Recombinant DNA-based molecular techniques

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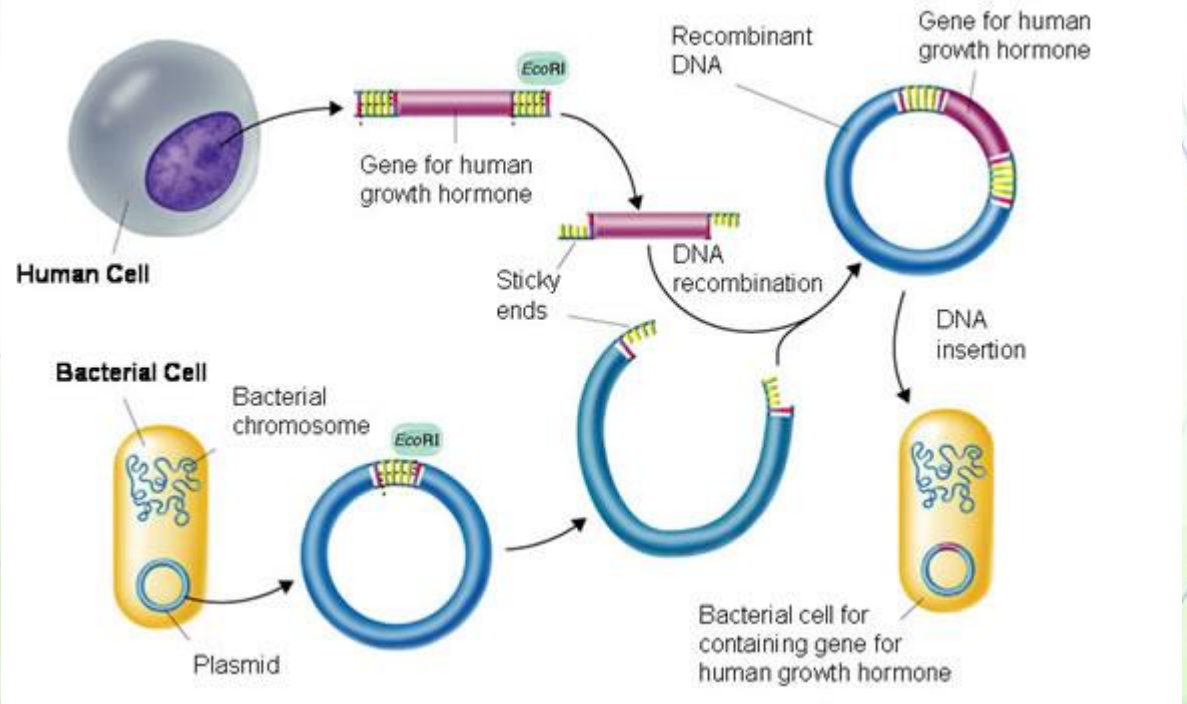
Cloning vectors

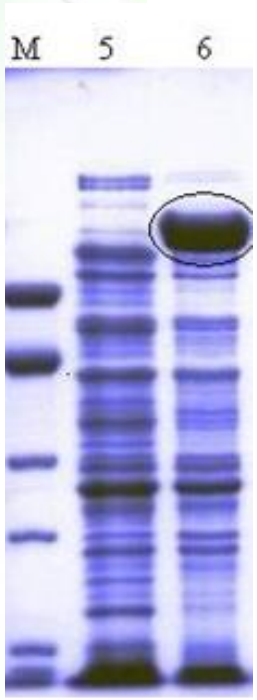
- Cloning plasmid vectors contain at least three essential parts required for DNA cloning:
  - Can replicate
  - Includes selectable marker-antibiotic resistance gene
  - Can insert a foreign DNA fragment

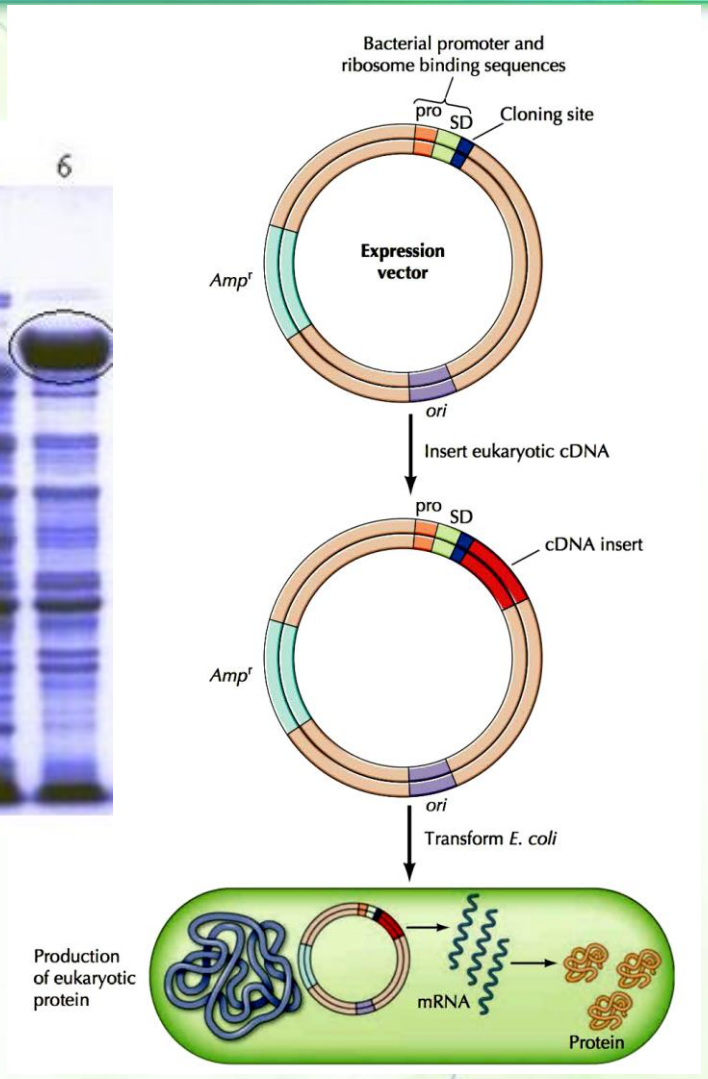


Expression vectors

- Expression vectors contain
  - Promoter sequences upstream of gene to be inserted
  - Ribosomal binding sequences (Shine-Dalgarno [SO] sequences
  - An insertion (cloning) site
  - A selectable marker (antibiotic resistance gene)
  - An Origin of replication

- The protein is expressed and purified.
- *Examples: insulin, growth hormone, plasminogen activator, erythropoietin*

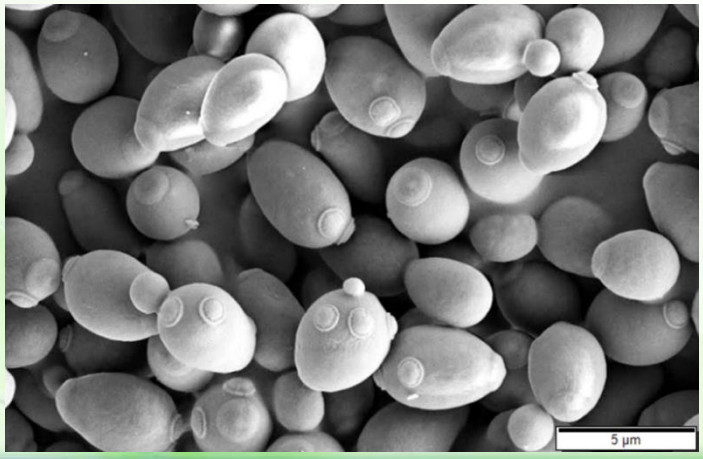




Challenges

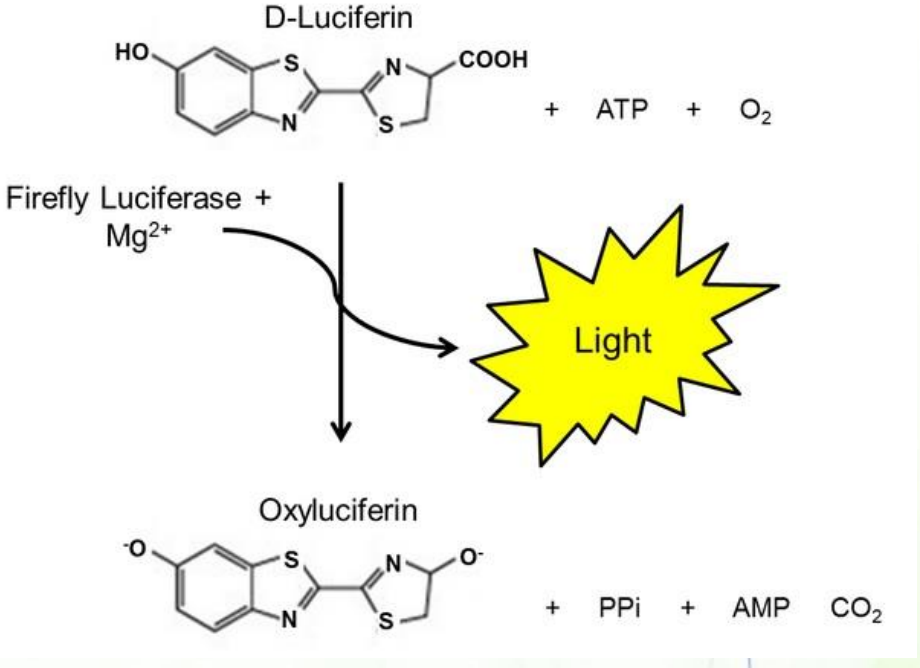
- Internal disulfide bonds in bacteria
- No post-translational modification (example: glycosylation)
- Misfolding
- Degradation

