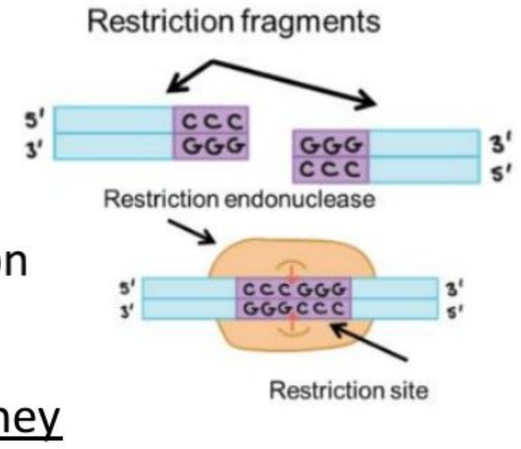
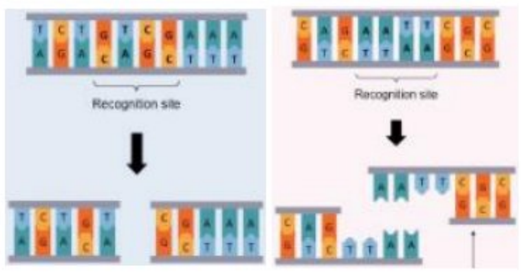
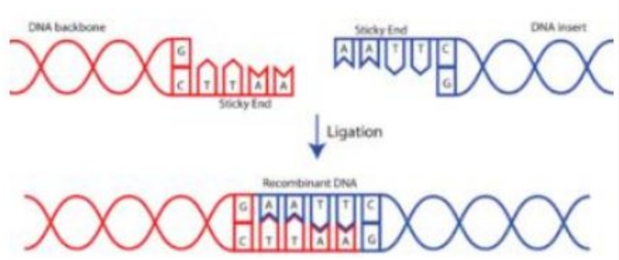
❖ Restriction endonucleases

- **Endonucleases** ➔ **Endo** = within molecule / **nucleases** = enzyme that degrade nucleic acid
- **Restriction endonucleases:** Bacterial enzymes that recognize and cut (**break**) the phosphodiester bond between **nucleotides** at a **restriction site** generating **restriction fragments**

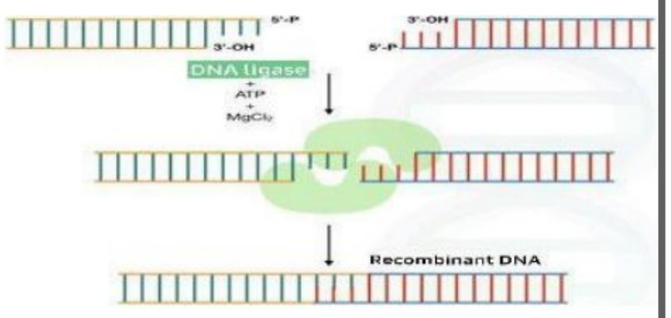


  - ➢ **Restriction site:** A **specific sequences** range (**4- to 8-**bp) where Restriction endonucleases cut, and they are **palindromic sequences**
  - ➢ **Palindromic sequence:** A sequence read **the same** from left to right as they do from right to left (the same sequence on the both strands)
- They are called **restriction** → because bacteria use them to **restrict the growth** of viruses attack them (bacteriophages) by cutting the DNA of the virus
- Examples on Restriction endonucleases:
  - ➢ **EcoRI**, its restrictions site → GAA*TTC / CTT*AAG
  - ➢ **HindIII**, its restrictions site → AAG*CTT / TTC*GAA
  - ➢ **SmaI**, its restrictions site → CCC*GGG / GGG*CCC
- Restriction endonucleases cut DNA in 2 ways:
  - ➢ **Blunt** ➔ Cut in the **same position on both strands** giving blunt-ended fragments
  - ➢ **Staggered (off-center)** ➔ Cut in **different positions on each strand** generating sticky or cohesive ends





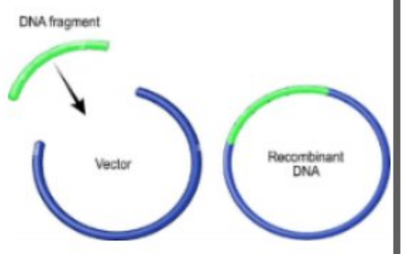
  - o Sticky ends have **single-stranded overhangs** at the end ➔ which can form **H-bonds** with other **complementary sequences** of nucleotides
  - o If these sequences are from **different sources** ➔ they are called **recombinant DNA**
  - o The **hydrogen bonds** are **non-covalent bonds** means that they are ➔ **unstable bond** (reversible bonds break & reform) ➔ so, the **2** sticky ends can be **released or disassociated** from each other
- **DNA ligase** ➔ an **ATP-dependent enzyme** that catalyzes the formation of **phosphodiester bonds** between the **3'-hydroxyl group** of one strand and the **5'-phosphate end** of another strand



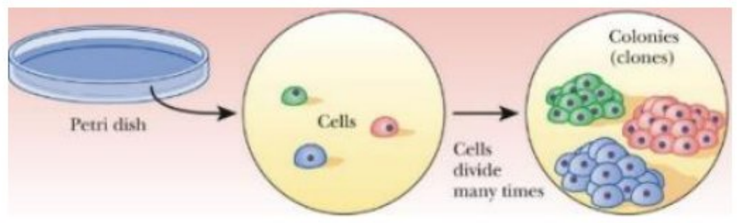
❖ DNA cloning

- Cloning means ➔ making several **copies** of one thing
- **DNA cloning** is a technique that allows for:
  - ➢ **Amplifying** a DNA segment into many, many copies in a **biological system**.
  - ➢ **Expressing a gene** inside a **biological system**
    - ✓ (such as: yeast, bacteria, cultured human cells or even the human body as a whole)
- **DNA cloning** usually involves:
  - ➢ The formation of a **recombinant DNA**
  - ➢ **Insertion into the cell** (such as bacteria) → it divides, grows and proliferates ➔ **amplifying** DNA

