Department of BSBE
Indian Institute Of Technology Guwahati

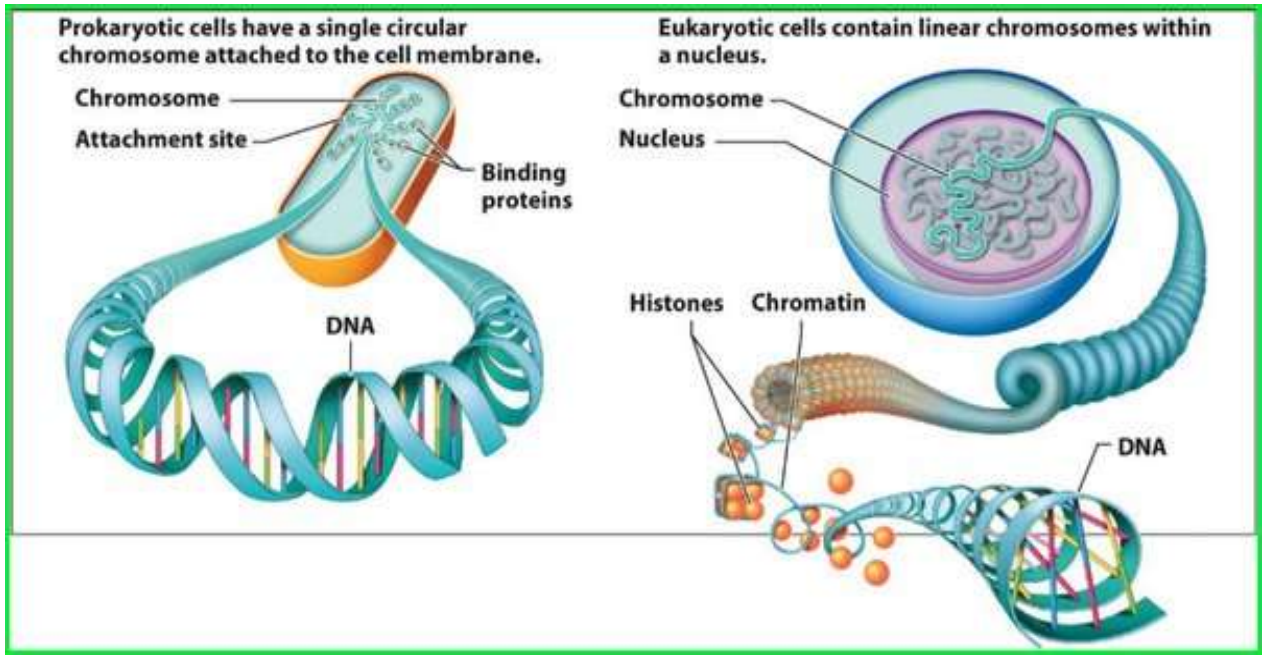
DNA/Protein Interactions: EMSA, Reporter Assays, Pull Down Assays, CHIP

Dr. Sanjukta Patra

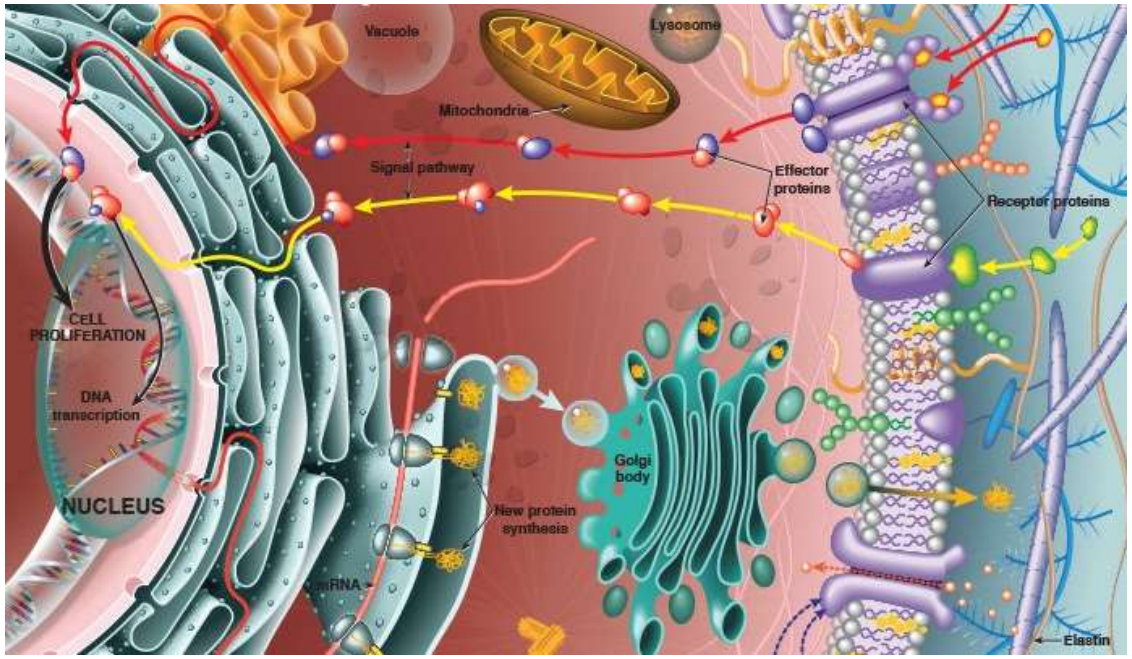
BT 207

Jan - May 2023

Location of DNA & Protein Inside Cell



Prokaryotic Cell



Eukaryotic Cell

Do DNA-Protein interact inside cell?

- Yes

Why?

DNA–protein interactions are essential for several molecular and cellular mechanisms, such as

1. transcription
2. transcriptional regulation
3. DNA modifications

What are the types of interaction(s)?

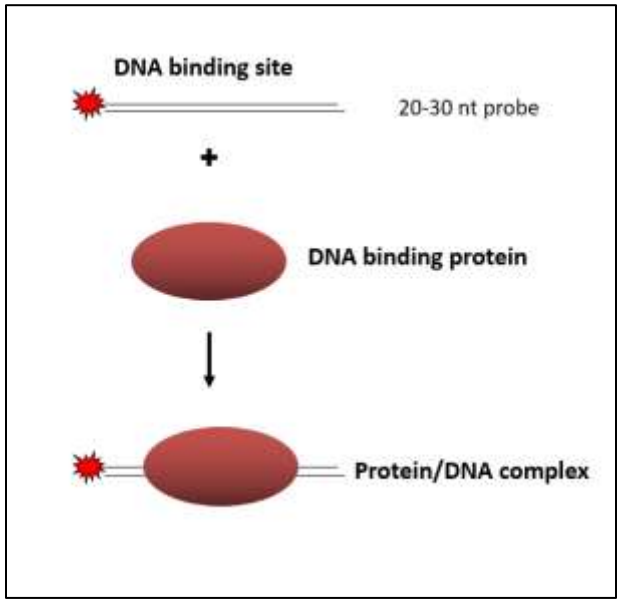
Proteins interact with DNA through

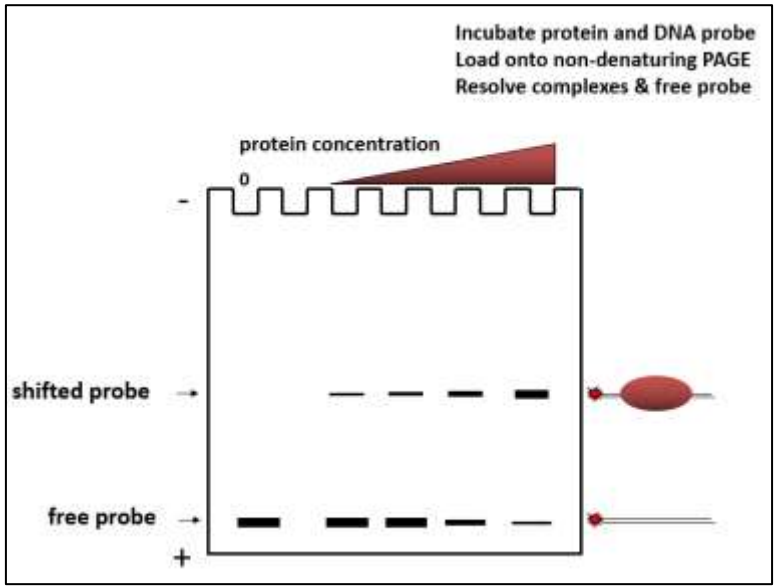
1. electrostatic interactions (salt bridges),
2. dipolar interactions (hydrogen bonding, H-bonds),
3. entropic effects (hydrophobic interactions)
4. dispersion forces (base stacking).