- Solution: use a eukaryotic system such as yeast



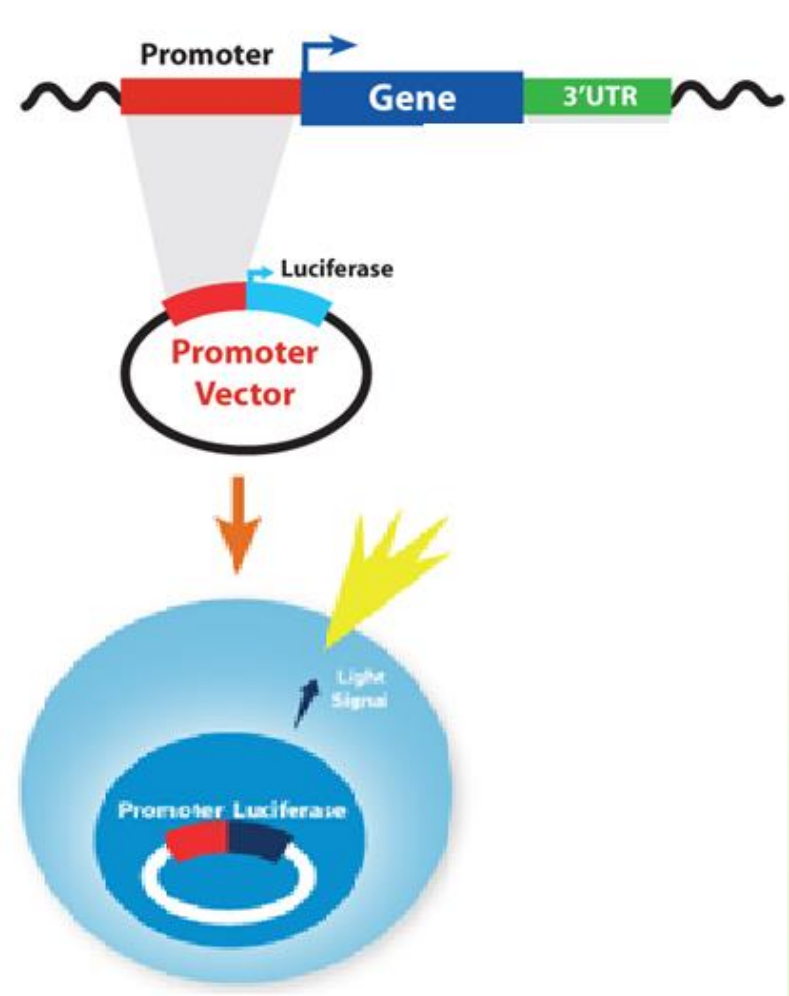
Promoter analysis:
Role of enzymes

Firefly luciferase

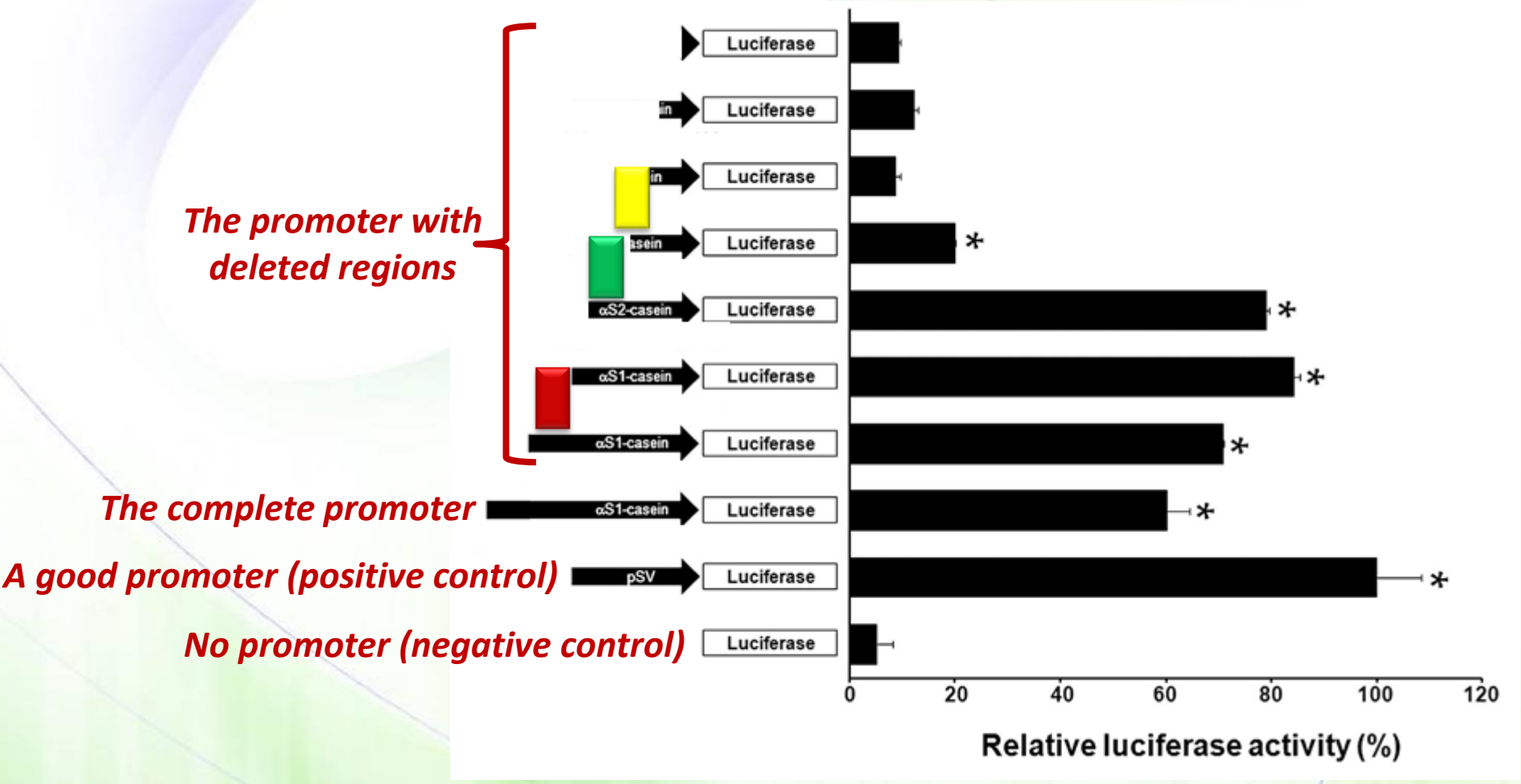


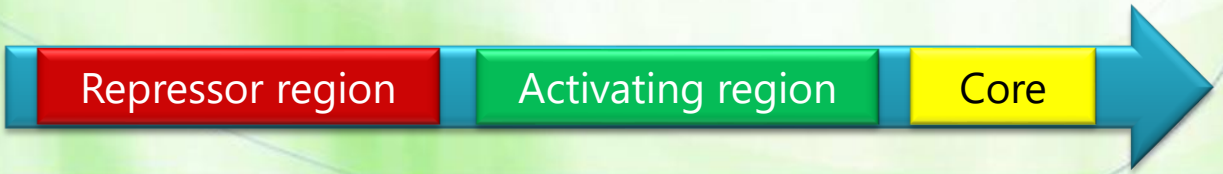
The purpose and procedures

- Purpose: study the activity of a gene at certain conditions or elucidate the function of certain regions of the promoter
- The promoter of the gene only is placed upstream of a "reporter gene" such as the luciferase gene in a plasmid, the plasmid is transfected (inserted) into the cells, and the expression level of luciferase (instead of the gene itself) is measured.