- **Recombinant DNA:** It is DNA molecule which **made up of 2 or more <u>different DNA from different sources</u>**, composed of:
    1) **A vector** ➔ a <u>carrier of the gene</u> or the DNA segment of interest (usually **a bacterial plasmid**)
    2) **A gene** (that encodes a protein **or** a non-coding RNA) using **restriction endonucleases**

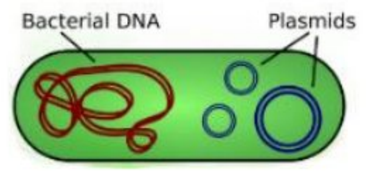


- **Clones:** They are <u>genetically **identical** populations</u> (copies) of organisms, cells, viruses, or DNA
- All the members of the clone are **derived from a single one** (cell, virus, or DNA molecule) ➔ so a single cell generates the whole identical population

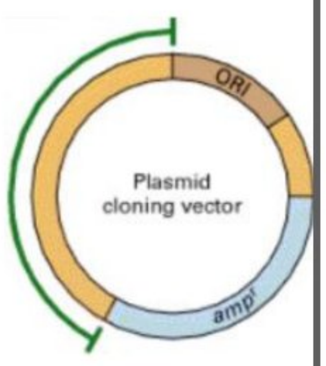


- **How do we clone a DNA molecule?**
    - ➢ We insert the DNA fragment of interest into the DNA carrier (vector), using **Endonuclease** & **<u>ligase</u>** enzymes → forming a **recombinant DNA molecule**
    - ➢ This procedure is known as ➔ **recombinant DNA technology**, which is part of **genetic engineering**

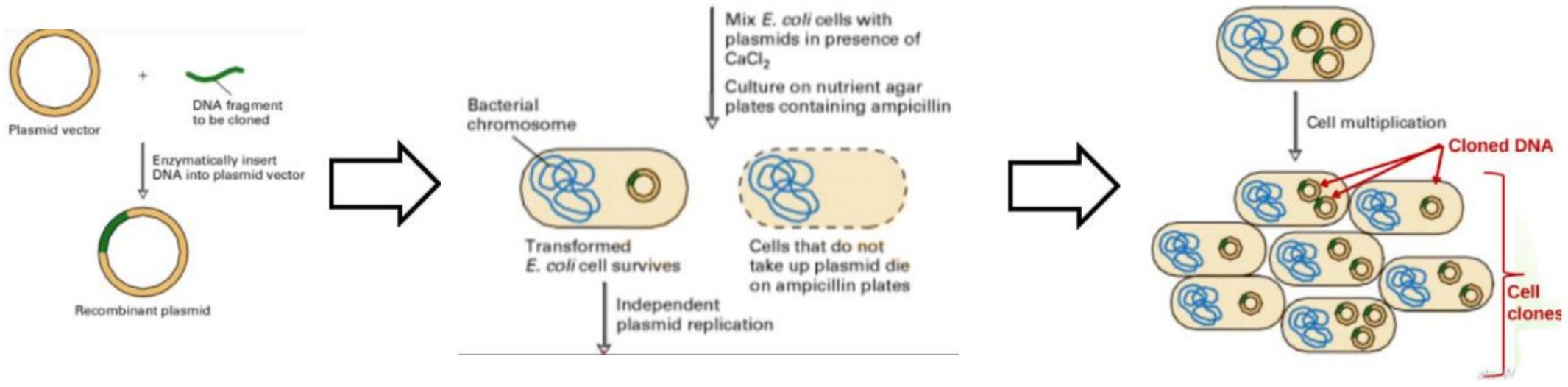
- **Bacterial plasmid:** natural bacterial **circular DNA** that is <u>not a part of the main circular DNA</u> chromosome, and it can **<u>replicate independently</u>** of the main bacterial genome, so:
    - ➢ A bacteria can have many plasmid & can be **transferred** from one bacterial cell to another
    - ➢ They can be used as **vectors** for **cloning** (cloning vectors) or **expression** (expression vectors)



- **Features of plasmid cloning vectors:**
- Plasmid cloning vectors <u>must have</u> the following three components:
    - **Their own origin of replication** (OriC) ➔ that allows them to **<u>replicate independently</u>** of the bacterial chromosome
    - **An antibiotic resistance genes** (**selectable** marker gene) ➔ allows for selecting the bacteria that can resist a certain antibiotic (such as ampicillin), others will die
    - **A restriction site** ➔ that allows for **insertion of the DNA segment** of interest into the plasmid

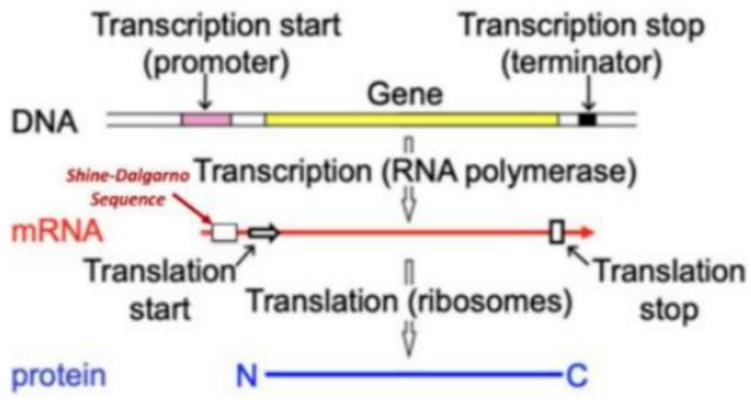


- Both **DNA fragments** to be cloned and **vector** ➔ are cut by **<u>the same restriction endonuclease</u>** that makes DNA fragments with **same** sticky-ends hybridize (annealing between the complementary sequences) to each other, when mixed ➔ then **DNA ligase** is added to close the plasmid ➔ we have **recombinant DNA or plasmid** → which is returned to bacteria ➔ **then cloning (amplifying)**



