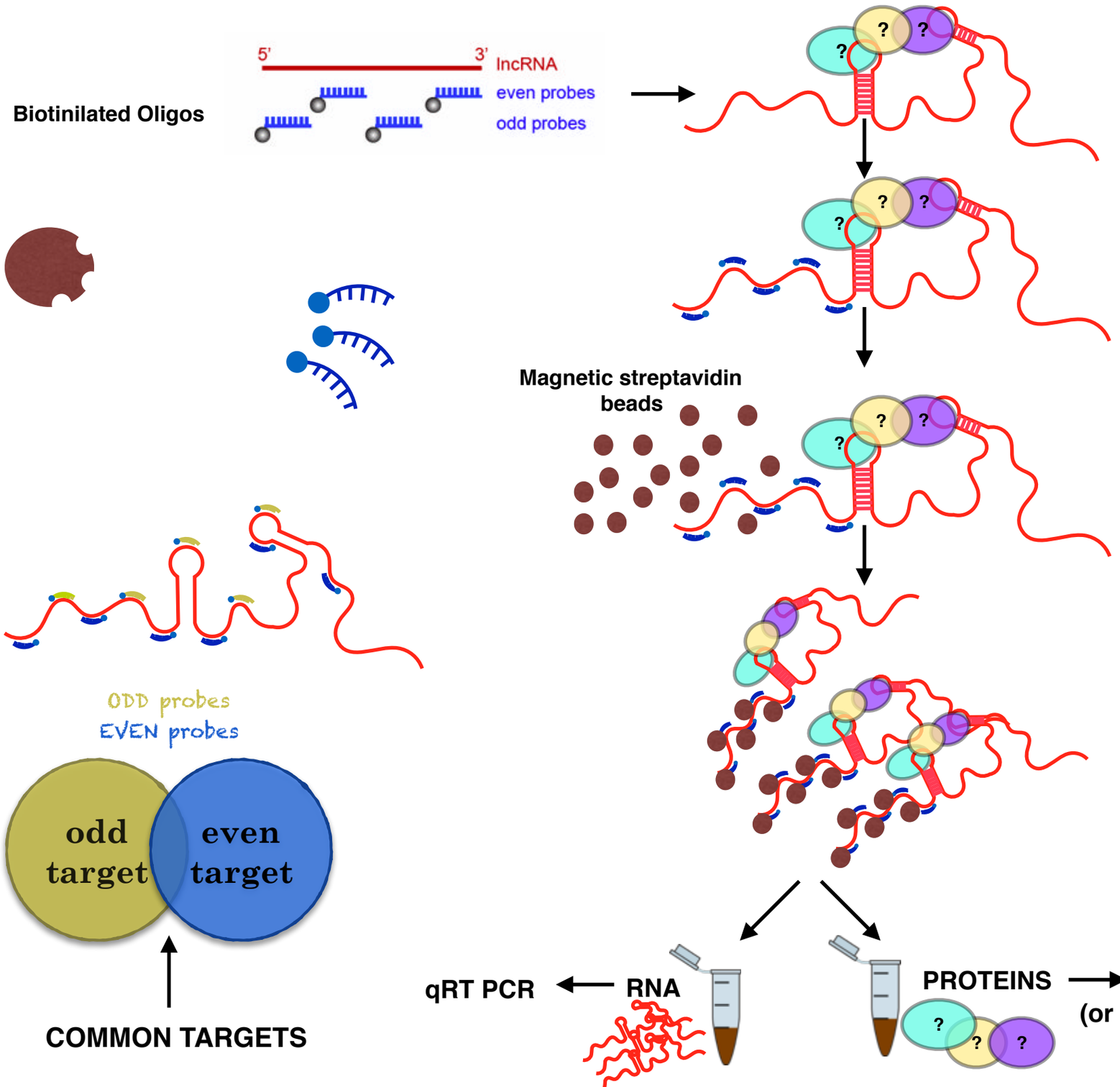
2 Collect cell extract

3. Binding step

4. Introduction of Streptavidin-magnetic beads

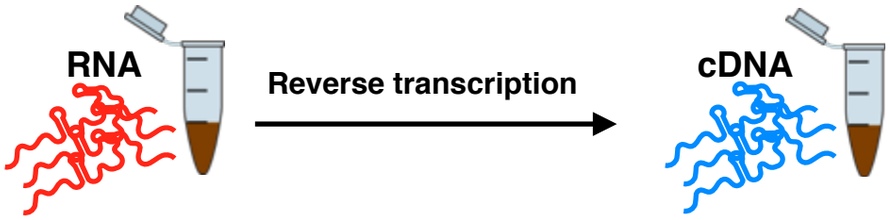
5. Pull down

6. Protein and RNA analysis

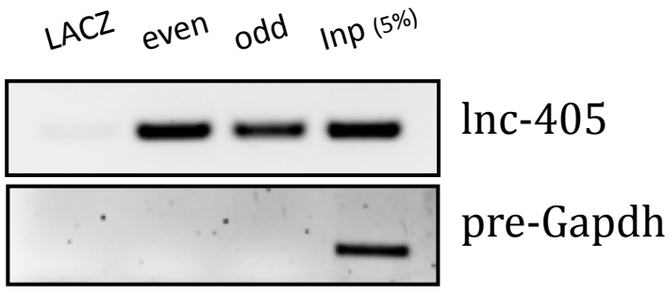


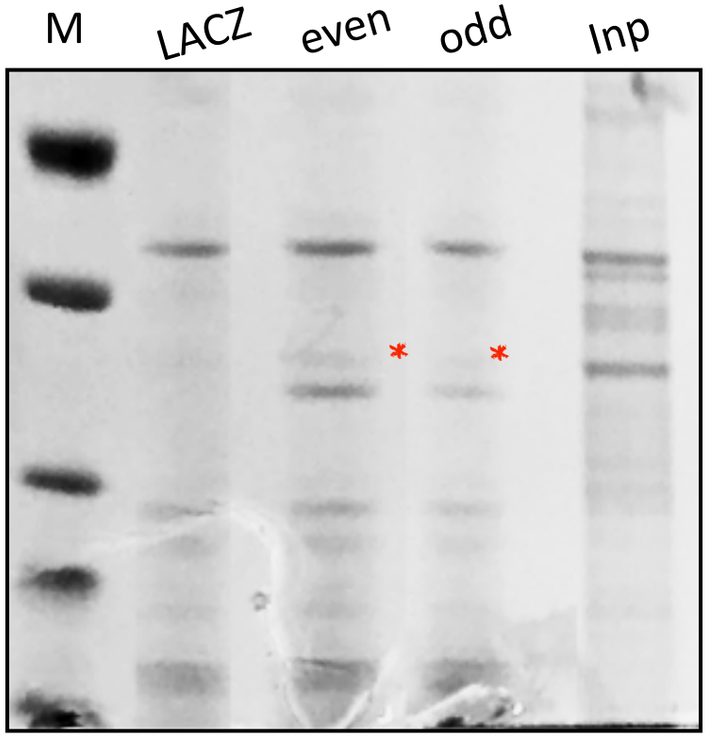
Endogenous RNA pull down

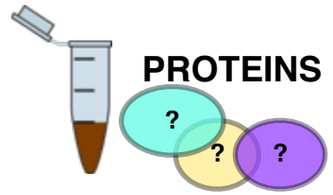
Lnc-405 endogenous pulldown



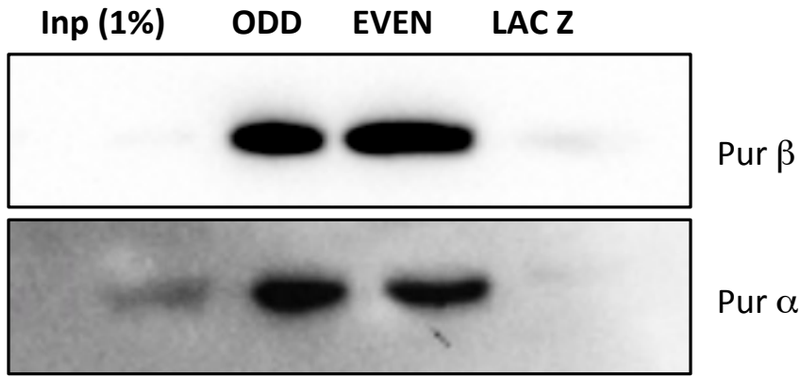
Semiq RT-PCR







Western Blot



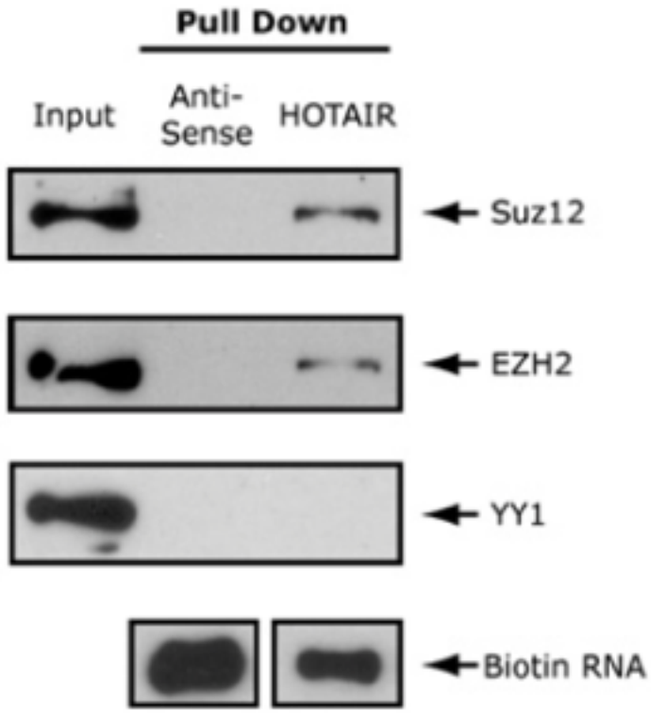
MASS SPECTROMETRY

Accession	Description
Q9QXS1	Plectin OS=Mus musculus GN=Plec PE=1 SV=3 - [PLEC_MOUSE]
Q8VDD5	Myosin-9 OS=Mus musculus GN=Myh9 PE=1 SV=4 - [MYH9_MOUSE]
P62843	40S ribosomal protein S15 OS=Mus musculus GN=Rps15 PE=1 SV=2 - [RS15_MOUSE]
P42669	Transcriptional activator protein Pur-alpha OS=Mus musculus GN=Pura PE=1 SV=1 - [PURA_MOUSE]
P62301	40S ribosomal protein S13 OS=Mus musculus GN=Rps13 PE=1 SV=2 - [RS13_MOUSE]
E9Q557	Desmoplakin OS=Mus musculus GN=Dsp PE=1 SV=1 - [DESP_MOUSE]
Q6P5H2	Nestin OS=Mus musculus GN=Nes PE=1 SV=1 - [NEST_MOUSE]
P62702	40S ribosomal protein S4, X isoform OS=Mus musculus GN=Rps4x PE=1 SV=2 - [RS4X_MOUSE]
P20152	Vimentin OS=Mus musculus GN=Vim PE=1 SV=3 - [VIME_MOUSE]
P31001	Desmin OS=Mus musculus GN=Des PE=1 SV=3 - [DESM_MOUSE]
O35295	Transcriptional activator protein Pur-beta OS=Mus musculus GN=Purb PE=1 SV=3 - [PURB_MOUSE]