Example

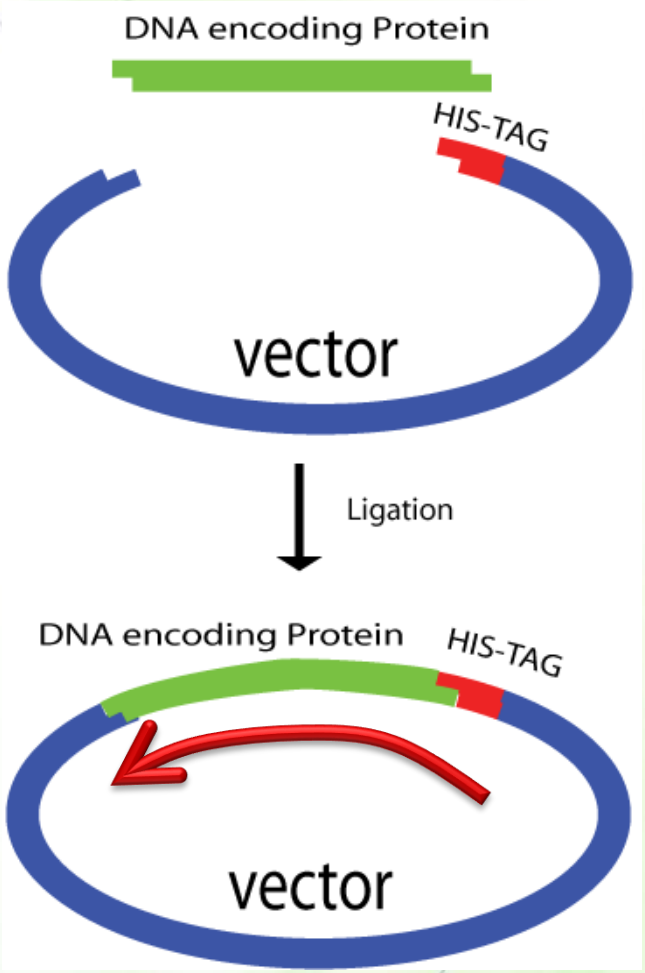

The promoter with deleted regions
The complete promoter
A good promoter (positive control)
No promoter (negative control)
Luciferase
αS2-casein
αS1-casein
αS1-casein
αS1-casein
pSV
0
20
40
60
80
100
120
Relative luciferase activity (%)


Repressor region
Activating region
Core

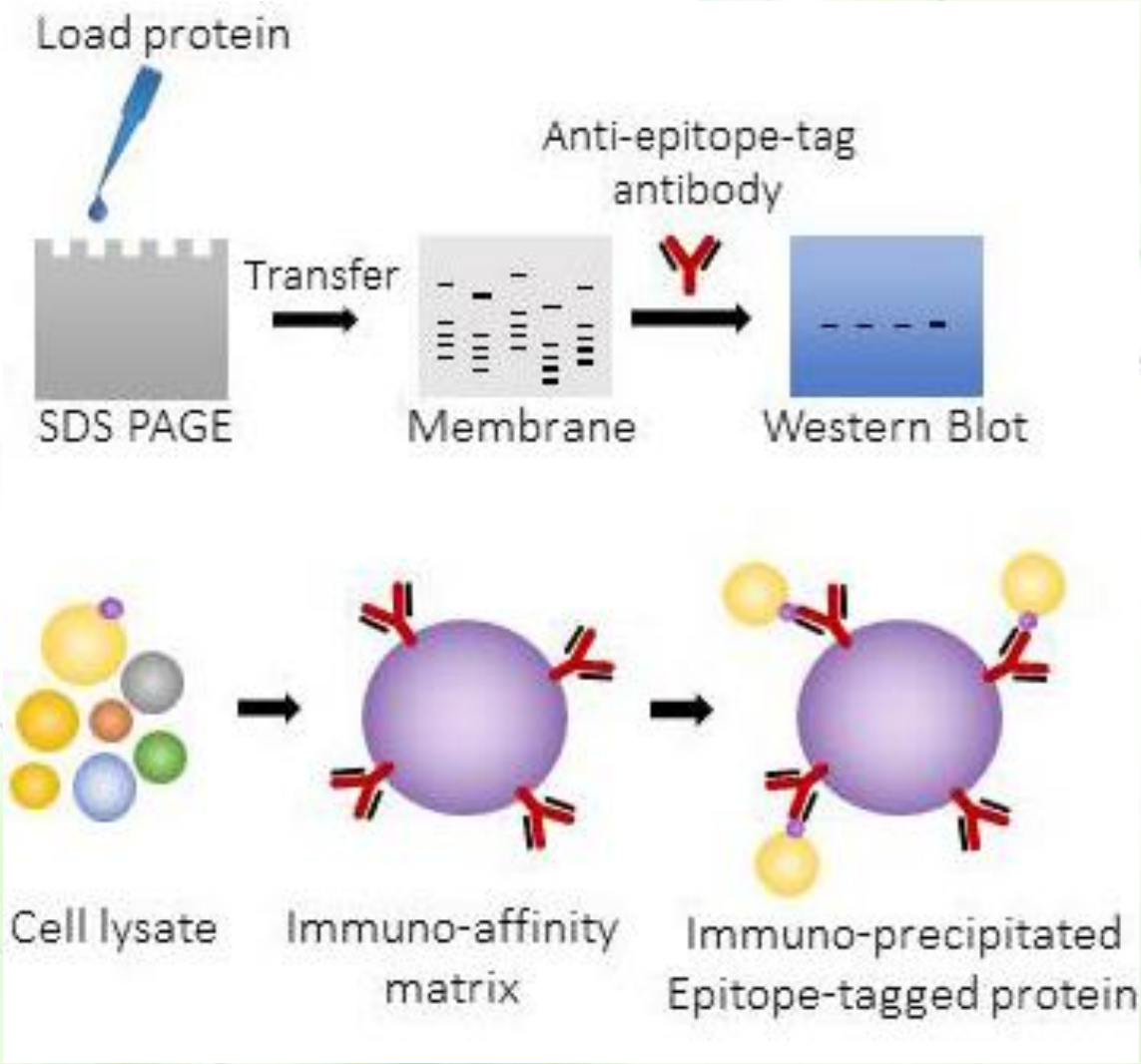
Protein tagging or creation of protein hybrids

Proteins can be "tagged"

- A protein-encoding gene is cloned in a special vector containing a tag gene producing a protein with an extra sequence of amino acids called tags.
- These tags allow easy protein purification and detection.



Uses of protein tags

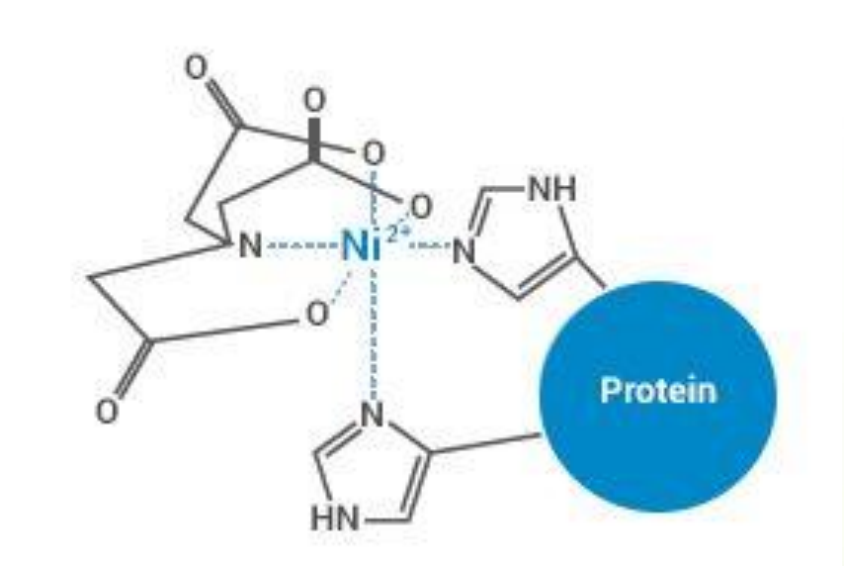


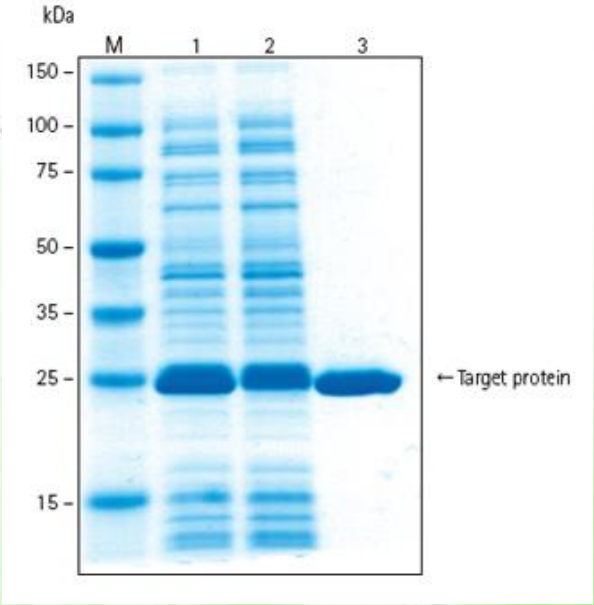
Major protein and epitope tags

Name	Amino acids	Detection	Purification
FLAG	DYKDDDDK	antibody	FLAG peptide
Green fluorescent proteins (GFP)	~220 aa protein	antibody or fluorescence	None
Glutathione S transferase (GST)	218 aa protein	antibody	glutathione
HA	YPYDVPDYA	antibody	HA peptide
Poly-His	HHHHHH	antibody	nickel, imidazole
Myc	EQKLISEED	antibody	Myc peptide
V5	GKPIPNPLLGLDST	antibody	V5 peptide