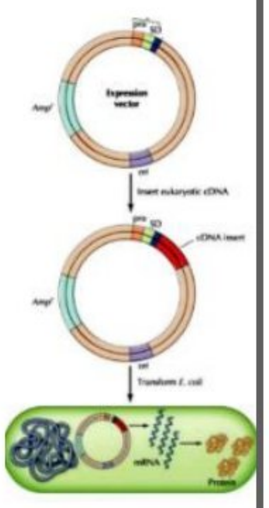
❖ Overview of gene expression

- In order to express gene we need:
  - **Promoter** (on **DNA**)➔ which is <u>the binding site of RNA polymerases</u>, (transcription initiation site)
  - **Termination sequence** (on **DNA**) ➔ to stop transcription producing mRNA
  - **Translation start site** (on **mRNA**) ➔ which is AUG
  - **Translation stop codon** (on **mRNA**) ➔ (UGA, UAG, UAA)

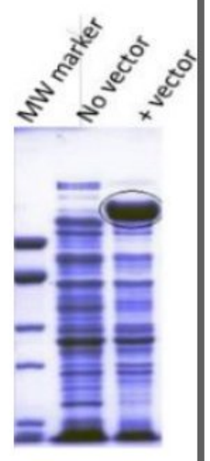


- The product of this process is a **polypeptide chain** that folds into its 3-dimintional structure

- **Expression vectors**
- The vector we use <u>to express a specific gene</u> (bacteria produce human protein)
- Expression vectors contain additional sequences:
  - **Promoter sequences** upstream of gene to be inserted ➔ to initiation transcription
  - **Ribosomal binding sequences** (**ShineDalgarno [SD] sequences** in bacteria) ➔ to initiate translation
  - **A transcription termination sequence** (encodes the stop codon) ➔to stop expression



- Examples of human proteins that can be produced inside bacteria cell: **insulin, growth hormone, plasminogen activator, erythropoietin**

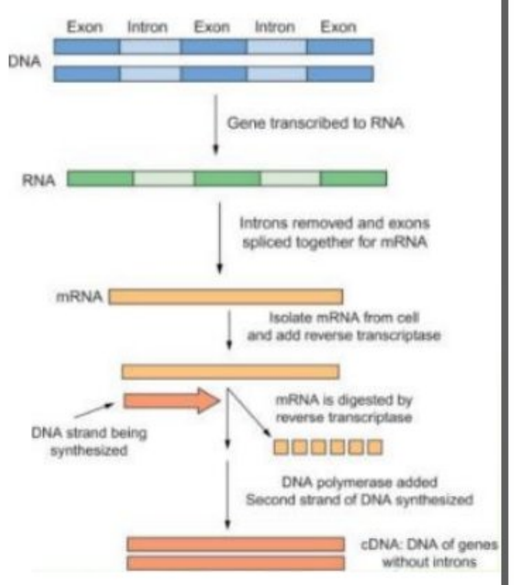


- This technique is **Gel electrophoresis for proteins** are separated according **the size:**
  - ➢ **Bacteria with no vector** ➔ there is **no expression** of the proteins.
  - ➢ **Bacteria with vector** ➔ there is **gene expression** of the protein on interest

- **How do we select for human mRNA?**
- When we comes to express human gene in bacteria, there are <u>two challenges</u>, and two solutions for each one:

1) The "intronic" <u>challenge</u>

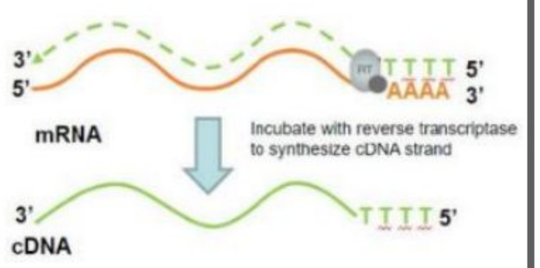
- Genes contain **introns (<u>non-coding</u> sequences),** can clone DNA molecule (gene) **without introns by:**
  - **The "reverse" <u>solution</u>** ➔ by using a **reverse transcriptase** to make a **cDNA** from the **<u>mRNA</u>** molecule then adding **DNA polymerase** to make a **double stranded DNA without introns** then it will be inserted in the plasmid



- **Reverse transcriptase:** Enzyme generates DNA from RNA (use RNA as a template)

2) The "many types of RNA" <u>challenge</u>

- Inside human cells we have **many types of RNA molecules** → and we need only mRNA
  - **So,** we use **reverse transcriptase** to produce cDNA
  - Reverse transcriptase requires a **<u>primer</u>** (a short nucleotide sequence to start replication)and we use a **poly-T primer** binds to the mRNA (which **<u>contains poly-A tail</u>**) then **reverse transcriptase** start make a copy of **cDNA**
  - The only type of RNA having Poly-A tail is mRNA (so it will be selected from others)

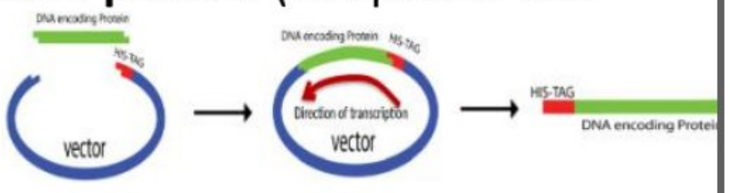


- **Challenges of protein expression in bacteria**
- Proteins that produced inside bacteria by expression vector will face these challenges:
  - **No internal disulfide bonds** (covalent bond that provide the **stability** for the proteins in eukaryotes such as disulfide bonds between the heavy & light chains of **antibodies**)
  - **No post-translational modification** such as **glycosylation** (adding sugars)
  - **Protein misfolding** ➔ the 3-dimensional structure **is not formed properly** inside bacteria because bacteria lacks modifier proteins (such as chaperones in human cells)
  - **Protein degradation** ➔ degraded by the bacteria (because it is **not** recognized as a normal bacterial protein)

- **The Solution of this challenge is:**
  - ➢ use a **eukaryotic system** such as yeast (Mono-cellular eukaryotic cell)