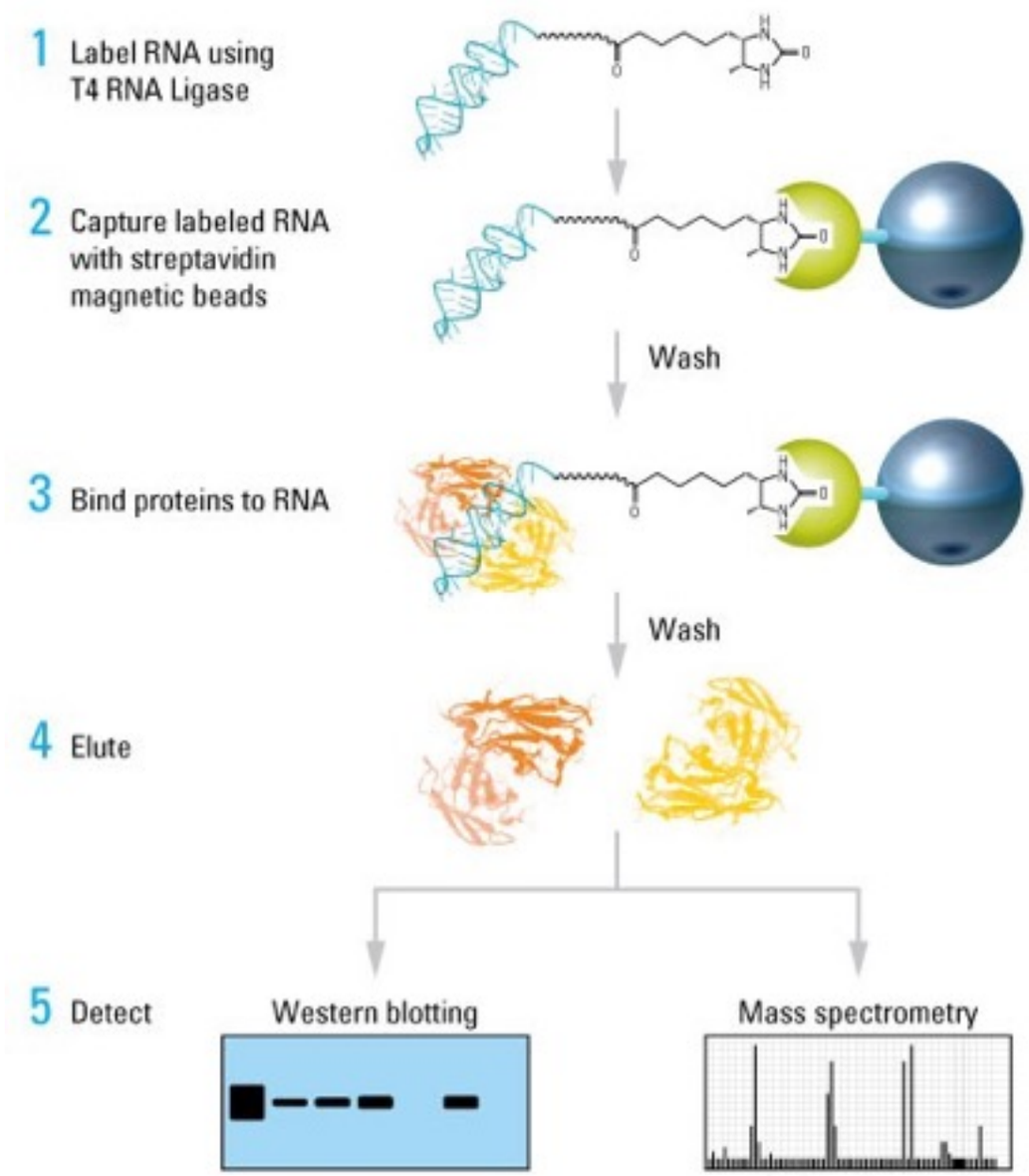
Exogenous RNA pull down

HOTAIR exogenous pulldown

Western Blot



Rinn et al. Cell 129, 1311–1323, June 29, 2007

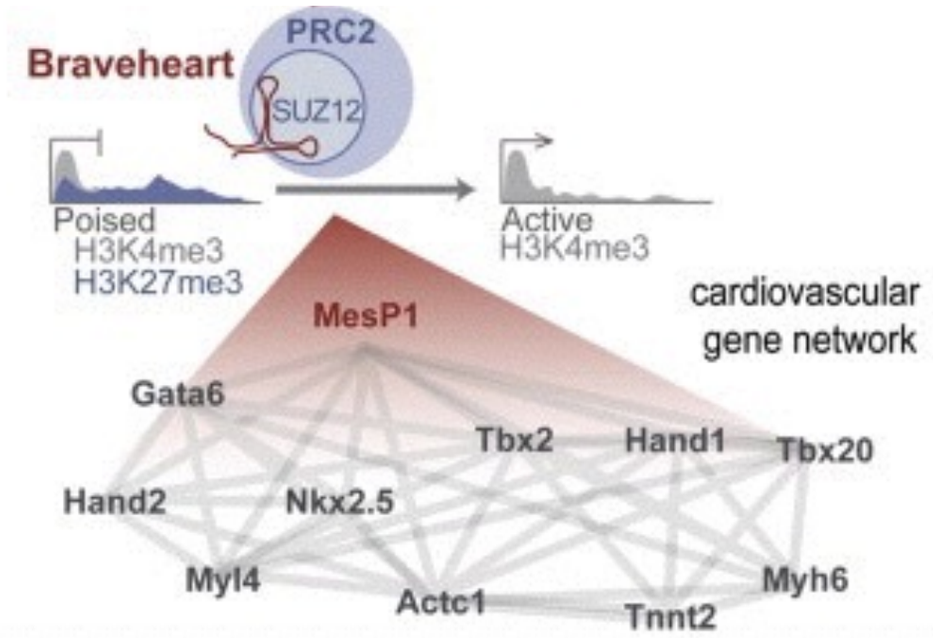


RNA-Protein Pull-Down Protocol
Summary of the procedure for end-labeling RNA with