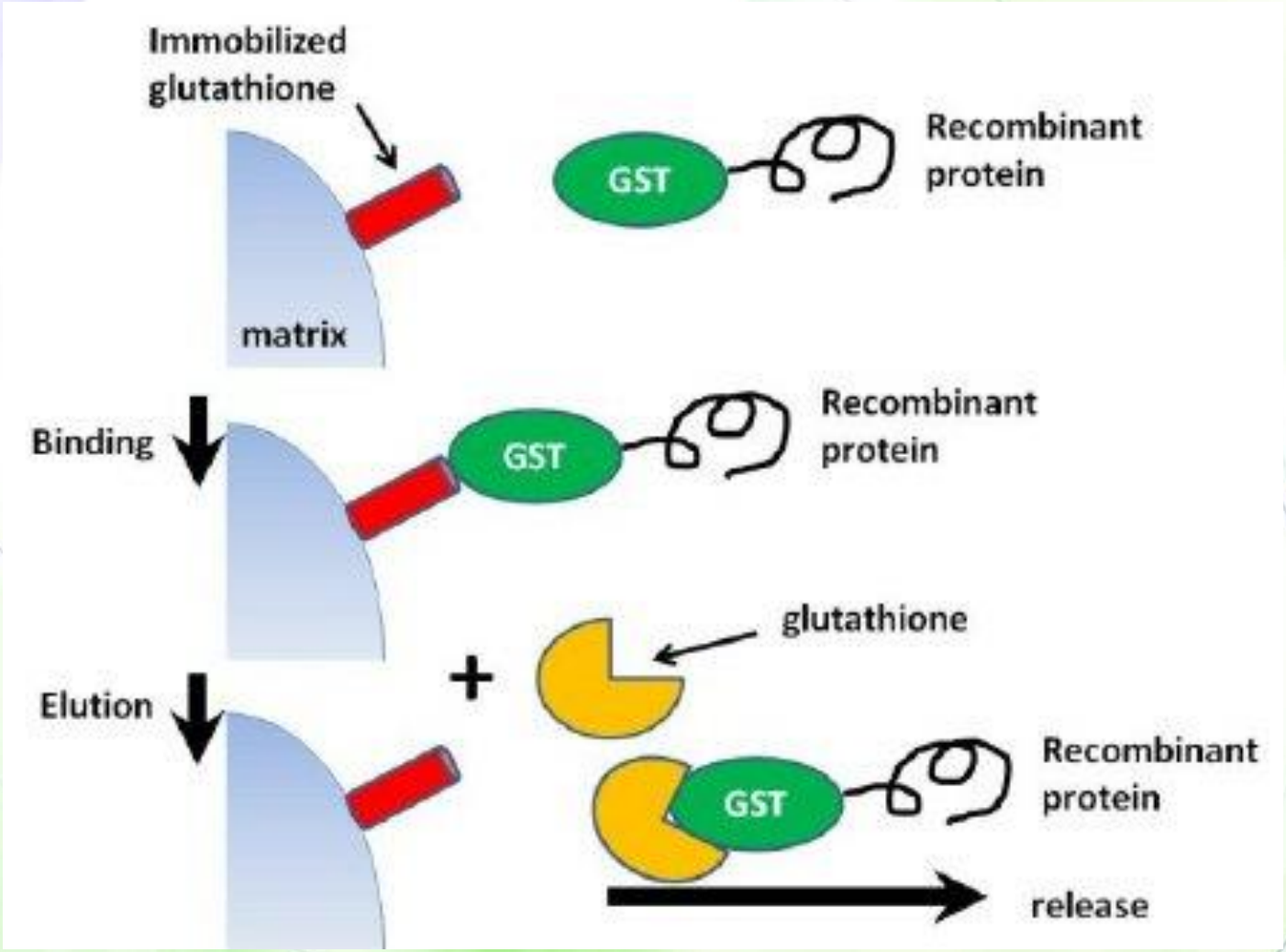
His tag

- The addition of six histidines to a protein would allow for purification using breads with bound nickel ions.