❖ Protein tagging and creation of protein hybrids

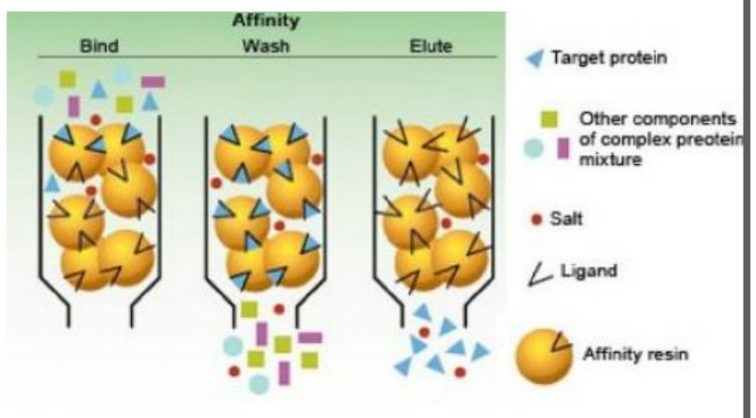
- Tagging is adding a tag or label → so, these tags allow easy protein purification & detection
- A protein-encoding gene is cloned in a special **vector containing a tag gene** → which will be expressed with the gene ➔ encodes into amino acids (tags) **present on the produced protein** (as apart of the recombinant protein)



- **Post-protein tagging**
- How we can **isolate** the protein that is tagged from other proteins?

1) Affinity chromatography

- We have a column (cylinder) that has a matrix contains specific ligands (that can bind only to the proteins of interest)
- **So,** proteins that have the affinity to these ligands will bind, while others will pass out from the column
- Finally, we can isolate the protein of interest from the column

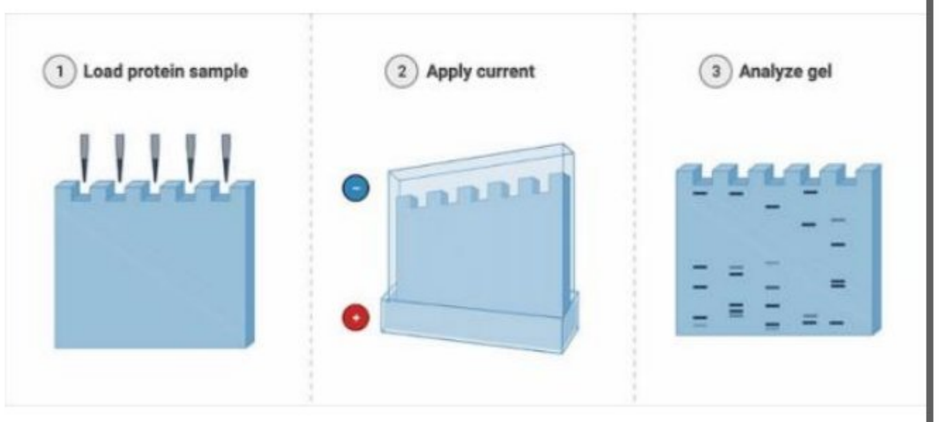


2) Immunoprecipitation

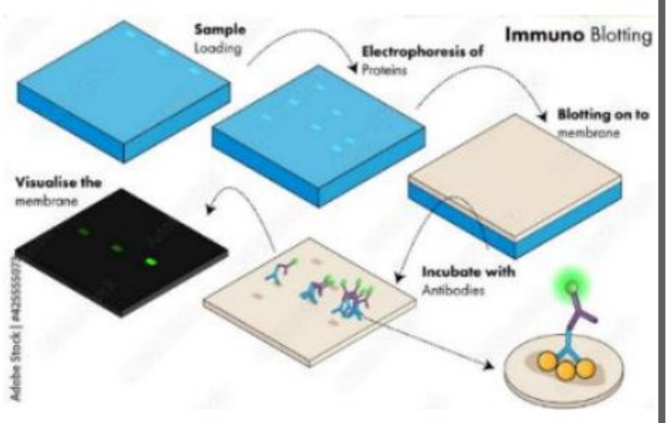
- Antibodies will bind to proteins highly specific
- We put a collection of proteins in tubes that contain resin and beads **with antibodies** on their surface → which **binds to the proteins of interest very specifically**
- These beads are **heavy** ➔ so they **precipitate** (go all the way down)
- Then proteins of interest will be isolated from beads and other proteins

3) Gel electrophoresis (SDS-PAGE)

- Separating proteins according **their size**
- **We use special gel** ➔ put our sample into **wells** ➔ applied an **electrical current** ➔ proteins will be separated according **their size** as bands➔ **each band** contain many copies of different protein with the **same size**



4) Immunoblotting



- Like southern blotting
- **Proteins** are separated through a gel according to size ➔ and then they are transferred to a membrane
- Then adding **antibodies** that have labels attached to them
- **Antibodies** binds **specifically** to proteins giving **a signal we can detect**

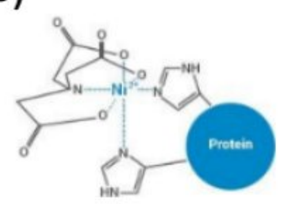
❖ Major protein tags

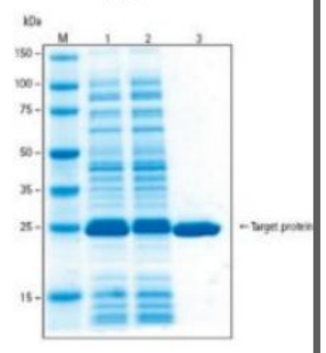
- There are different types of tags we can use with different purposes:

Detection: only seeing the protein (detecting it)
Purification: extracting the protein from a sample contains other molecules

➢ Poly-HIS:

- ✓ a small tag composed of **6 residues of an amino acid** known as **histidine (H)**
- ✓ Tagged proteins can be detected by **Antibodies** (specific to the 6 histidine residues)
- ✓ Can be purified by **Affinity chromatography** using breads with bound **nickel** (or imidazole) on their surface → Ni binds to the 6 H residues and so only the tagged protein will be purified