desthiobiotin and then capturing and enriching specific RNA binding proteins using the T**hermo Scientific Pierce Magnetic RNA-Protein Pull-Down Kit**.

Cell

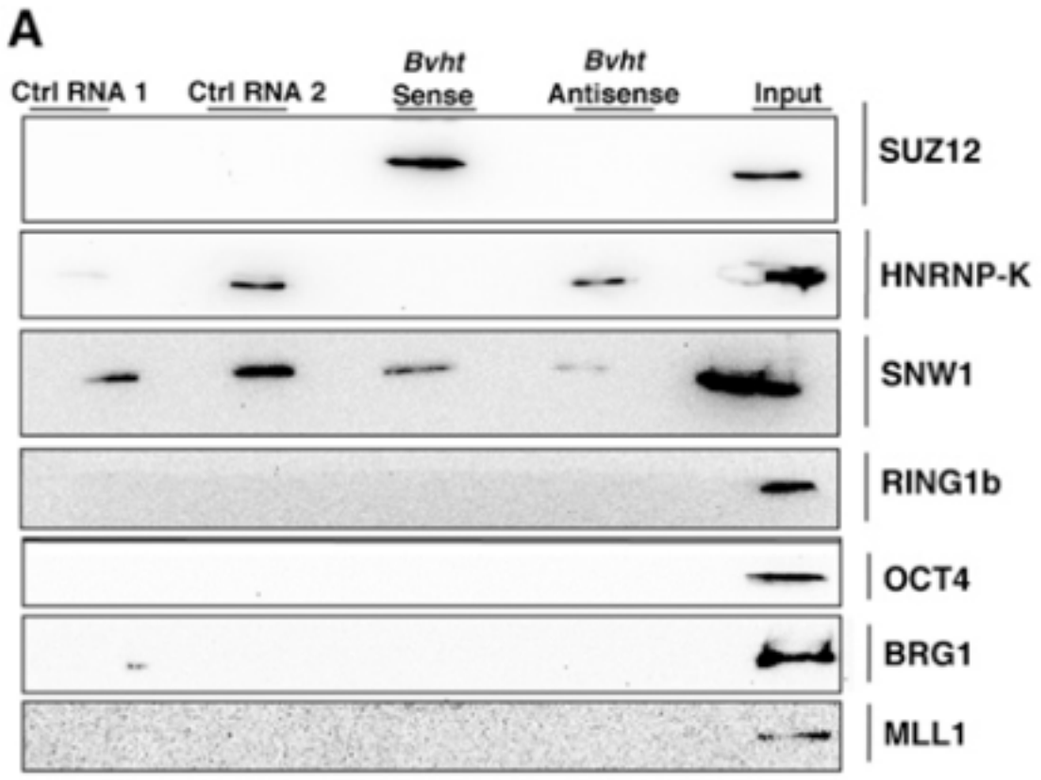
Braveheart, a Long Noncoding RNA Required for Cardiovascular Lineage Commitment