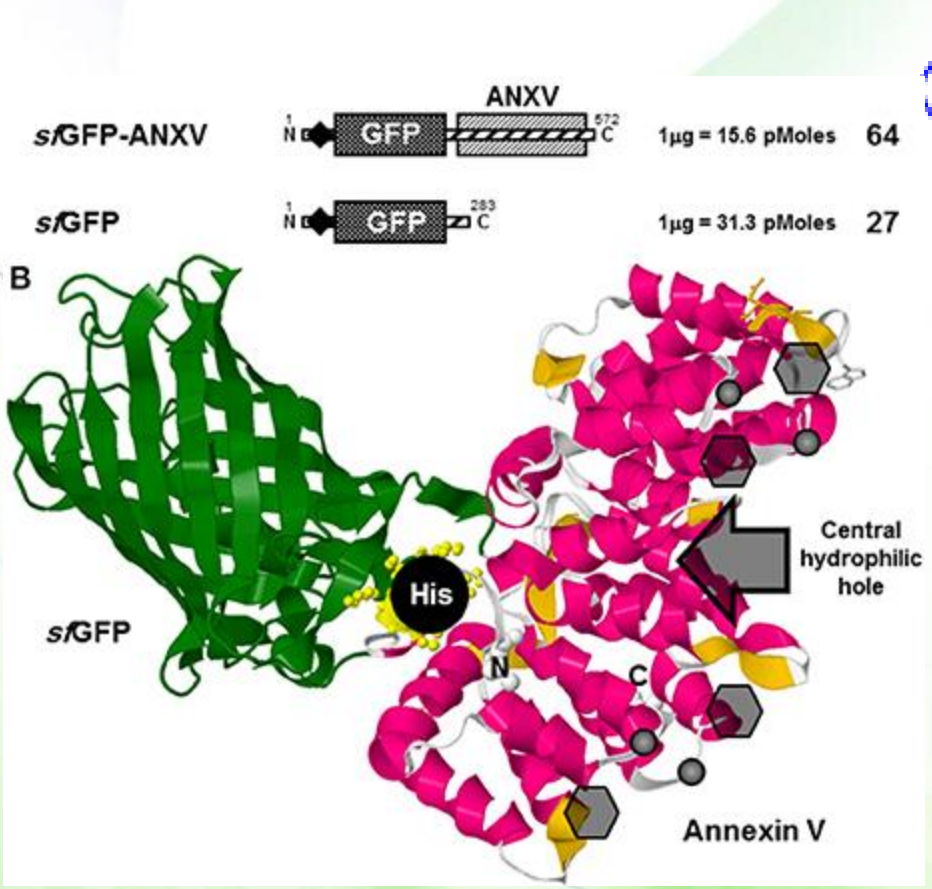
Purification of GST-tagged proteins



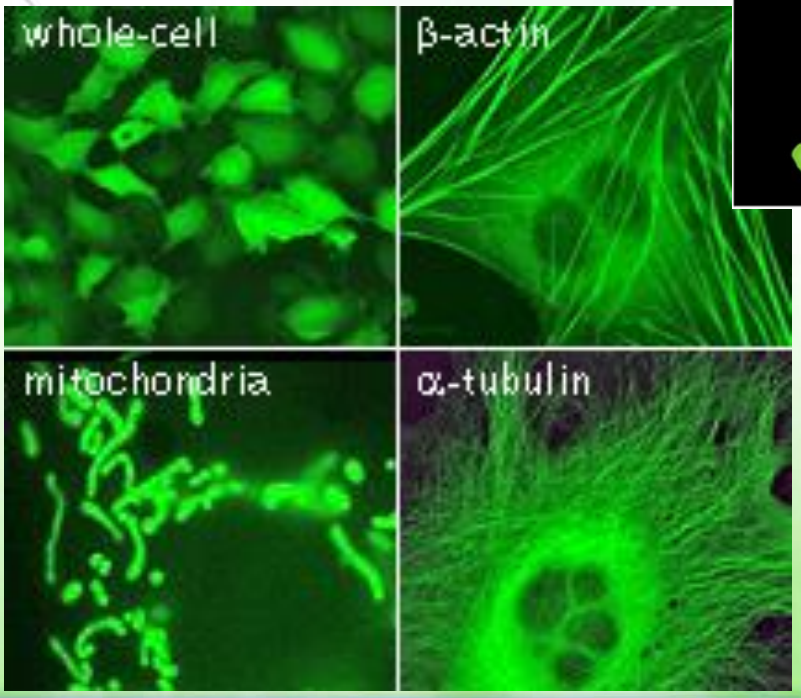
GFP-tagged proteins

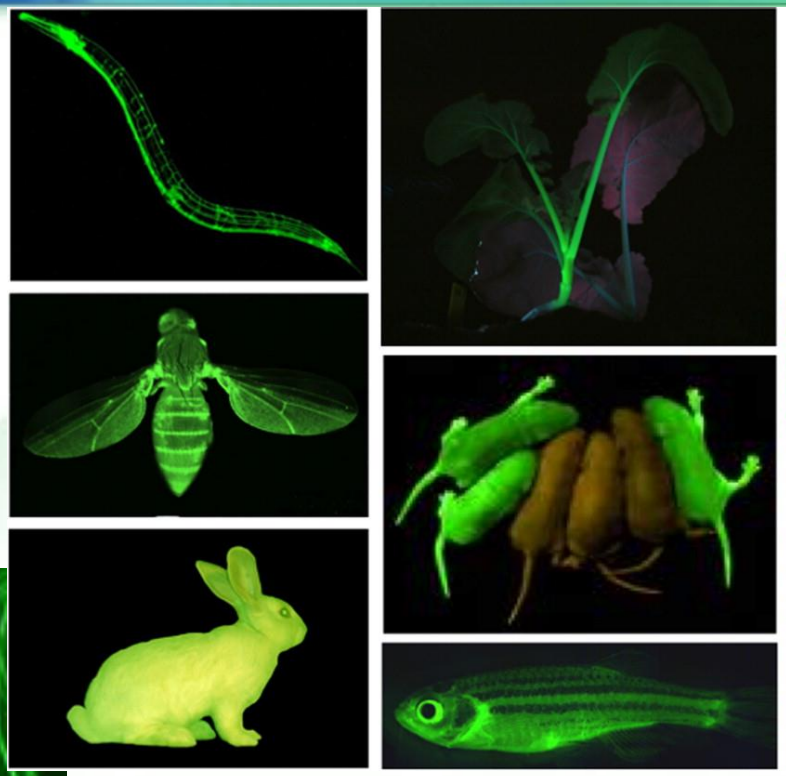
The power of domains

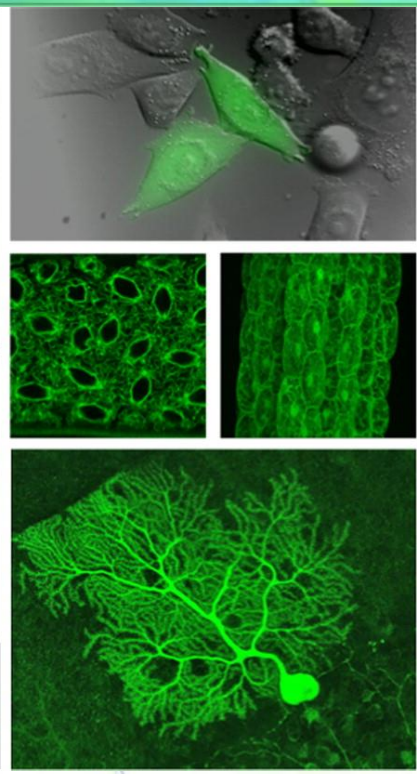
- GFP allows for protein detection rather than for purification purposes.

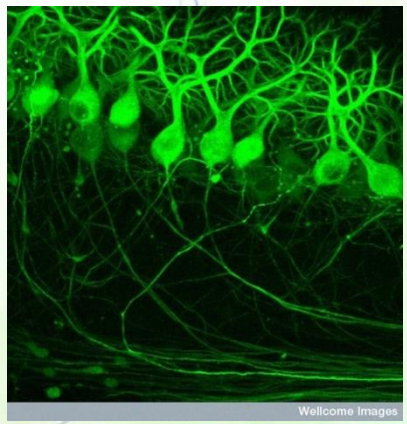


A world of possibilities





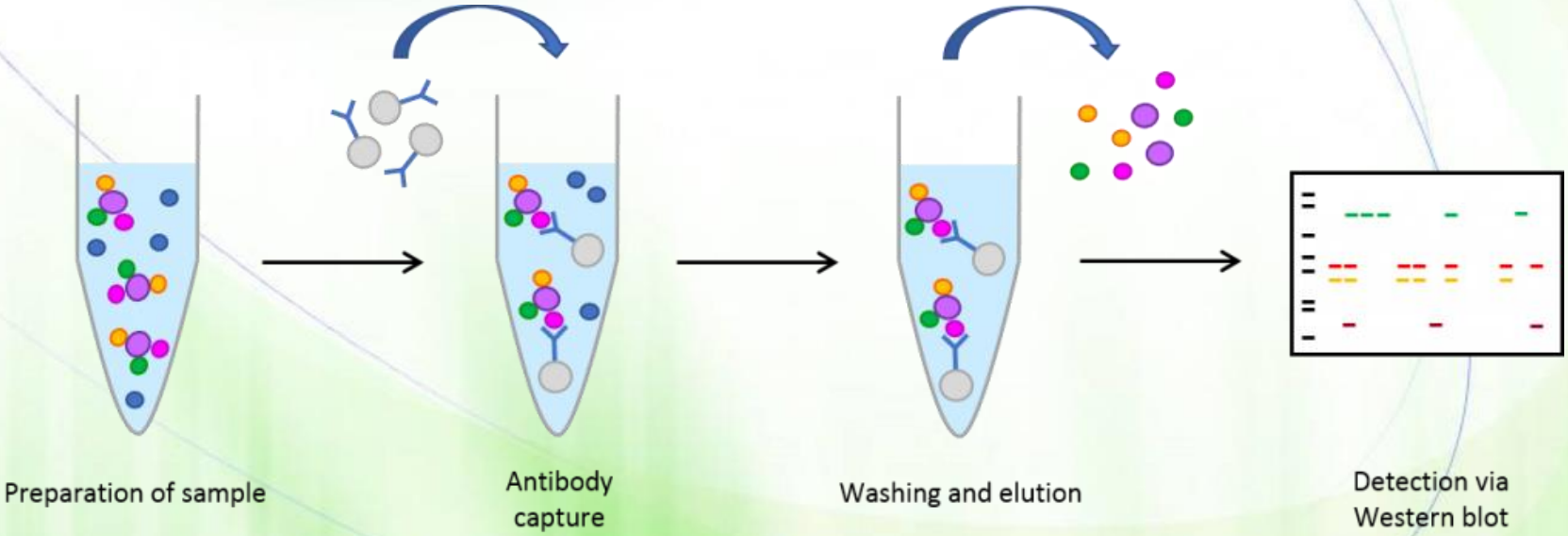




Protein-protein interaction

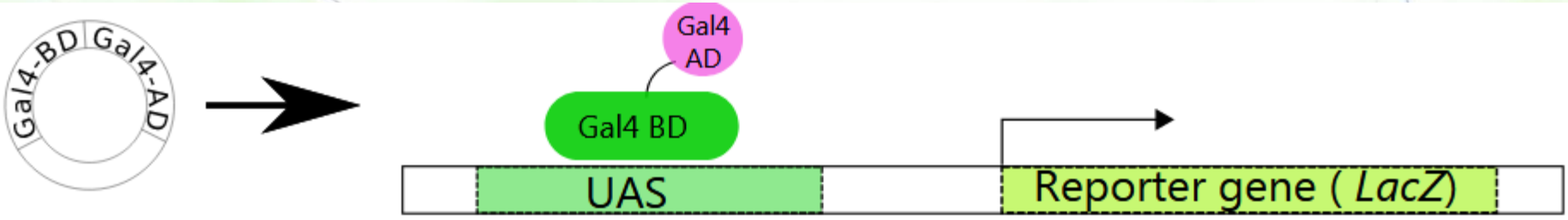
(*Co*)-Immunoprecipitation

- Antibody molecules that target a specific protein are conjugated to special beads.
- A mixture of cell proteins are added to the beads.
- Only the protein of interest is precipitated as well as other proteins bound to it (co-precipitated).