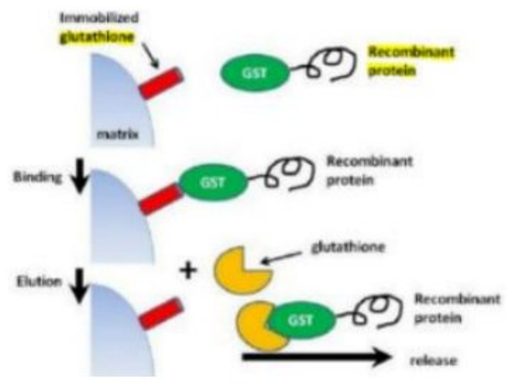




- ✓ So, we clone the gene in the bacterial cell → then it will be expressed producing proteins which we purify & analyze them by gel electrophoresis showing that it is highly expressed (due to cloning)

➢ GST (Glutathione S transferase)

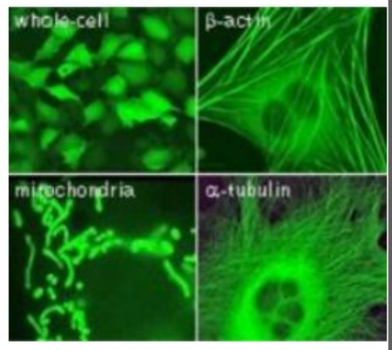
- ✓ composed of **218 residues of an amino acids**
- ✓ Tagged proteins can be detected by **Antibodies**
- ✓ Can be purified by **Affinity chromatography** using breads with bound **Glutathione** (substrate of GST) on their surface → recombinant proteins bind to glutathione → then it can be released



➢ GFP (Green Fluorescent proteins)

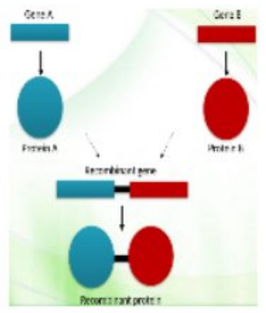
- ✓ It is a protein produced by **jellyfish** allows for protein detection (natural florescence) rather than for purification purposes
- ✓ composed of **220 residues of an amino acids**
- ✓ Tagged proteins can be detected by **Antibodies or Fluorescence**
- ✓ **No purification**
- ✓ Proteins tagged by GFP will fluoresce → so we can know where the protein goes **inside the cell and also can in the organism** (such as the stem of the plant, mouse)

- ✓ GFP can be linked to different cell proteins such as actin/tubulin

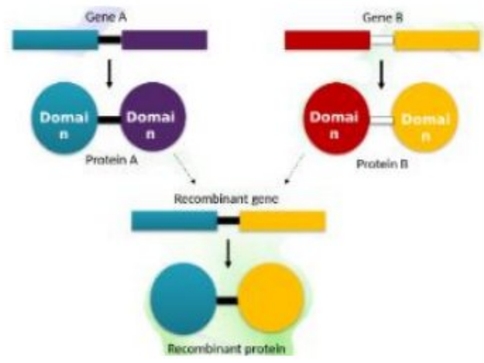


Name	Amino acids	Detection	Purification
FLAG	DYKDDDDK	antibody	FLAG peptide
Green fluorescent proteins (GFP)	~220 aa protein	antibody or fluorescence	None
Glutathione S transferase (GST)	218 aa protein	antibody	glutathione
HA	YPYDVPDYA	antibody	HA peptide
Poly-His	HHHHHH	antibody	nickel, imidazole
Myc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

- **<u>Production of a recombinant protein</u>**
- **Recombinant protein:** Protein produced from **different domains** of proteins using <u>genetic engineering</u>
- **Protein domain:** A compact region (part) of the protein which has a **defined 3D-structure and function**
  - ✓ A domain is a **self-stabilizing** region can **fold independently** from the other parts, and so it can **function** probably if it is **disconnected** from the rest of the protein
  - ✓ A protein can have several domains which determine its structure and function



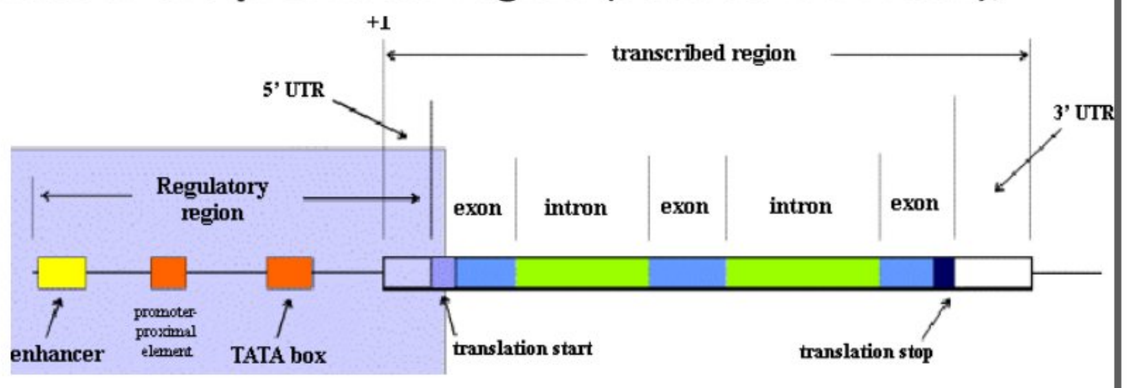
➢ Each protein produced from its gene → if these **two genes expressed all together at the same time** ➔ being transcribed together into a single mRNA → being translated as a single unit forming the recombinant protein



➢ Here we have **gene A** which produce **protein A** with <u>2 domains</u>, also we have **gene B** produce **protein B** with <u>2 domains</u> ➔ we can make a protein with a <u>domain from protein A and different domain from protein B</u> resulting in **Recombinant protein**

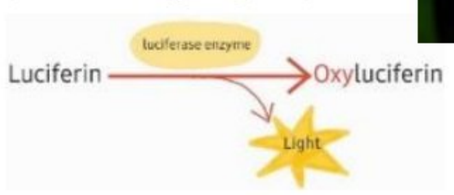
❖ Analysis of transcriptional regulatory sequences