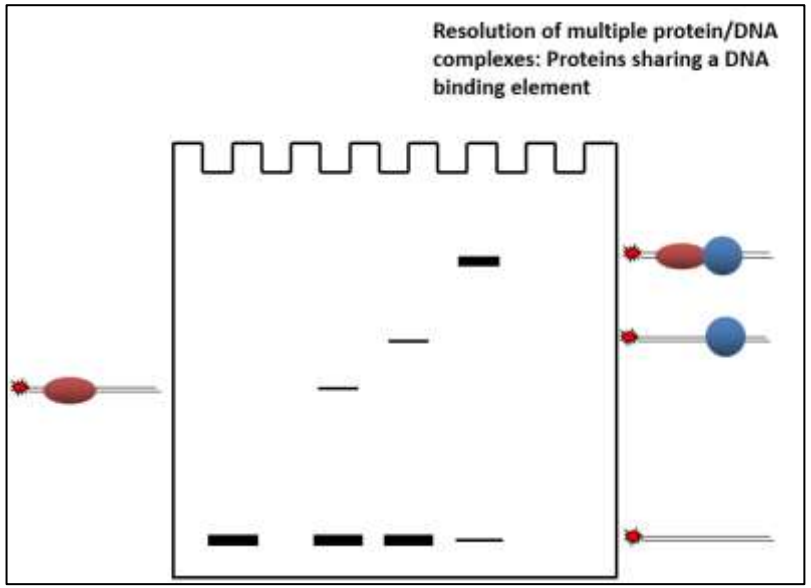
EMSA

EMSA-Electrophoretic Mobility Shift Assay

- The EMSA is used to study proteins binding to known DNA oligonucleotide probes and can be used to assess the degree of affinity or specificity of the interaction
- This technique is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected **to non-denaturing polyacrylamide or agarose gel electrophoresis**
- Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay
- Adding a protein-specific antibody to the binding components creates an even larger complex (antibody-protein-DNA) which migrates even slower during electrophoresis. The slow migration is known as a super-shift and can be used to confirm the protein identity.







EMSA: Overview

- The ability to resolve protein–DNA complexes depends largely upon the stability of the complex during each step of the procedure. During electrophoresis, the protein–DNA complexes are quickly resolved from free DNA, providing a "snapshot" of the equilibrium between bound and free DNA in the original sample.
- The gel matrix provides a "caging" effect that helps to stabilize the interaction complexes: even if the components of the interaction complex dissociate, their localized concentrations remain high, promoting prompt reassociation.
- Additionally, the relatively low ionic strength of the electrophoresis buffer helps to stabilize transient interactions, permitting even labile complexes to be resolved and analysed by this method.
- Protein–DNA complexes formed on linear DNA fragments result in the characteristic retarded mobility in the gel.
- However, if circular DNA is used (e.g., mini-circles of 200–400 bp), the protein–DNA complex may actually migrate faster than the free DNA, similar to what is observed when supercoiled DNA is compared to nicked or linear plasmid DNA during electrophoresis.
- Gel shift assays are also good for resolving altered or bent DNA conformations that result from the binding of certain protein factors. Gel shift assays need not be limited to protein–DNA interactions.
- Protein–RNA and protein–peptide interactions have also been studied using the same electrophoretic principle.

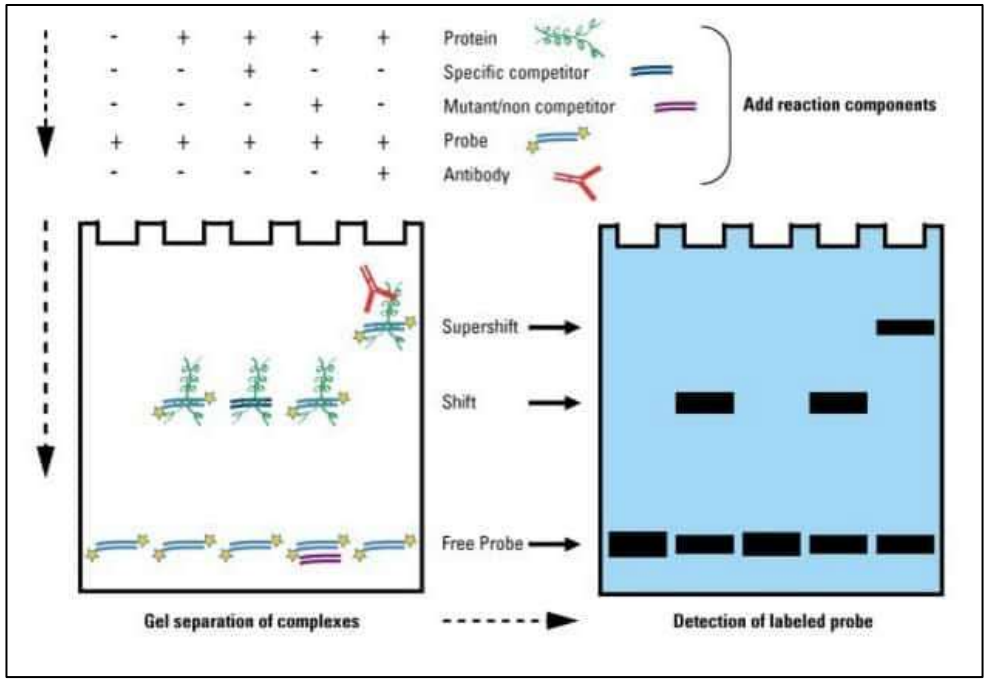


Figure: The gel shift assay consists of three key steps: (1) binding reactions, (2) electrophoresis, (3) probe detection. The order of component addition for the binding reaction is often critical. Completed binding reactions are best electrophoresed immediately to preserve potentially labile complexes for detection. This idealized example shows complete elimination of the protein–probe complex with the addition of a specific competitor or protein-specific antibody. However, only a reduction in intensity is observed rather than the complete elimination of bands.

EMSA: Application(s)

- Detect low abundance DNA binding proteins from lysates.
- Test binding affinity through DNA probe mutational analysis.