Taking advantage of domains

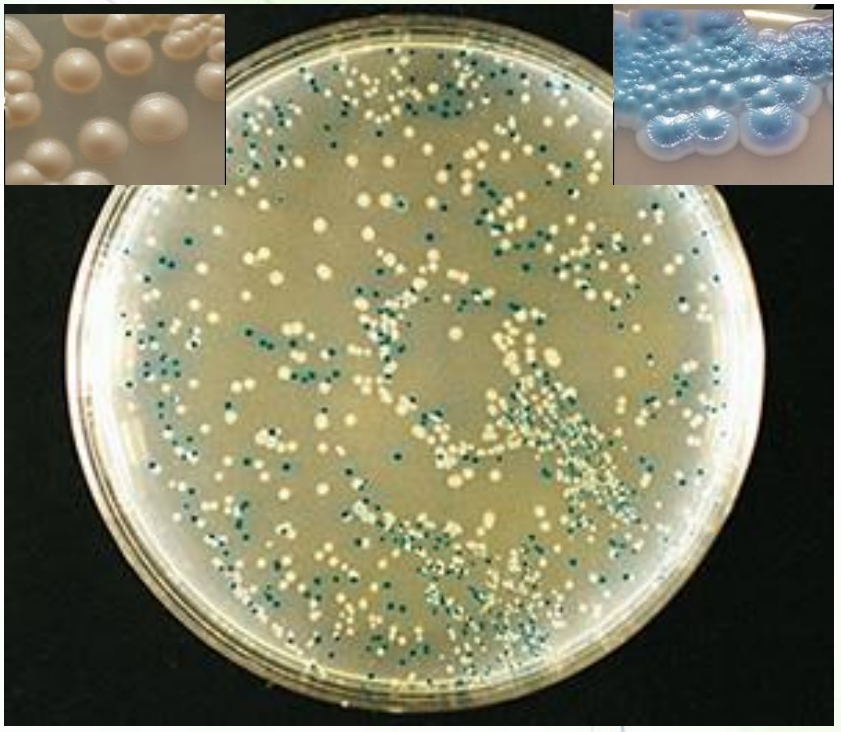
- In yeast, an upstream activating sequence (UAS) exists.
- UAS is controlled by a transcription factor that is made of two domains
  - A DNA-binding domain (BD)
  - An activating domain (AD) that is responsible for the activation of transcription.
  - Both must be close to each other in order to transcribe a reporter gene such the LacZ gene.

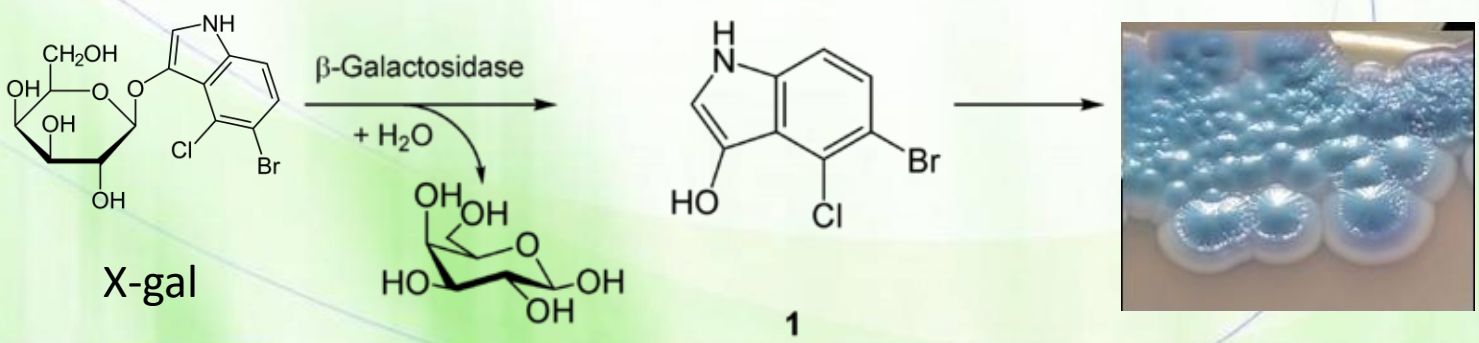


A. Regular transcription of the reporter gene

Why is the LacZ gene used?

- Yeast are grown in the presence of a lactose analog called X-gal, which produces a blue product when cleaved.
- When the LacZ gene is activated, beta-galactosidase is produced, which cleaves X-gal generating blue colonies.

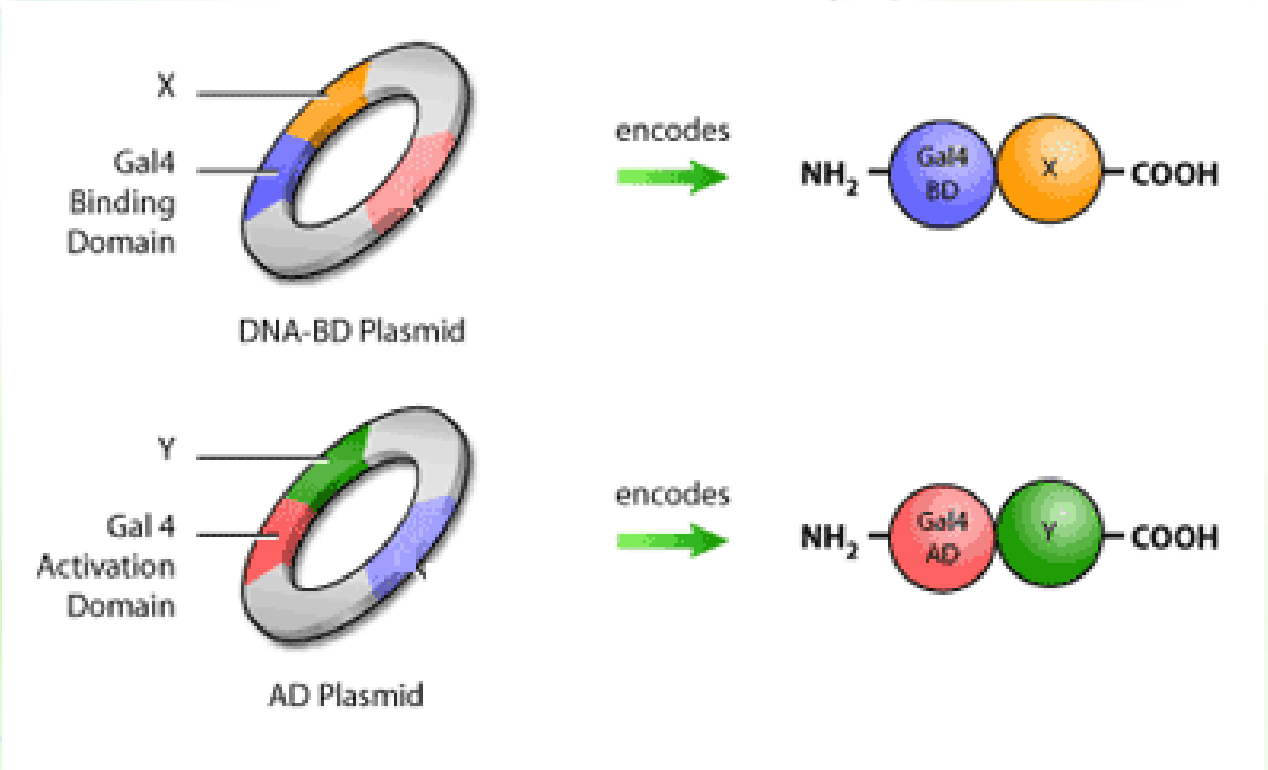




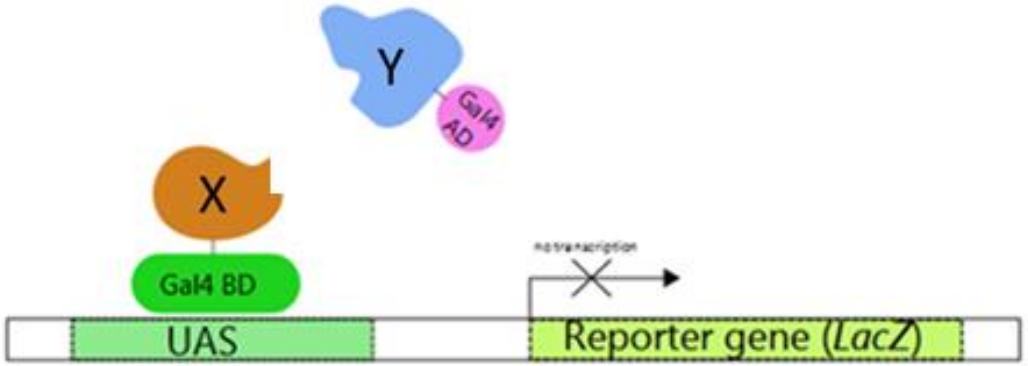
Cloning of hybrid proteins

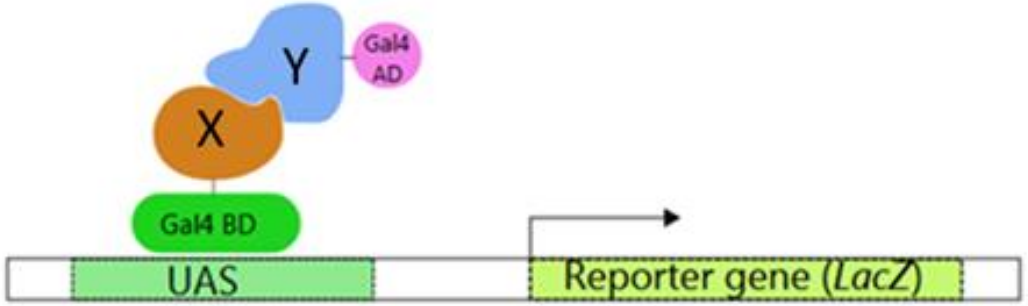
- In order to discover proteins (Y) that interact with a known protein (X), the X gene is cloned so it is produced integrated with the DB domain and the unknown genes are separately cloned so they are produced integrated with AD.