- A eukaryotic gene Contains:
  - <u>Transcribed region</u> ➔ consist of **transcriptional initiation site** (+1 site), **exons** and **introns**
  - <u>Regulatory region</u> (non-coding sequences): sequences **that regulate the gene activity**, either positive regulation or negative regulation ➔ consist of the **promoter region** (such as TATA box), **promoter proximal elements** (like operon sequences), **enhancers** (<u>positively regulate</u>) & **silencers** (<u>negatively regulate</u>)



❖ Firefly luciferase

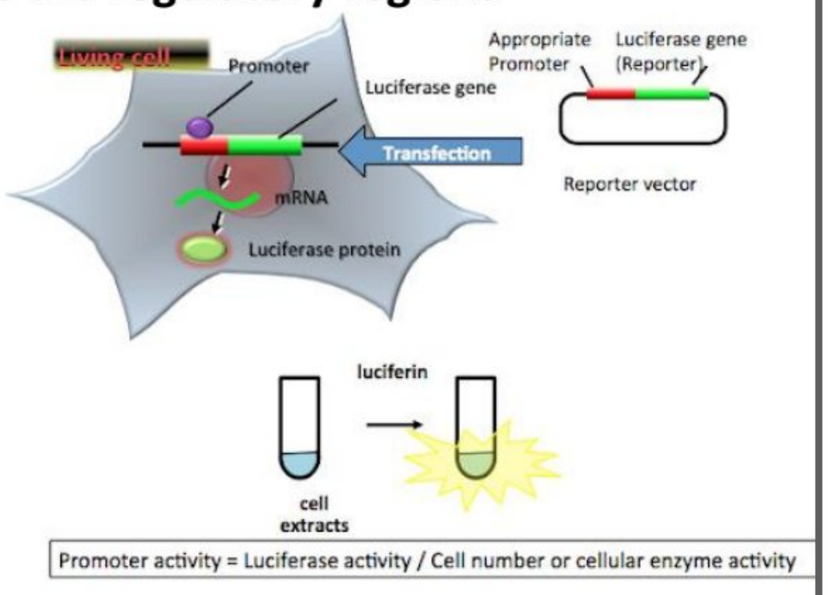
- **Luciferin:** A molecule present in the **firefly** body which make them <u>fluoresce</u> (emitting **light**) at night when it converted into **Oxyluciferin** by **Luciferase enzyme**



- **Luciferase reporter assay:**
  - ➢ We use this technique **(the purpose)** ➔ to study the <u>activity of a gene</u> at certain conditions or elucidate (explain) the <u>function of certain regions of the promoter</u>

- **Reporter gene:** Gene used to know the <u>importance of a certain region</u> (**regulatory**) under certain conditions in **regulating gene expression** ➔ examples: Luciferase gene
  - ➢ The only sequences present <u>upstream to the reporter gene</u> are the **regulatory regions**

- To measure the activity of the regulatory elements of any gene, we extract the **<u>regulatory region</u>** of this gene and place it in the plasmid **<u>upstream to the reporter gene</u>** (Luciferase gene)
- The plasmid is transfected (inserted) into the cell and then the **level of luciferase** (not the original gene) expression is <u>measured</u> and analyzed

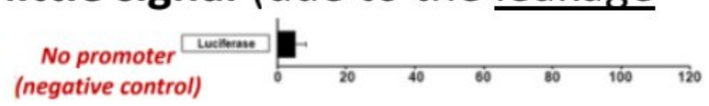


- We analyze the level of luciferase as the following:
  - If the **gene of interest** should be **highly expressed** ➔ more activation by the regulatory elements → **luciferase gene** would be highly expressed → producing **more luciferase enzyme** → converting more luciferin molecules into **Oxyluciferin** ➔ producing more stronger color (the report/signal)

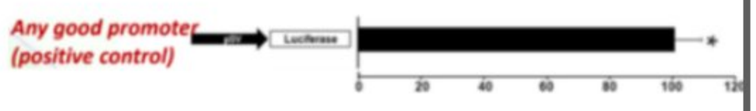
- We study regulation of the gene of interest ➔ by measuring the **amount of light** produced under certain conditions, such as:
  - **Activation (+ regualtion)** → more expression → more light
  - **Inhibition (- regualtion)** → less expression → less light

- The complete promoter contain repressor, activator and the core regions, When there is:

  - **No promoter** → almost **no expression** of the luciferase → **very little signal** (due to the leakage of expression or just a background color) ➔ **Negative control**



  - **Any good promoter** → The **maximum expression** of luciferase → **maximum signal** (light) ➔ **Positive control**

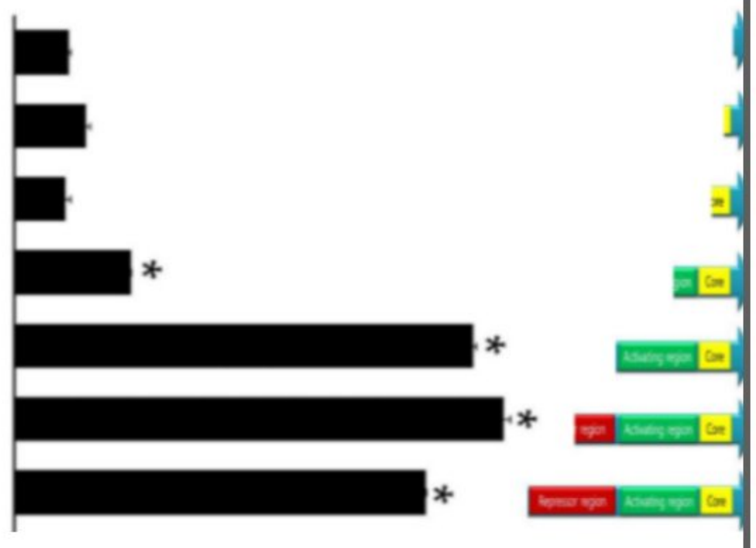


  - **The complete promoter** of the gene of interest → some **expression** of luciferase → **some signal**

  - **The promoter** of the gene of interest with deleted regions:

✓ Remove a **repressor region** → removing a negatively regulating region → **increasing the expression** of luciferase gene → **more the signal** ➔ **Positive control**
  - More removing → more increasing in the expression