EMSA: Limitation(s)

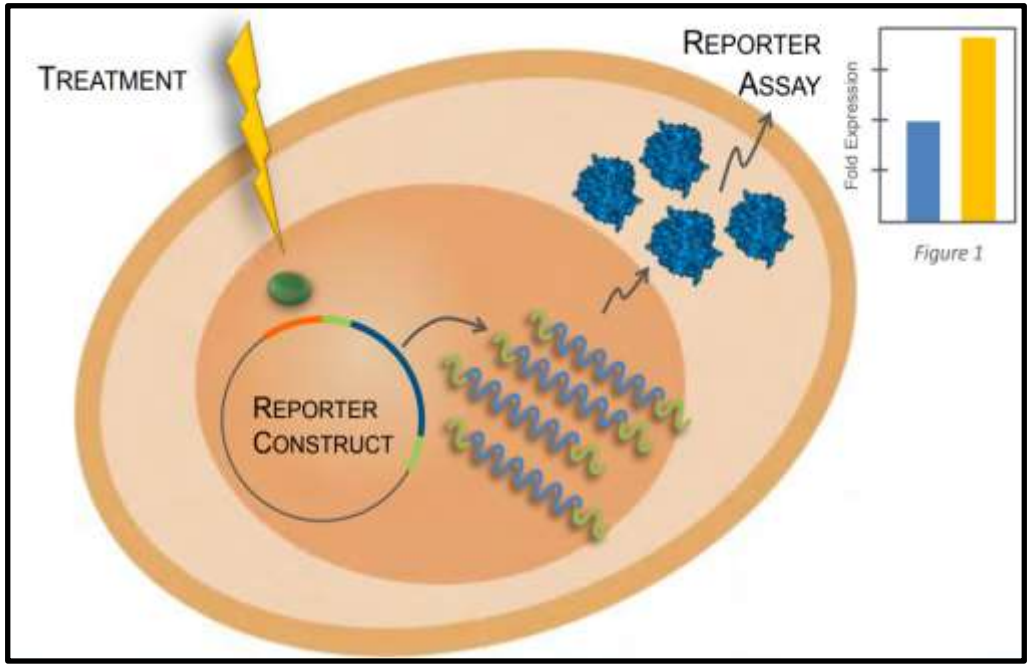
- Difficult to quantitate.
- Need to perform super-shift assay with antibody to authenticate protein identity in a complex(High cost).

Reporter Assays

Reporter Assay:

What is a Reporter?

- Genetic reporters are indicators of gene expression or cellular events coupled to gene expression.
- Reporters can mark any gene product:
  - Transcriptional Fusion – Reports on transcriptional and post-transcriptional regulatory inputs & events.
  - Translational Fusion – Reports on post-translational regulatory inputs & events.
- Reporters may be used in cells, tissues, or whole organisms.



Principle:

- Reporter gene assays are used to measure/locate ability of an unknown DNA-sequence.
- This is done by linking the unknown promoter sequence to an easily detectable reporter gene whose product can be easily detected and quantifiably measured.

S.No	**Gene name**	**Gene product**	**Assay**
1	*lacZ*	β-galactosidase	Enzyme assay
2	gfp	Green fluorescent protein	Fluorescence/Microscopy
3	luc	Luciferase enzyme	Fluorescence/Microscopy

Reporters Can Be Used For A Variety Of Applications

Gene expression:

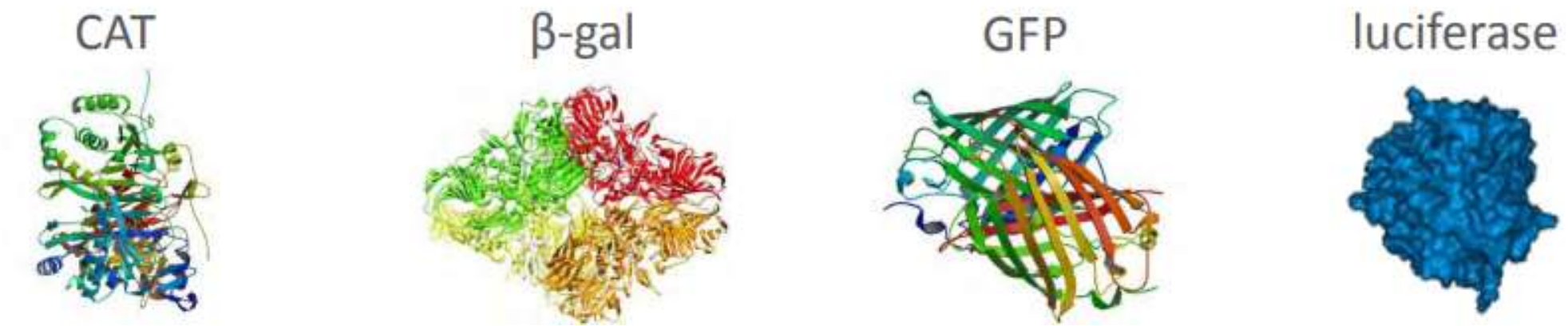
- Transcription and post-transcriptional regulation
  - Promoters/response elements
  - Enhancers
  - 5’- and 3’-UTRs
  - Transcriptions factors
  - RNA binding proteins & miRNAs
- **Post-translational regulation**
  - Protein stability
  - Protein localization
  - Protein: Protein interactions

Cellular Events:

- Receptor activation/signalling
  - Receptor ligands, agonists & antagonists
  - Nuclear receptors
- **Pathway analysis**
  - Defining pathways
  - Protein: Protein interactions
- **Disease/Immune responses**
  - Cellular response to infection
  - Cellular response to therapy
  - Infectious agent replication/response to therapy