- Both recombinant plasmid are transformed (transferred) into yeast.



The possibilities

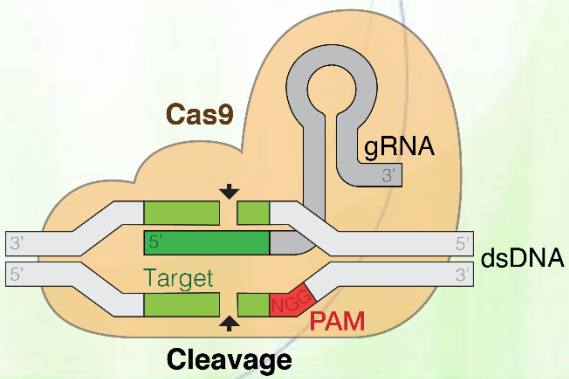




CRISPR-CAS9 system

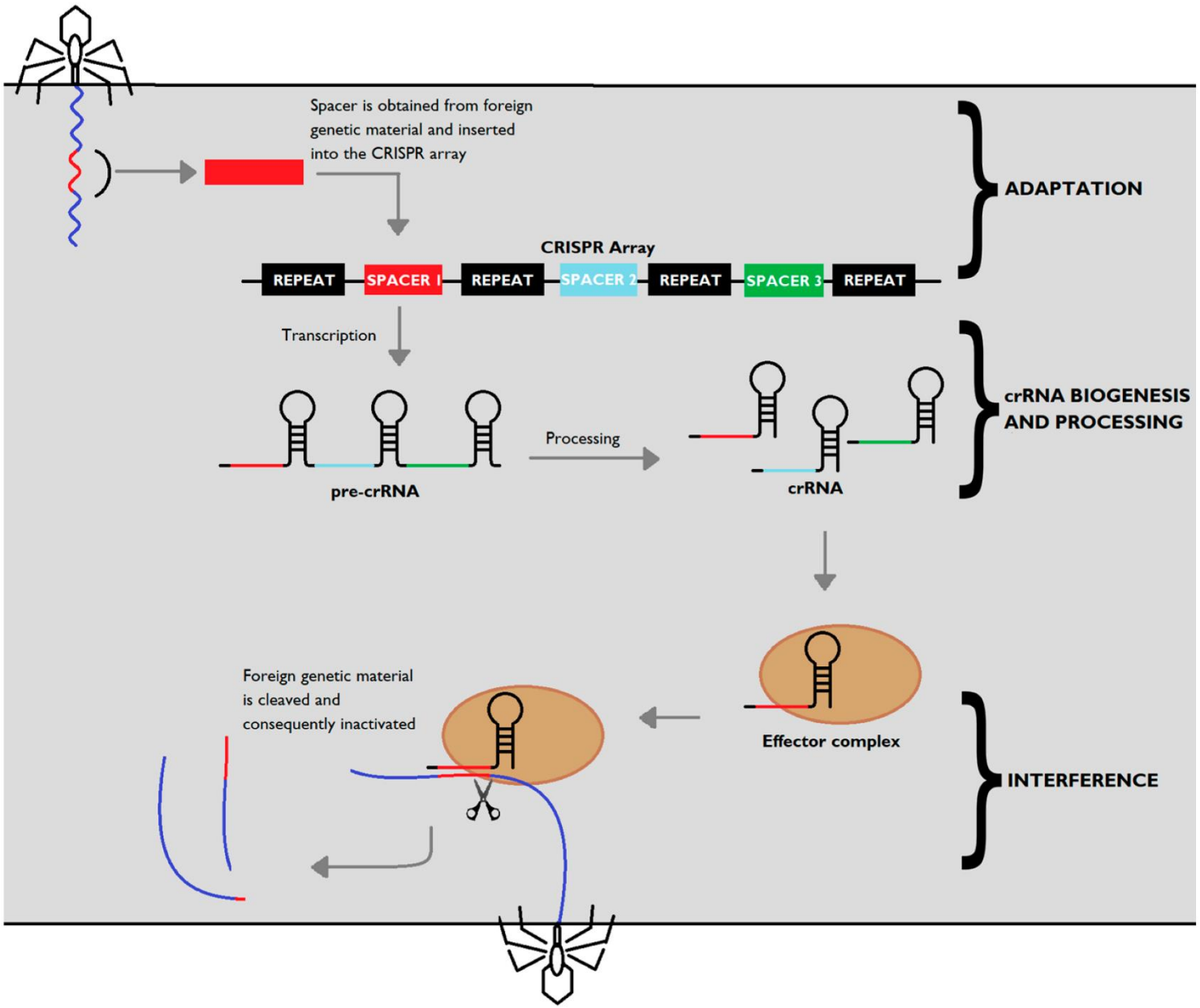
Genome editing by CRISPR/Cas9

- CRISPR is clustered regularly interspaced short palindromic repeats
  - It is a bacterial genetic system that constitutes the immune system of bacteria against phages.
- Cas9 is a RNA-guided nuclease that can either create single or double strand breaks.
  - The nuclease is directed to its target sequence by a short RNA fragment known as a guide RNA (gRNA) or single guide RNA (sgRNA), which is complementary to the target segment of the genome.

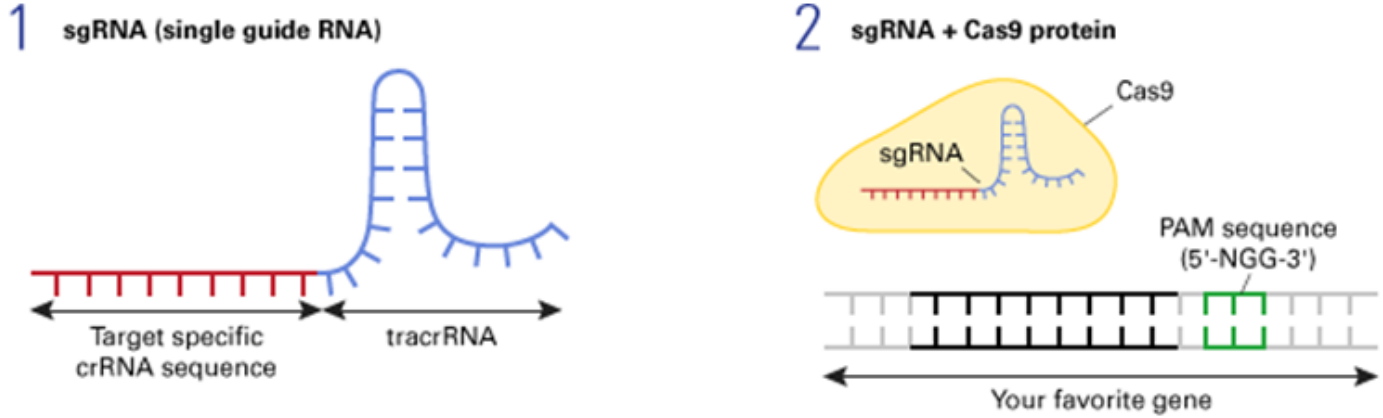


The concept

- When a phage infects a bacterial cell, the cell chops off the phage DNA into smaller pieces and integrates one of these fragments into the CRISPR cluster.
- When the phage infects the cell again, the cell transcribes the DNA into RNA (gRNA), which is integrated into the Cas9 nuclease and guides it to the phage DNA to degrade it.