Carla A. Klattenhoff,[1,6] Johanna C. Scheuermann,[1,6] Lauren E. Surface,[1] Robert K. Bradley,[1,2,7] Paul A. Fields,[1] Matthew L. Steinhauser,[3] Huiming Ding,[1] Vincent L. Butty,[1] Lillian Torrey,[1] Simon Haas,[1] Ryan Abo,[1] Mohammadsharif Tabebordbar,[1,4,8] Richard T. Lee,[3,5] Christopher B. Burge,[1,2] and Laurie A. Boyer[1,*]



RIP

Bvht exogenous pulldown



Molecular techniques for the study of the interaction between:

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

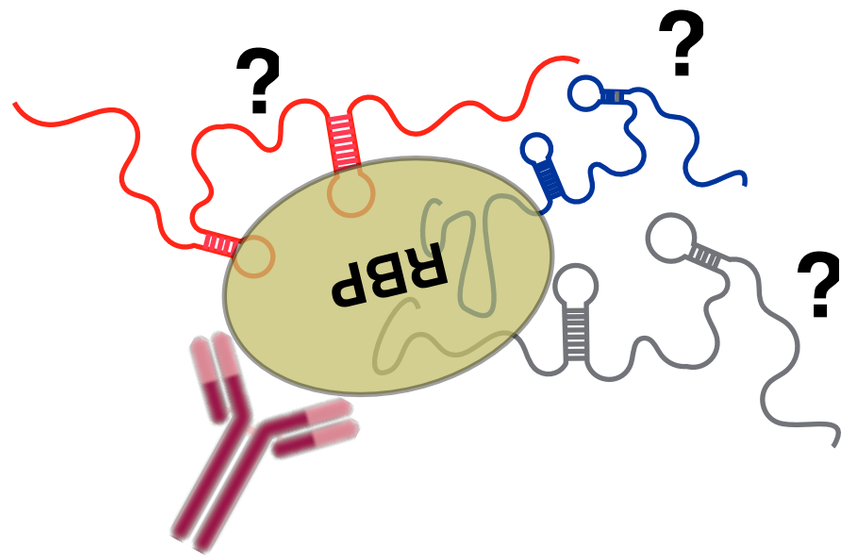
RNA-Protein: **RNA pull down**

Protein-RNA: **RIP** (RNA immunoprecipitation)

RIP

(Rna Immunoprecipitation)

AIM: Identification of the RNAs bound to a known Protein



- Cytoplasmic or Nuclear extract
- Isolation of Ribonucleoprotein complexes

Variants:
CLIP (UV-RIP)