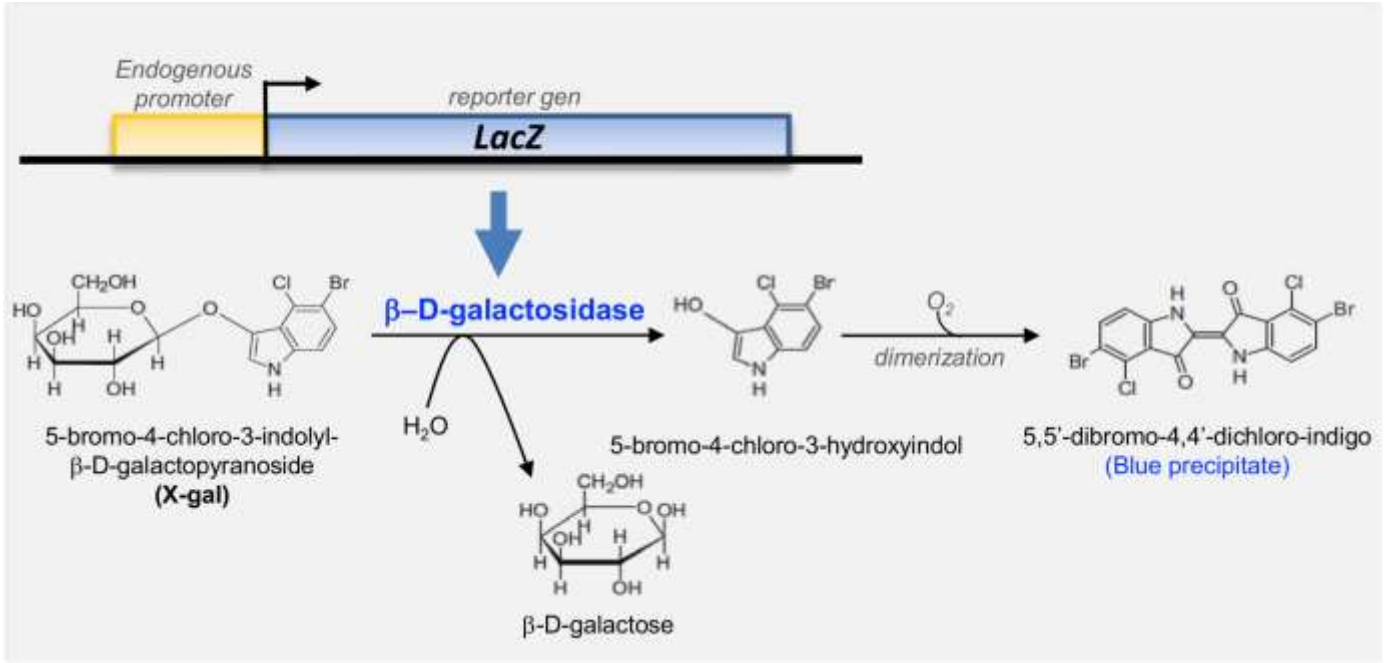
Properties Of A Good Reporter

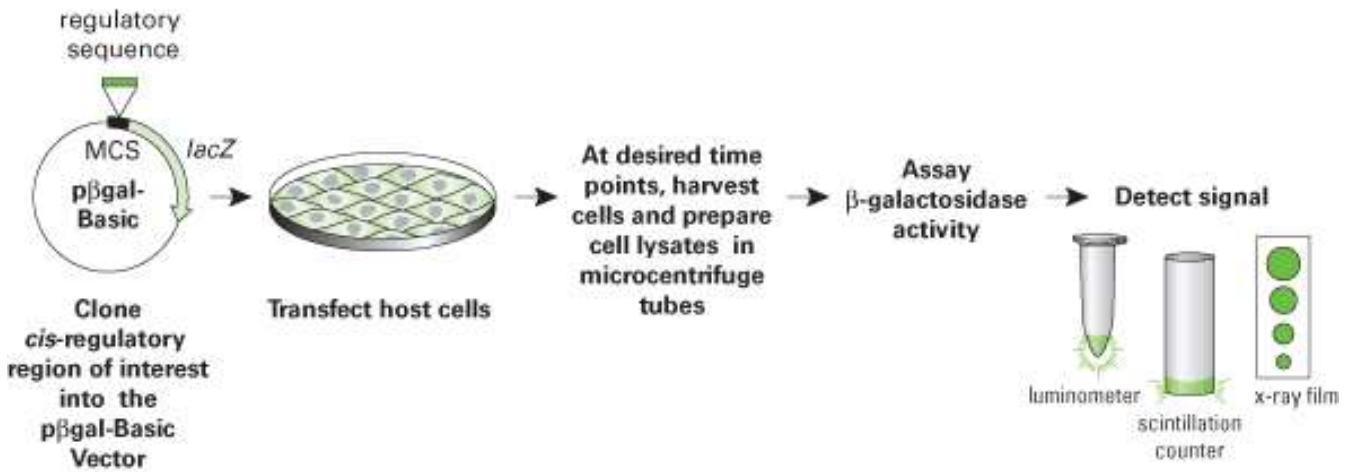
- A good reporter should have:
  - Enzyme - signal amplification.
  - Active upon synthesis - no processing or assembly
  - No endogenous analog (protein or substrate) – low background
  - Convenient assay
  - Quantitative, sensitive & wide dynamic range



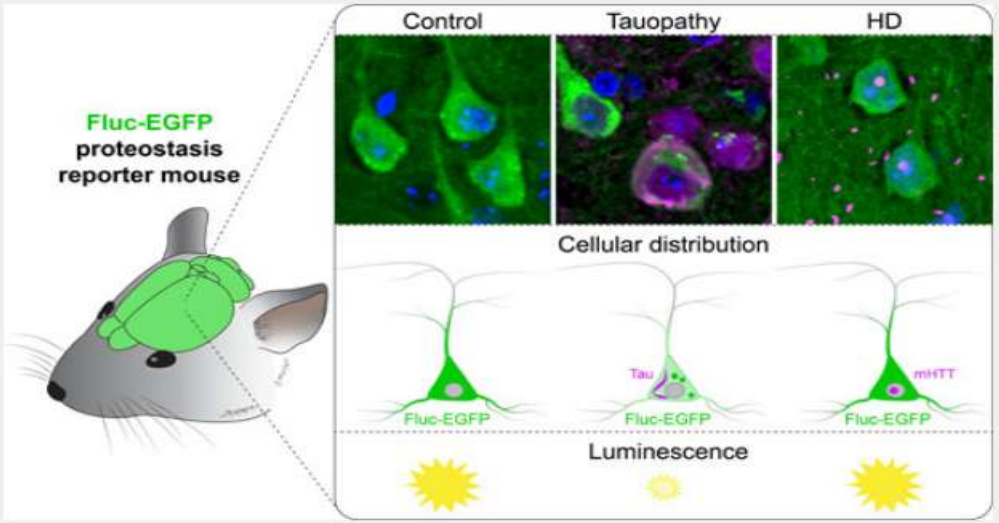
*Chloramphenicol acetyltransferase (CAT); Beta-galactosidase (β-gal); Green fluorescent protein (GFP)

Beta-galactosidase as a Reporter





GFP as a Reporter



This study describes a new reporter mouse line for monitoring neuronal proteostasis. The reporter reveals that protein aggregates associated with neurodegenerative diseases differ in their impact on the cellular protein quality control system.

- Fluc-EGFP reporter mouse allows studying neuronal proteostasis alterations in aging and disease.
- Fluc-EGFP reporter detects proteostasis impairments in tauopathy mice, but not in Huntington's disease mice.
- Mechanistic studies in cultured neurons show that different aggregating proteins cause distinct cellular compartment-specific defects of proteostasis.

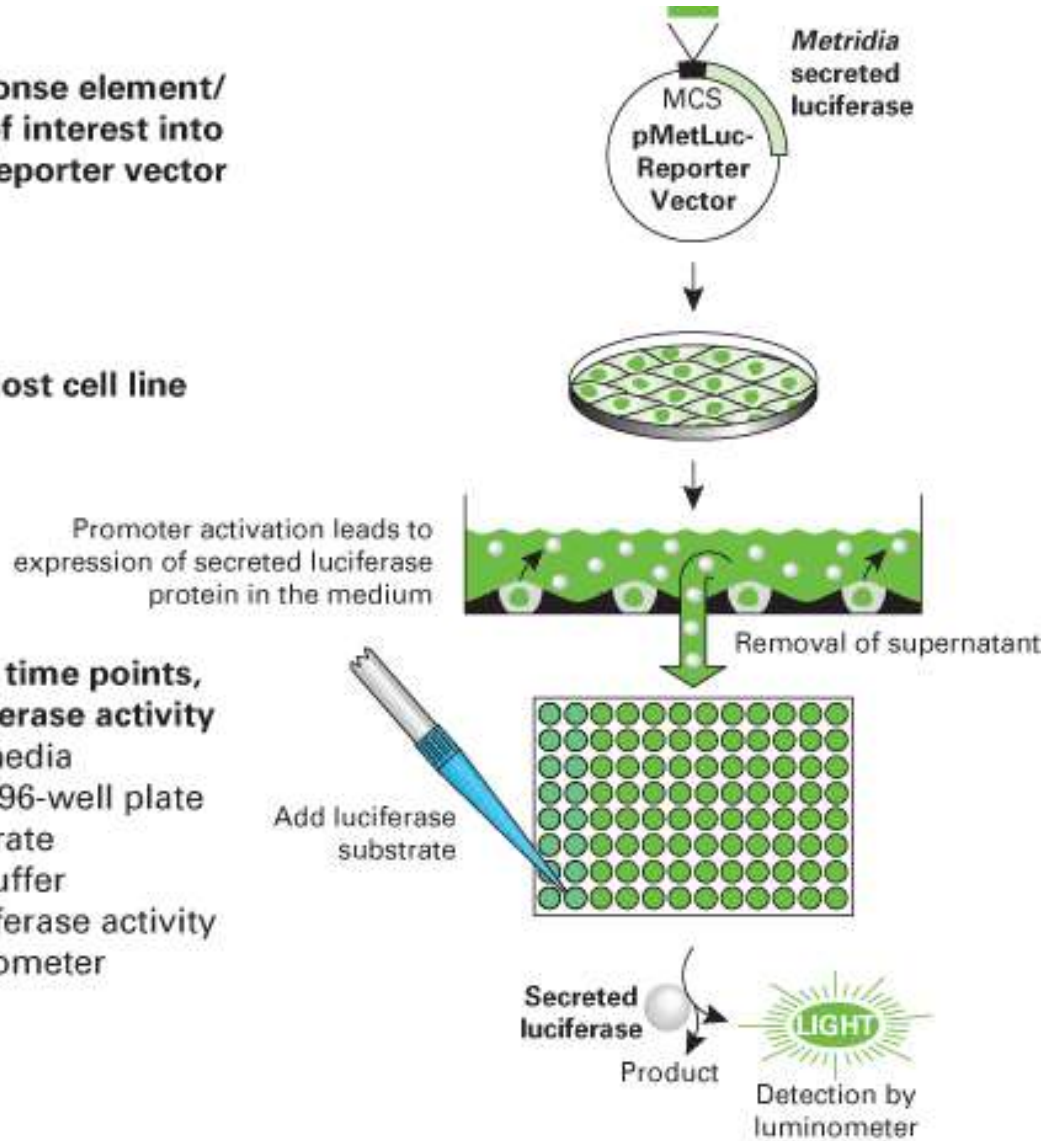
Paper Link: https://www.embopress.org/doi/full/10.15252/embj.2020107260