✓ Remove an **activator region** → removing a positively regulating region → **decreasing (drop) the expression** of luciferase gene → **less the signal** ➔ **Negative control**
  - More removing → more decreasing (drop) in the expression



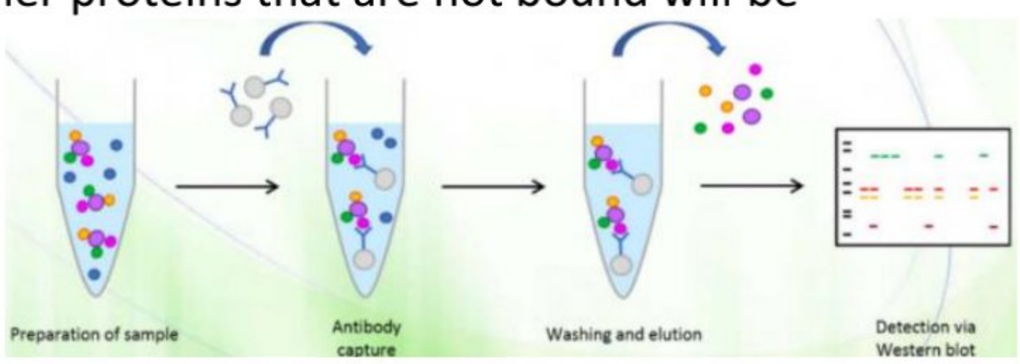
✓ Remove the **core promoter** → almost **no expression** ➔ **Negative control**

❖ Protein-protein interaction

- Proteins interact with each others in order to produce an effect on cells, different domains in the same protein can interact with different proteins

❖ (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 & other proteins bound to it will precipitate (co-precipitated), and the other proteins that are not bound will be removed
- We can use **immunoblotting** (called **western blot**) or **SDS-page** ➔ to study our sample containing the protein of interest & other protein that bound to it



✓ **NOTE :**

- **Southern blot** ➔for **DNA** **Northern blot** ➔ for **RNA** **Western blot** ➔for **proteins**

- **<u>What is a DNA library</u>?**
- It is a **library** can be created **for DNA fragments** (just like book libraries)
- We can have **clones of bacteria** each containing a **specific piece of DNA** ➔ we can save these clones in the **freezer** and take whichever clone we want to study

- **<u>There are 2 types of DNA libraries:</u>**

Genomic DNA library

- The **whole genome** of a cell or a group of cells
- The whole genome contains (coding & non-coding regions): introns, exons, enhancers, silencers ...
- So, each **recombinant DNA (plasmid)** contains fragments from the **whole genome** (everything **coding and non-coding**)

cDNA library

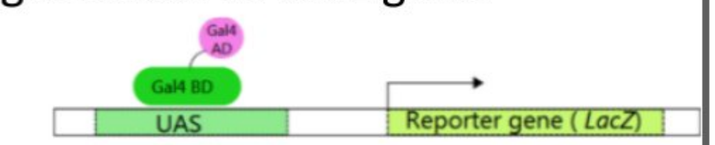
- Contains only **cDNA** (made from mRNA using reverse transcriptase enzyme
- cDNA contains only exons (coding, simpler) including translated and untranslated regions
- So, each **recombinant DNA (plasmid)** contains a specific gene of **only exons** (coding) including **translated and untranslated regions**

The recombinant DNA (plasmid) is inserted into a bacterial cell to be cloned and amplified → then isolating DNA (cDNA or genomic DNA) into collections

- If we want to create a library from cells from different tissue; skin cell, neuron cell, muscle cell :
  - **Genomic DNA libraries** ➔ <u>will be identical libraries</u> because they all have the **same genome**
  - **cDNA library** ➔ <u>will be different libraries</u> because each cell express **genes at different levels**

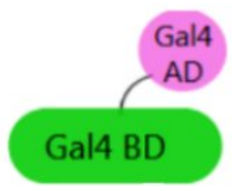
❖ Yeast two-hybrid system

- In **yeast** → there is a <u>regulatory element</u> known as **UAS element** which exist **upstream of the activating sequences** ➔ The **UAS element** is a <u>binding site of a transcription factor</u> **Gal4**
- The binding between **Gal4** & **UAS element** **induces the expression** of a gene such as LacZ gene (reporter gene) in some yeast cells



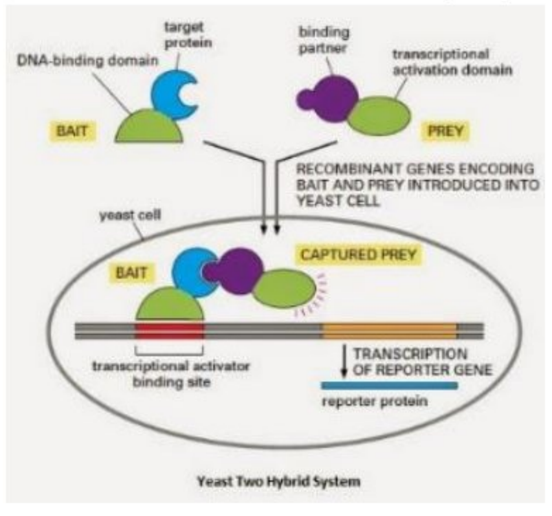
A. Regular transcription of the reporter gene

- **Gal4** protein consist of <u>2 domains</u> :-
  - **A DNA-binding domain** (**BD**) ➔ that binds to the UAS system
  - **An activating domain** (**AD**) ➔that is responsible for the activation of transcription (stimulates <u>RNA polymerase</u> to transcribe the gene)



- Both domains <u>must be close to each other</u> in order **to induce transcription of a reporter gene**