RIP

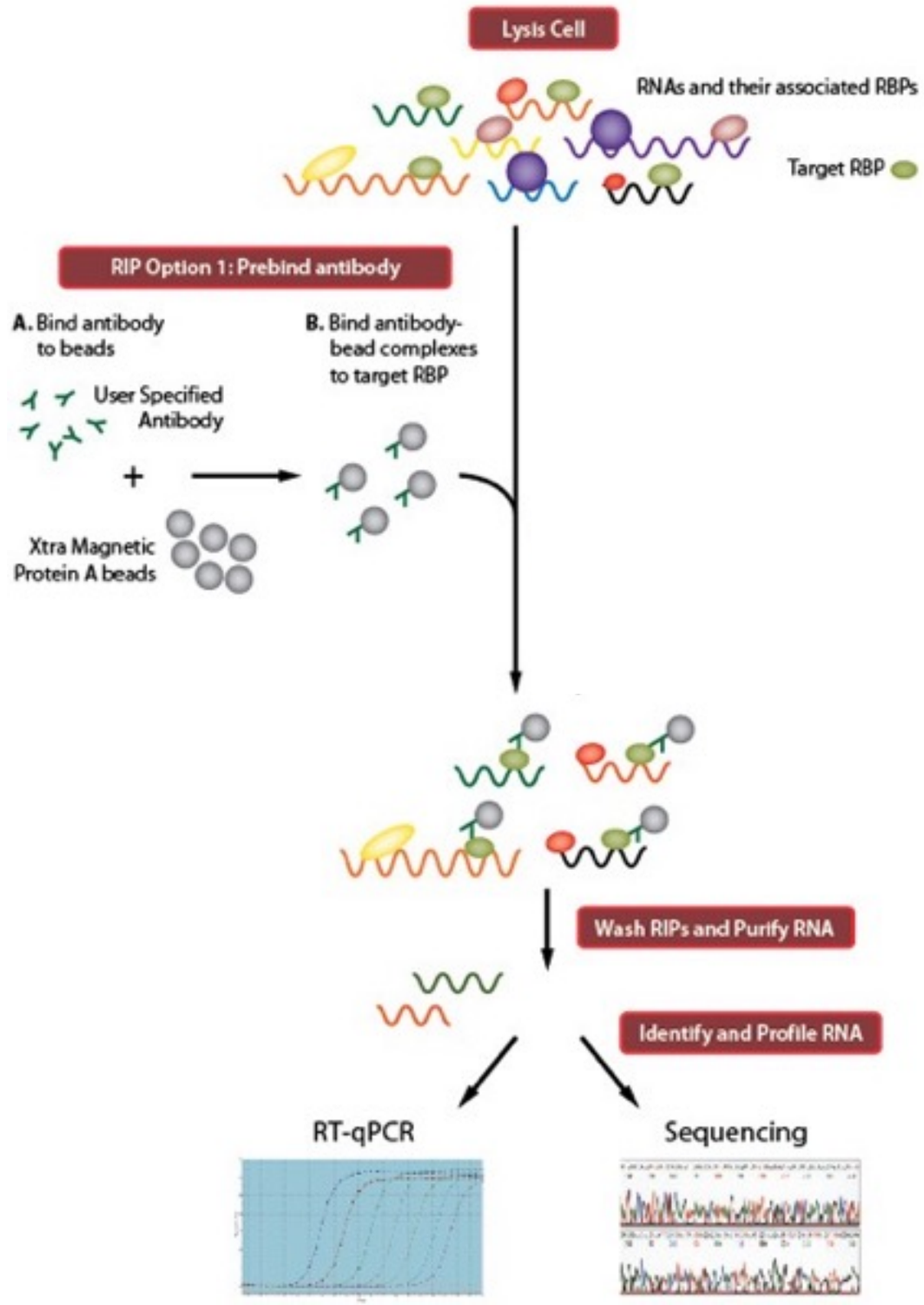
WORK FLOW

1. Lysis Cells and collect cell extract

2 Prebinding between AntiBody and Beads

3. Introduction of cellular extract (Binding step)

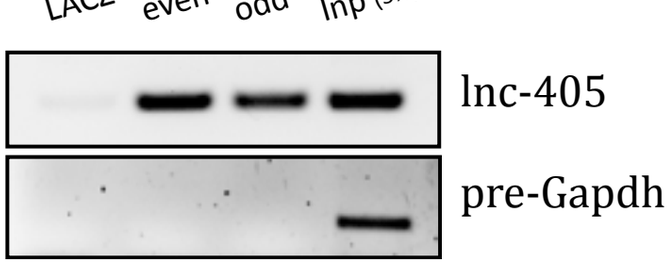
4. Wash and Purification of RNA-protein complexes