Luciferase as a Reporter

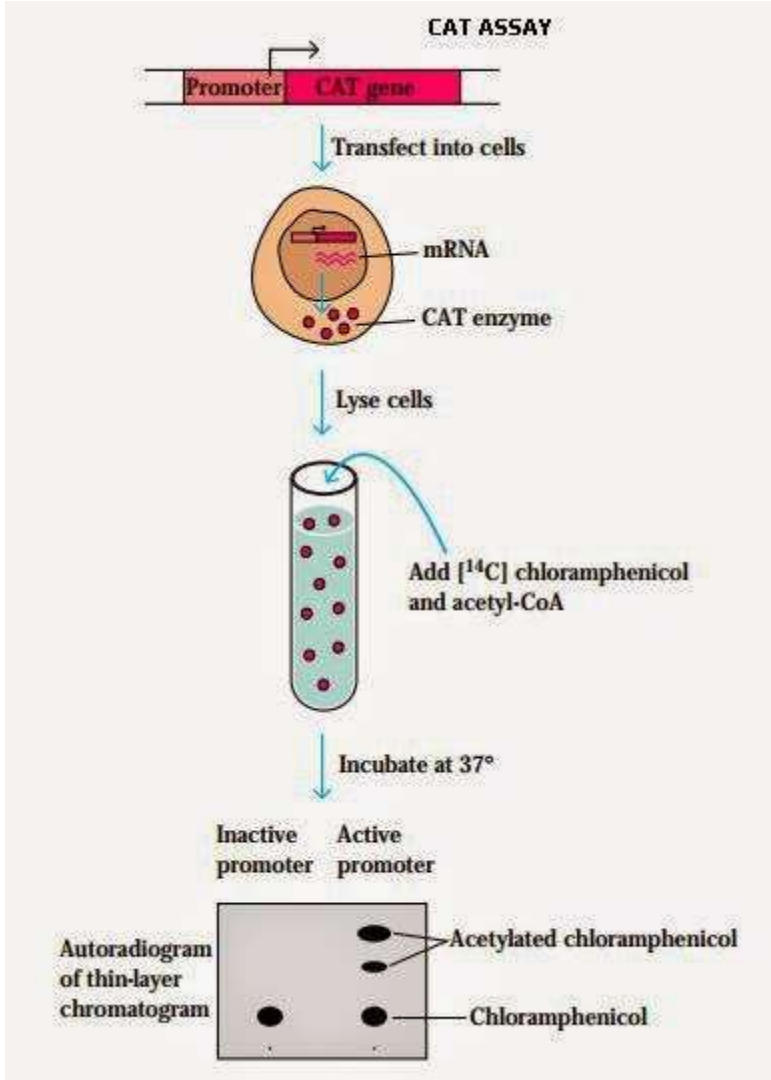
1. **Clone response element/ promoter of interest into pMetLuc-Reporter vector**

2. **Transfect host cell line**

3. **At desired time points, assay luciferase activity**
   - Transfer media sample to 96-well plate
   - Add substrate reaction buffer
   - Assay luciferase activity in a luminometer