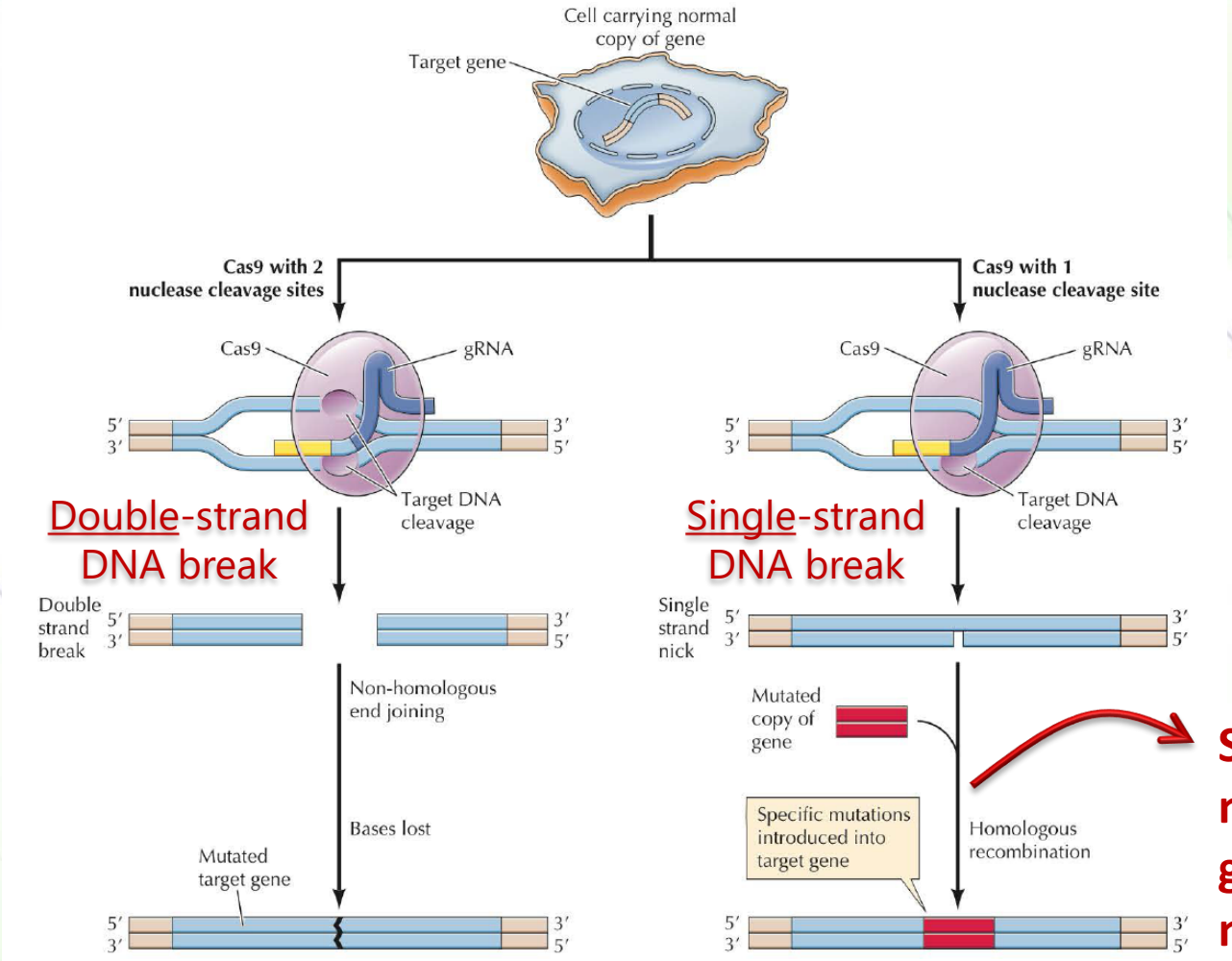

Spacer is obtained from foreign genetic material and inserted into the CRISPR array
ADAPTATION
CRISPR Array
REPEAT
SPACER 1
REPEAT
SPACER 2
REPEAT
SPACER 3
REPEAT
Transcription
pre-crRNA
Processing
crRNA
crRNA BIOGENESIS AND PROCESSING
Effector complex
Foreign genetic material is cleaved and consequently inactivated
INTERFERENCE

The steps of action



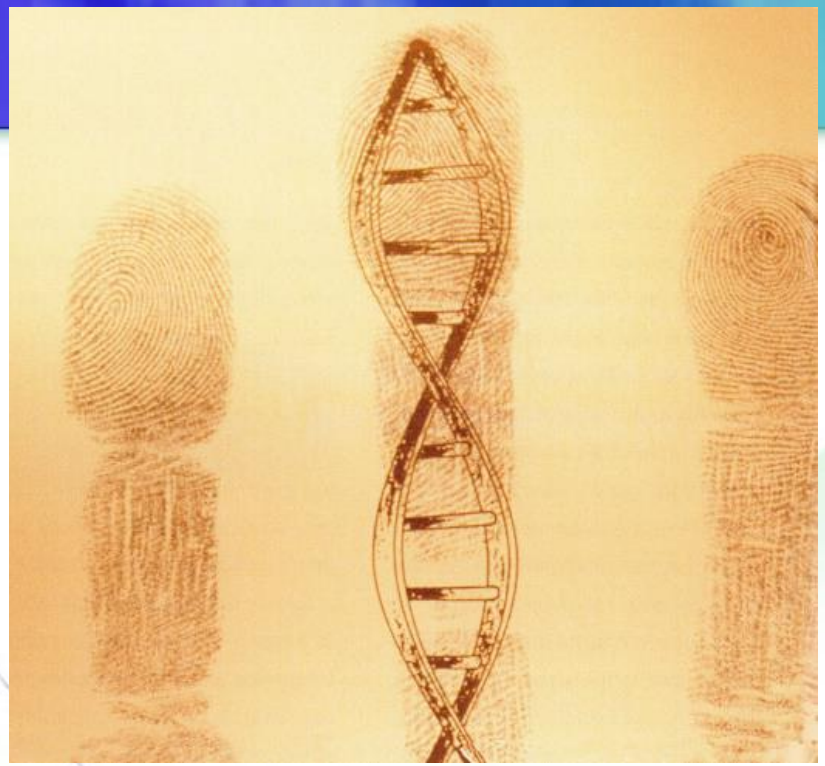
Both the gRNA and Cas9 gene can be introduced into human cells as genes cloned into plasmid vectors.

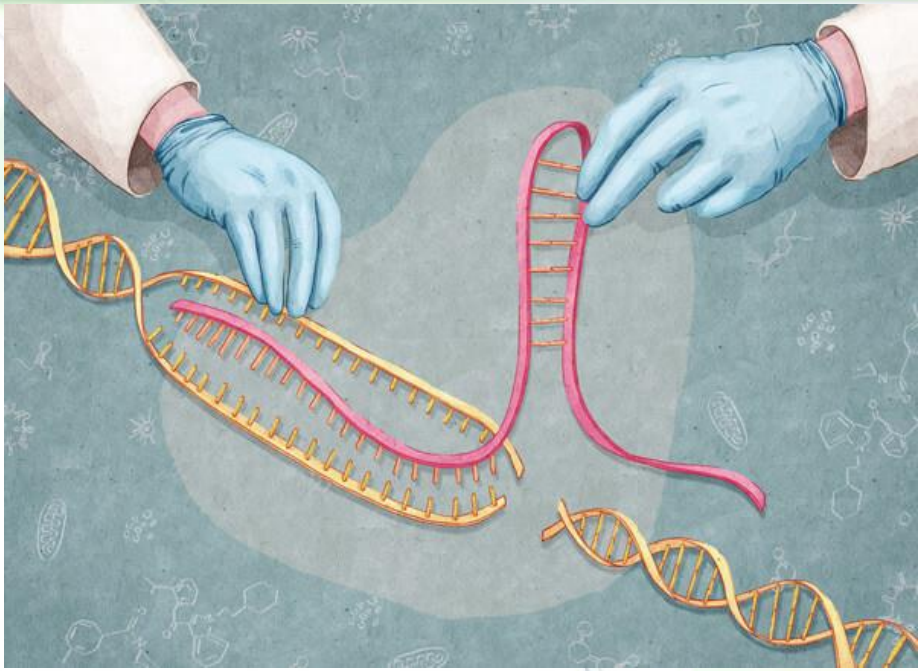
The actions of Cas9

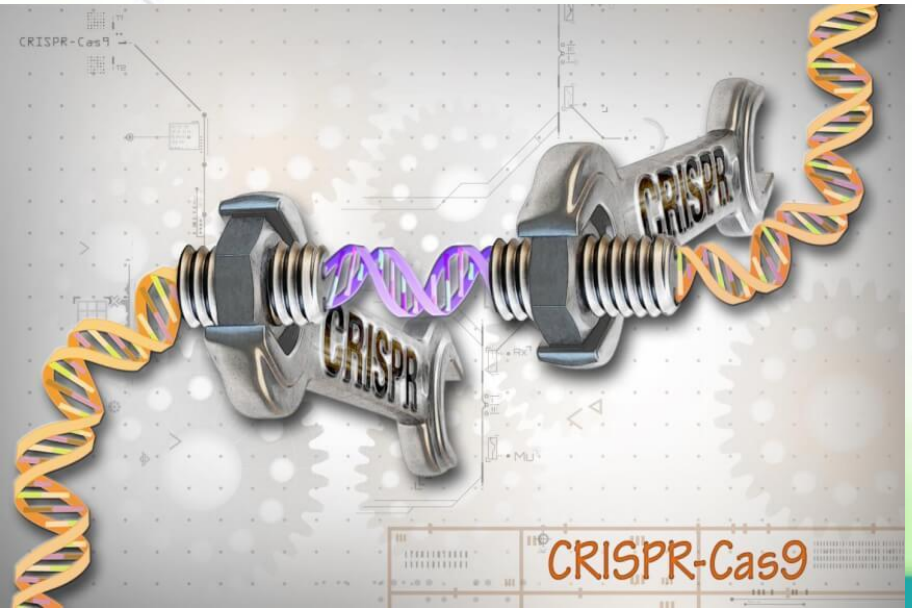


Either mechanism, the function of a gene can be studied by mutating it.

Specifically in this mechanism, a mutated gene is replaced by a normal one is a possibility (or the opposite.






CRISPR-Cas9
CRISPR
CRISPR
CRISPR-Cas9