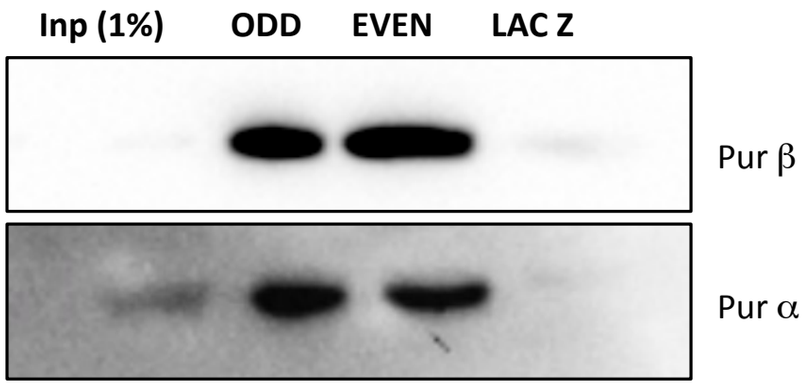


RNA Pulldwon and RIP

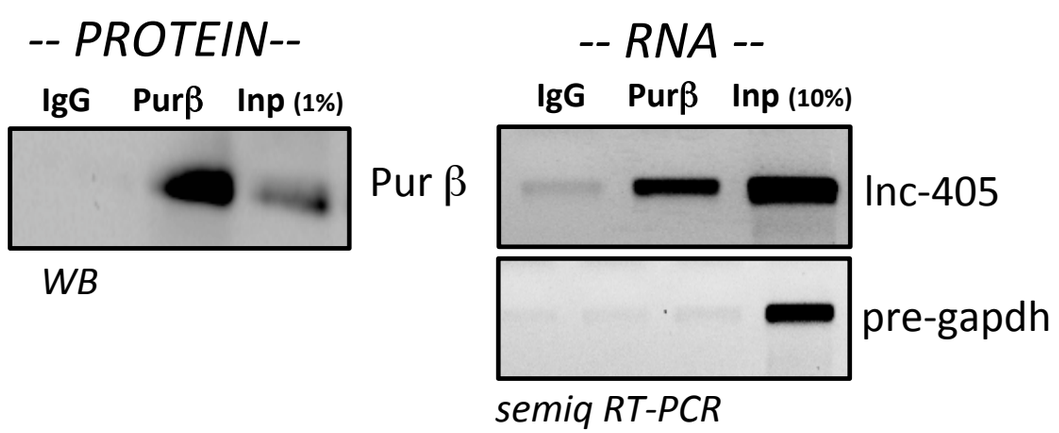
lnc-405
RNA PULLDOWN

Semiq RT-PCR





PurB
RIP



CLIP (UV-RIP)