CAT as a Reporter

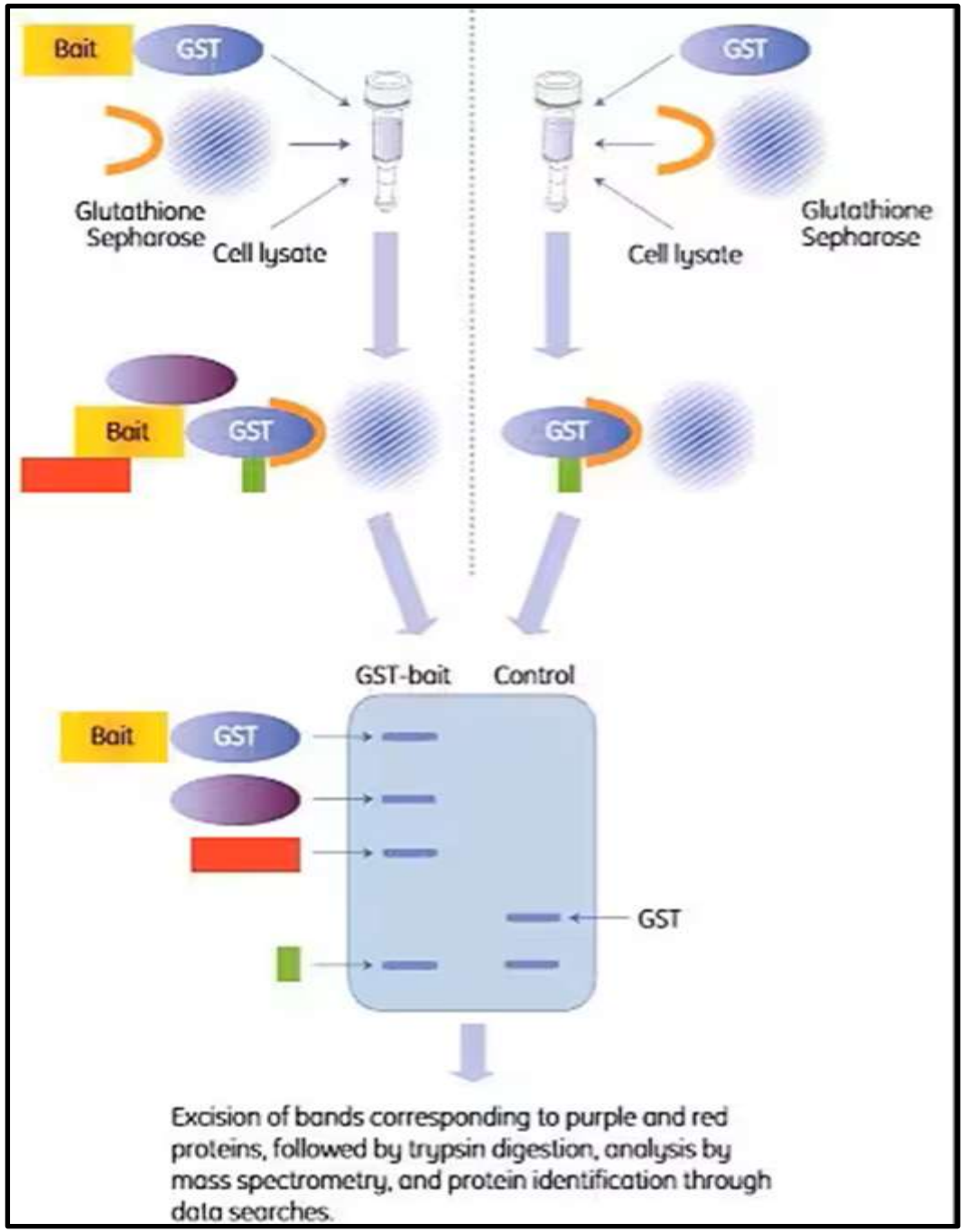


Pull Down Assay

- The pull-down assay is an in vitro method used to determine a physical interaction between two or more proteins.
- Pull-down assays involve isolation of a protein complex by adsorbing the complex onto beads.
- Immobilized ligands on the beads bind specifically to a component of the complex, either via an affinity tag (e.g., GST, histidine, maltose binding protein, etc.) or an antibody.
- Pull-down assays are often used for the isolation of low µg amounts of complexes, mainly for the purpose of identifying individual subunits.
- They have also been used to isolate material for limited functional studies, but other methods appear more suitable for the production of larger (mg) amounts. The components of the captured complex are often eventually analyzed by mass spectrometry to identify the subunits.
- Pull-down assays are important tools for mapping protein-protein interaction networks.

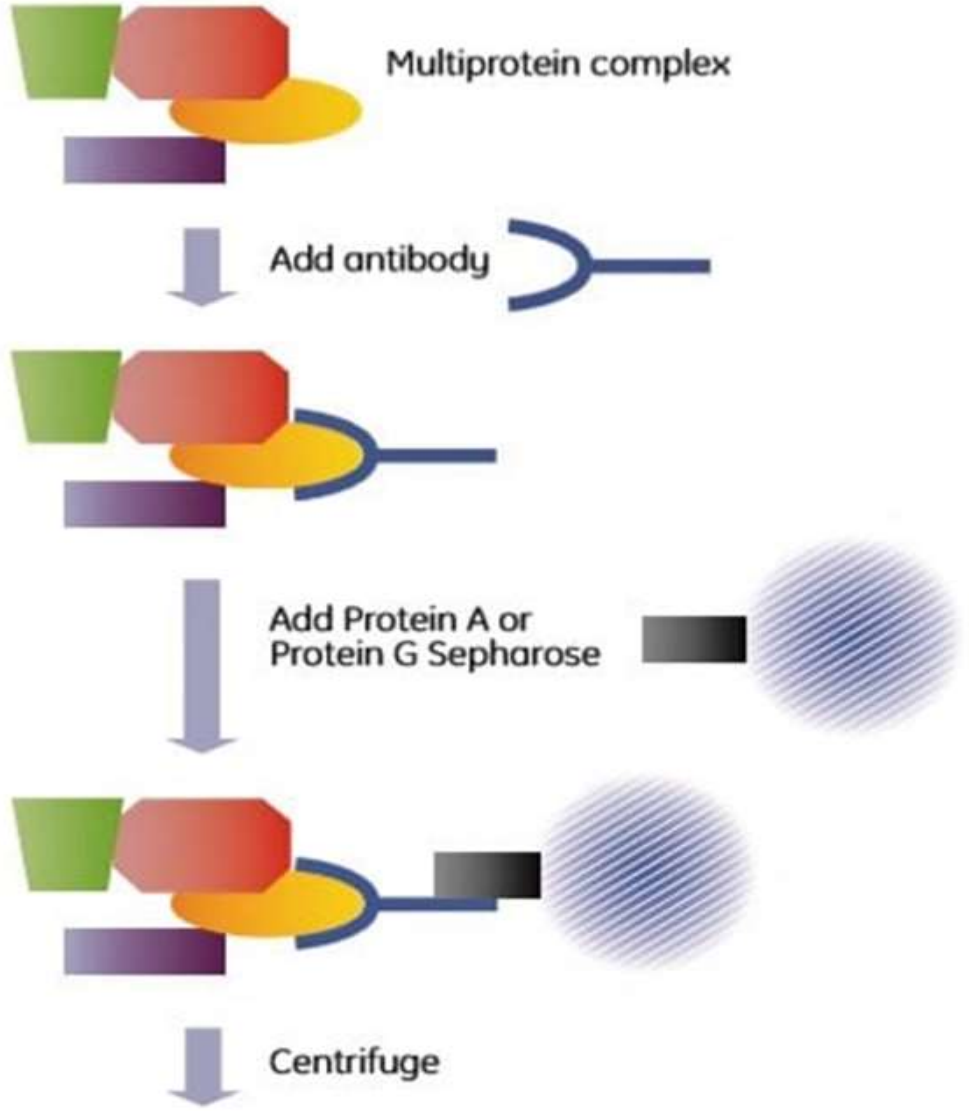
Pull Down Assay: Overview

- Pull-down assays are a form of affinity purification and are similar to immunoprecipitation, except that a "bait" protein is used instead of an antibody.
- Affinity chromatography (i.e., affinity purification) methodologies greatly enhance the speed and efficiency of protein purification and simultaneously provide the technology platform to perform a pull-down, or co-purification, of potential binding partners.
- In a pull-down assay, a bait protein is tagged and captured on an immobilized affinity ligand specific for the tag, thereby generating a "secondary affinity support"' for purifying other proteins that interact with the bait protein.
- The secondary affinity support of immobilized bait is then incubated with a protein source that contains putative "prey" proteins, such as a cell lysate.
- The source of prey protein at this step depends on whether the researcher is confirming a previously suspected protein–protein interaction or identifying an unknown interaction.
- The method of protein elution depends on the affinity ligand and ranges from using competitive analytes to low pH or reducing buffers.



CO-IMMUNOPRECIPITATION

- Immunoprecipitation is a well-established technique that uses antibodies to isolate antigens from crude biological samples.
- The name originates from analysis methods based on the precipitation reaction obtained when mixing antibody and antigen at the correct ratio.
- The method described here is a pull-down assay rather than a precipitation reaction.
- Co-immunoprecipitation uses antibodies directed towards one (known or supposed) component of a complex.
- The antibody binds to its antigen, which is part of a multiprotein complex. The antibody-protein complex assembly is then captured through the addition of protein A or protein G Sepharose beads to the mixture.



Elution and separation with SDS-PAGE followed by trypsin digestion, analysis by mass spectrometry and protein identification through database searches.

Method	Advantages	Disadvantages
Affinity pull-down	Generic Ability to purify low-abundant protein complexes	The presence of a protein tag may influence results Competition with the endogenous complex
Tandem affinity purification (TAP)	Generic Ability to purify low-abundant protein complexes Mild conditions used throughout	The presence of a protein tag may influence results Competition with the endogenous complex
Co-immunoprecipitation	Does not require cloning and heterologous expression. Rapid if antibody is available	Not generic - requires access to specific antibodies

Table: Advantages and disadvantages with different pull-down assays for the retrieval of protein complexes

CHIP-Chromatin Immunoprecipitation

Structure and function of chromatin

- In eukaryotes DNA is found in vivo in complex with proteins and RNA. It is divided between heterochromatin (highly condensed) and euchromatin (less extended).
- The major components of chromatin are DNA and histone proteins, although many other chromosomal proteins have prominent roles too.
- The fundamental unit of chromatin is the nucleosome, which consists of 2 copies each of H2A, H2B, H3 and H4 histones, and approximately 147 bp of DNA wrapped almost two times around the octamer.
- The function of chromatin is to package DNA to enable it to fit in the cell, strengthen DNA to assist with mitosis and meiosis, and serve as a mechanism to control gene expression, DNA repair, and DNA replication. Histone proteins play an important role in the regulation of these processes.

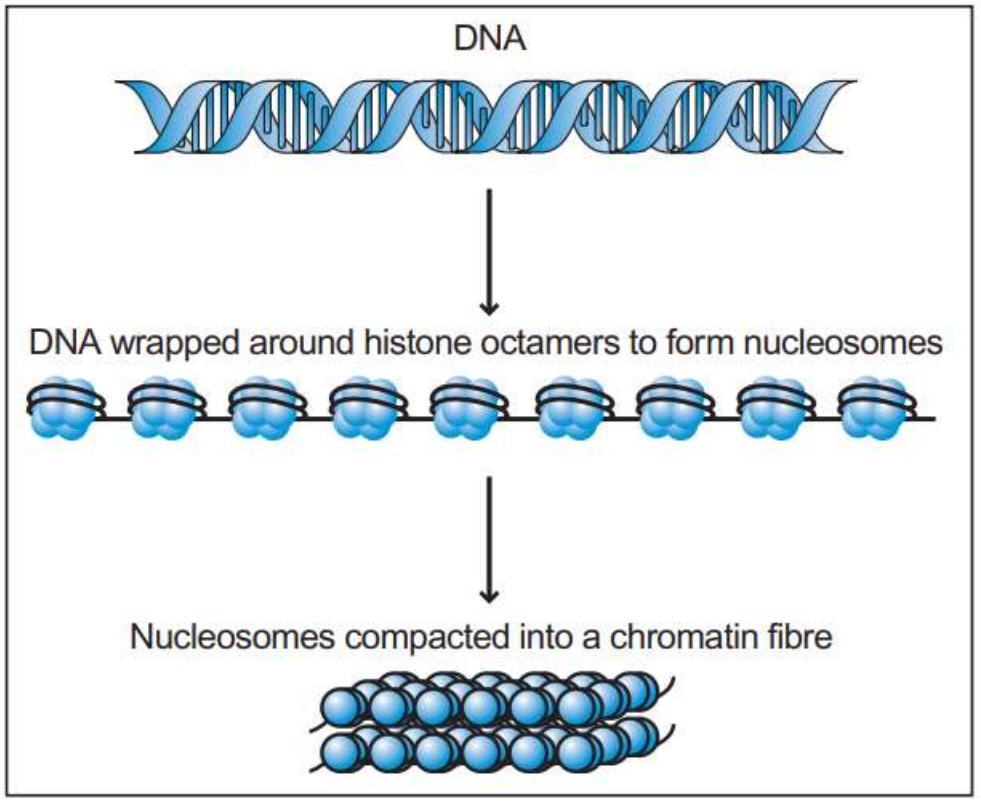


Figure: DNA wraps around histone proteins to form nucleosomes; these in turn couple to become the chromatin fiber

- A large number of residues found on the histones can be covalently modified with chemical groups by processes such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation and deimination (Kouzarides et al, 2007).
- Furthermore, cis-trans proline isomerisation while not strictly a modification results in a conformational change (Nelson et al, 2006). These modifications function either by disrupting chromatin contacts or by affecting the recruitment of non-histone proteins to chromatin (Kouzarides et al, 2007). A number of enzymes have been identified which catalyze the addition of these modifications to histone proteins.

History

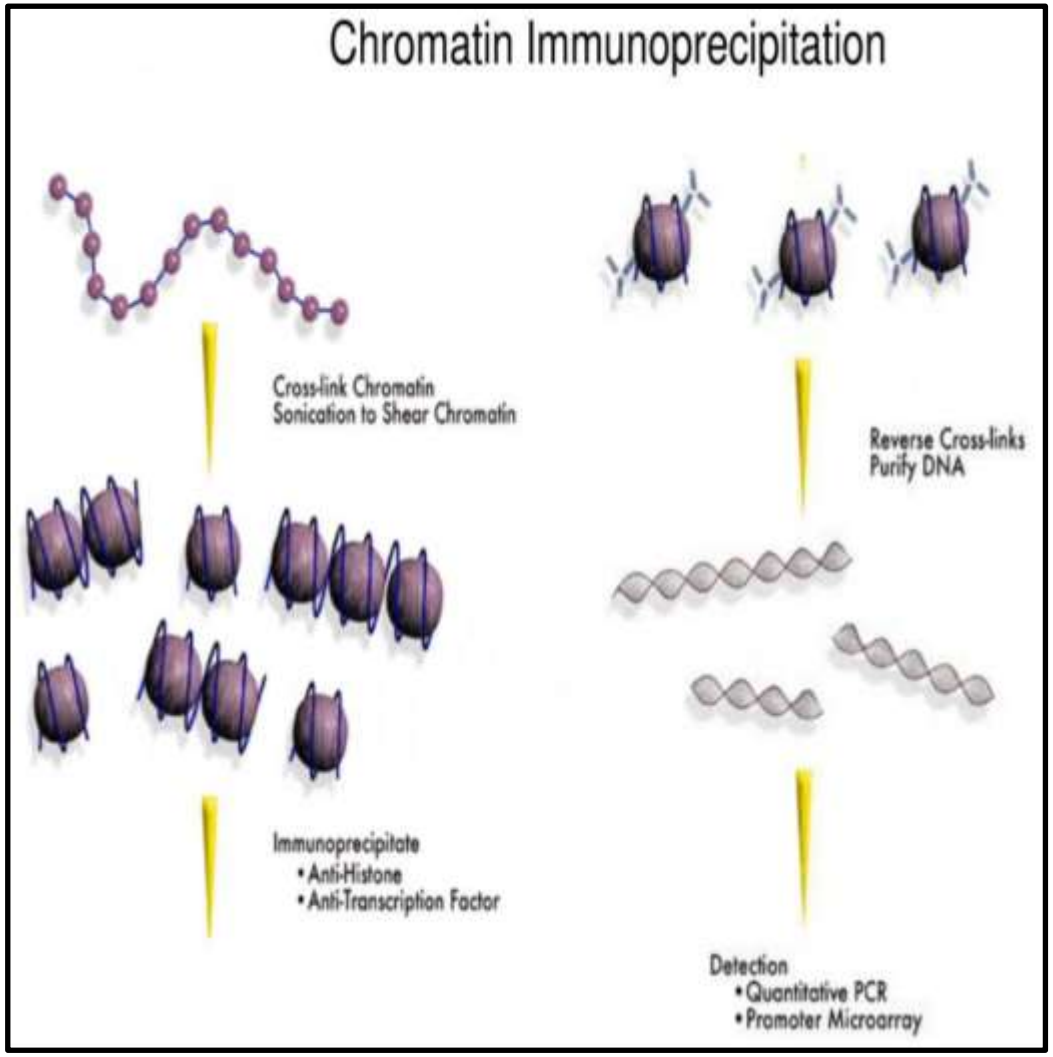
- The technique now referred to as ChIP - 1980’s by Gilmour and colleagues.
- They demonstrated an association of RNA polymerase II and topoisomerase I with active genes in Drosophila cells (Gilmour and Lis, 1985; Gilmour et al., 1986).
- The first account of an antibody against a histone modification being used in ChIP was in 1988 by Hebbes et al. An antibody recognizing N-acetyl-lysine was used to immunoprecipitate nucleosomes containing acetylated histones from 15-day-old chicken erythrocyte nuclei.
- The precipitated chromatin was then probed with β-globin and ovalbumin DNA sequences.
- Specific enrichment of the ß-globin locus, but not the ovalbumin gene, demonstrated a link between histone acetylation and an active transcriptional state in vivo (Hebbes et al., 1988)

What Is Chromatin Immunoprecipitation?