WORK FLOW

1. UV crosslink Cells or Tissue

2. Partial RNA digestion

3. Immunoprecipitate RBP and phosphorylate 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

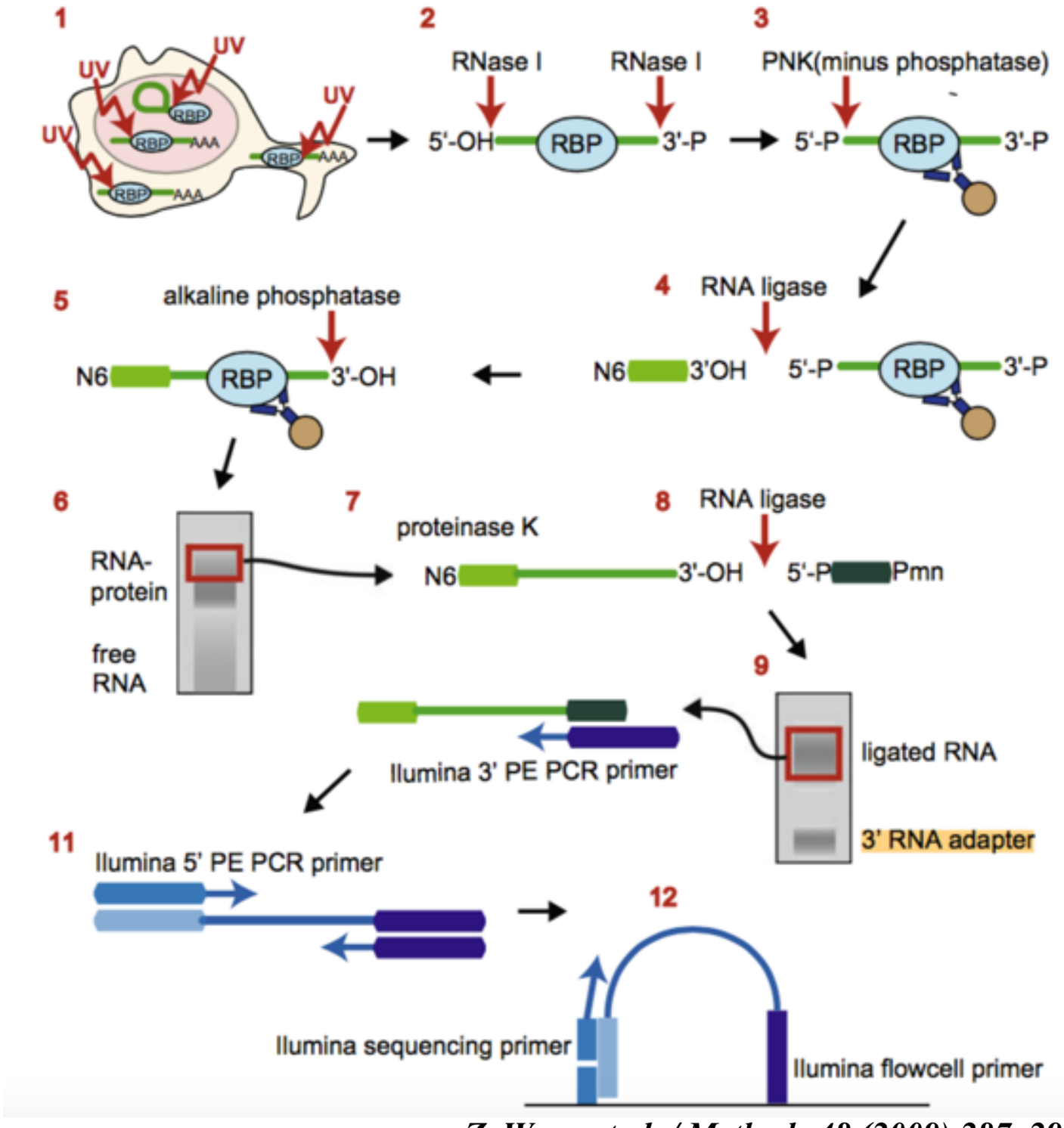
8. Ligate the 3' RNA adapter

9. Purify RNA on urea-TBE gel

10. Reverse transcription

11. PCR

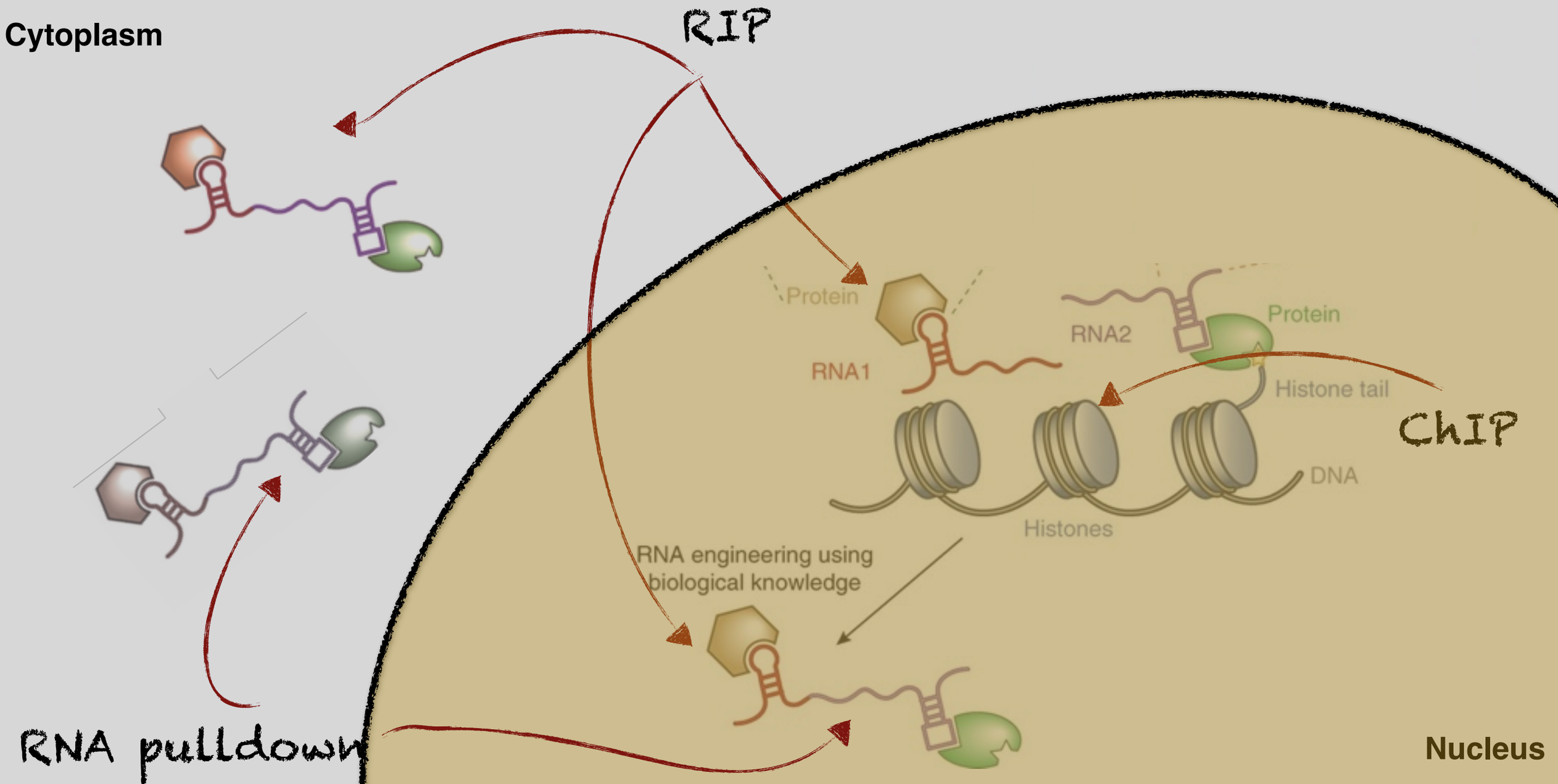
12. Illumina paired-end sequencing



Z. Wang et al. / Methods 48 (2009) 287–293

SUMMARY

	PRECIPRITATION	CHECK FOR
ChIP	PROTEIN	DNA
RNA pulldown	RNA	RNA/PROTEIN
RIP	PROTEIN	RNA/PROTEIN



THE END

Questions??

“Human mind is like a parachute, it only works when it is open” A.E.