- Not only do proteins interact with one another, they can also interact with DNA. Chromatin immunoprecipitation (ChIP) is a technique that determines whether a protein of interest interacts with a specific DNA sequence.
- This technique is often used to study the repertoire of sites on DNA that are bound by particular transcription factors or by histone proteins,
- and to look at the precise genomic locations of various histone modifications (including acetylation, phosphorylation, or methylation).

CHIP: Working

- ChIP can be used to examine the presence of protein-DNA interaction at steady state, or to quantify changes in interaction at specific phases of the cell cycle, or following a treatment of interest.
- Protein and associated chromatin are temporarily cross-linked in live cells or tissues (using formaldehyde or UV) and sheared using enzymatic digestion or sonication to yield ~300-1000 bp fragments of DNA.



- The protein of interest, along with any associated DNA fragments, is immunoprecipitated from the cell debris using a specific antibody.
- The cross-link is then reversed and DNA fragments are purified.
- The amount of eluted DNA can be assessed through quantitative real-time PCR (qRT-PCR) using primers flanking the genomic locus of interest.
- DNA amplification is an indication of enrichment in binding of the protein of interest.

Map proteins/histone modifications to genomic loci

Chromatin and associated proteins.

Cross-link

Start with 2 X 150 cm^2 dishes of confluent cells
(1 X 10^7 - 5 X 10^7 cells per dish)
Add formaldehyde to a final conc of 0.75%
Incubate 2 - 30 min RT
Add glycine to a final conc of 125 mM
Shake gently for 5 min

Cross-linking fixes proteins to DNA.

Cell collection

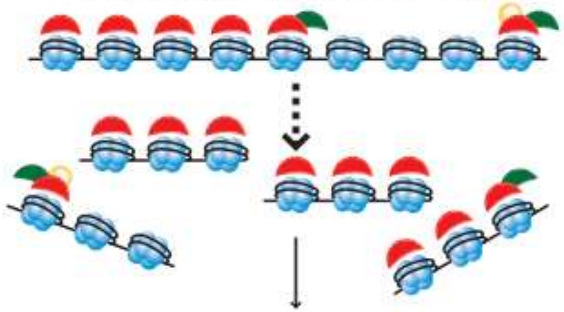
Wash cells with ice cold PBS
Scrape and collect cells into 5 ml ice cold PBS and transfer to a new tube
Wash dishes with 3 ml PBS to ensure all cells are collected

Cell lysis

Centrifuge 5 min 1,000 g and remove supernatant
Add FA lysis buffer to cell pellet (750 µl per 1 X 10^7 cells)

Sonication

Sonicate to give a fragment size of 200 - 1000 bp



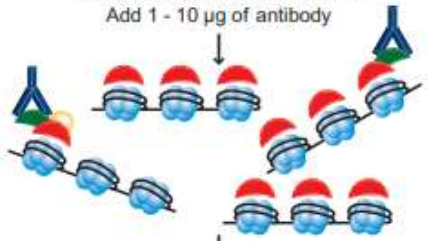
Sonication generates sheared, soluble chromatin. Optimize by performing a time course. Purify DNA, and analyse fragment size on 1.5 % agarose gel.

Centrifuge 30 sec 4˚C 8,000 g to pellet cell debris
Transfer supernatant containing chromatin to a new tube

Remove 50 µl (INPUT) and purify DNA to calculate the DNA concentration

Immunoprecipitation

Use an amount of chromatin equivalent to approximately 25 µg of DNA per IP
Dilute 1:10 with IP Dilution buffer
Add 1 - 10 µg of antibody



Antibody binds to target and associated DNA is isolated. DNA fragments not associated are removed during washes.

(Continued Overleaf)

Add 20 µl of protein A/G beads
Rotate overnight 4˚C
Centrifuge 1 min 2,000 g
Wash 3X with wash buffer
Wash 1X with final wash buffer

Elute protein / DNA complex

Add 120 µl of Elution Buffer to the protein A/G beads
Rotate 15 min 30˚C
Centrifuge 1 min 2,000 g
Transfer supernatant containing protein/DNA complex to a new tube

Reverse cross-links and purify DNA

EITHER

PCR purification kit

Add 2 µl RNase A (0.5 mg/ml)
Heat 65˚C 4 - 5 hr

Purify DNA using PCR purification kit according to manufacturer's instructions

Phenol:chloroform extraction

Add 5 µl proteinase K (20 mg/ml)
Heat 65˚C 4 - 5 hr

Extract DNA using phenol:chloroform
Precipitate with ethanol and 10 µl glycogen (5 mg/ml)
Resuspend pellet in 100 µl H_2O

DNA Analysis

Analyze DNA isolated by:
quantitative PCR, sequencing (ChIP-seq), microarray (ChIP-chip).

Modified ChIP Techniques

DNA fragments purified by ChIP can be utilized for a number of downstream analysis techniques. Furthermore, the basic ChIP protocol described above can be modified to answer additional biological questions.

- ChIP-on-chip: Genome-wide analysis of protein binding sites using microarray analysis of purified DNA fragments.
- ChIP-Seq: Genome-wide analysis of protein binding sites using deep sequencing of purified DNA fragments.
- Native ChIP: Omits the cross-linking step and uses micrococcal nuclease digestion to cut DNA at histone linkers to examine the DNA target of histone modifying proteins.
- ChIP-exo: Addition of an exonuclease digestion step to obtain increased resolution of protein binding sites, up to a single base pair.
- ChIA-PET (chromatin interaction analysis by paired-end tag sequencing): A technique that combines the principles of ChIP with chromosome conformation capture (3C) to detect long-range chromatin interactions mediated via a protein of interest.
- iChIP (indexing-first chromatin immunoprecipitation): A high-sensitivity technique that reduces the number of cells required for a ChIP experiment by initially barcoding total cellular chromatin.
- enChIP (engineered DNA-binding molecule-mediated chromatin immunoprecipitation): A technique which employs the CRISPR/Cas9 system to target specific genomic regions. A guide RNA complementary to the desired genomic region is expressed in combination with a tagged, enzymatically inactive Cas9 protein. ChIP is then performed using an antibody against the modified Cas9. This technique can help evaluate cis- and trans-interacting chromosomal looping events.
- RIP-Chip/RIP-Seq: Similar techniques used to analyze protein-RNA interactions.

Limitations of ChIP

- As with all molecular biology techniques, ChIP is not without its own set of limitations. ChIP assays often yield low signals as compared with controls, leading to inconclusive data.
- The assay is limited to a resolution relative to the size of the DNA fragments generated following shearing, which makes it difficult to determine the exact binding site of a protein.
- While ChIP will infer the presence of a protein at a given genomic locus, it cannot determine functional significance of the protein's binding at that DNA region.
- Cross-linking may additionally include proteins that transiently interact with DNA or DNA-binding proteins and have no functional significance. Similarly, DNA interactions of proteins with short residence time (as little as several seconds for some transcription factors) may not be fully captured.
- Additionally, interacting proteins may mask the epitope of the protein of interest. Finally, the ChIP technique is extremely dependent on the quality and specificity of the antibody employed and may not discriminate between different DNA-binding protein isoforms.