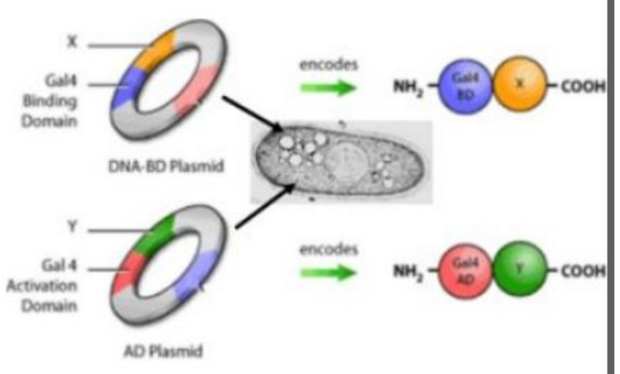
- The purpose of **the yeast two-hybrid system** is ➔ **investigate** if 2 proteins interact with each other, by:
  - ➢ Creating **2 recombinant protein** using **recombinant DNA technology**
  - The 1st protein composed of **DNA binding domain (BD)** of **Gal4** transcription factor & **one protein**
  - The 2nd protein composed of **activating domain (AD)** of **Gal4** transcription factor & **another protein**



Yeast Two Hybrid System

- ➢ If these **2 proteins interact** with each other ➔ then the **2 domains** will be close to each other lead to → a **stimulation or induction of transcription of reporter gene** (LacZ gene)
- ➢ If they **don't interact** ➔the **2 domain** will not be close to each other ➔ no transcription

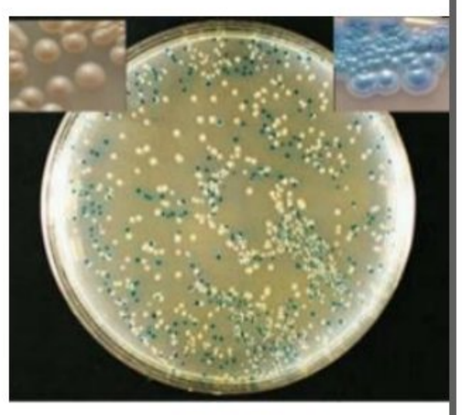
- In order to discover/ identify **unknown proteins** (**Y**) that interact with a **known protein** (**X**)
  - Creating **recombinant plasmid** contains **protein (X) gene** & **DB domain** of **Gal4** protein ➔ the **X** gene is cloned so it is produced recombined with the **DB domain**
  - Creating another **recombinant plasmid** contains **proteins (Y) gene** & **activating domain** of **Gal4** protein ➔ **cloned** so it is produced integrated (recombined) with **AD domain**



- Both **recombinant plasmids are transferred into yeast cell (each yeast cell has the 2 plasmids)** so all of them express the **X-DB hybrid**, but each one expresses a different **Y-AD hybrid** (Y1, Y2, Y3...)

- Yeast cells are grown in the presence of a **lactose analog called X-gal**, which generates a **blue product when cleaved by β-Galactosidase**.

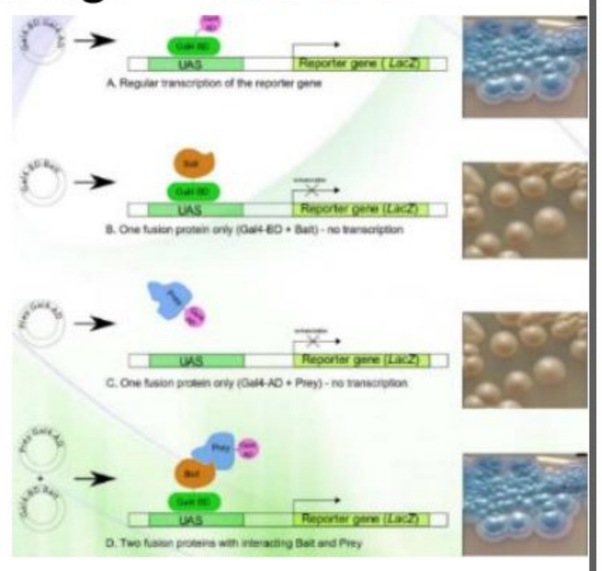
- If the 2 protein (**X & Y**) interact with each other ➔ then they will **stimulate transcription of LacZ gene** ➔ producing **β-Galactosidase** ➔ which cleave **X-gal** (instead of lactose) ➔ generating **blue colonies**.



- If the 2 protein (**X & Y**) **don't** interact with each other ➔ **NO transcription of LacZ gene** ➔ **NO β-Galactosidase** ➔ **NO cleavage** of **X-gal** ➔ **NO** blue color (the colonies would look in **white color**).

➢ **The possibilities and outcomes of yeast cell that produce :**

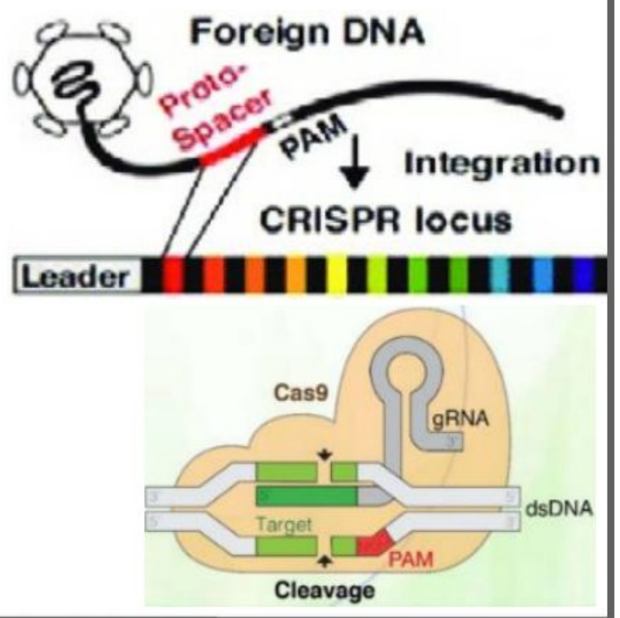
1) **Normal Gal4 (with its 2 domain)** ➔ (transcription) → **β-Galactosidase** ➔ **cleavage** ➔ **blue color**
2) Only **the DNA binding domain** with **X gene** ➔ **NO** transcription ➔ **white color**
3) **Only the activation domain** with **Y gene** ➔ **NO** transcription ➔ **white color**
4) **X gene** with **Y gene** together :
   - If the X & Y interact with each other ➔ **blue color**
   - If the X & Y **don't** interact with each other ➔ **white color**



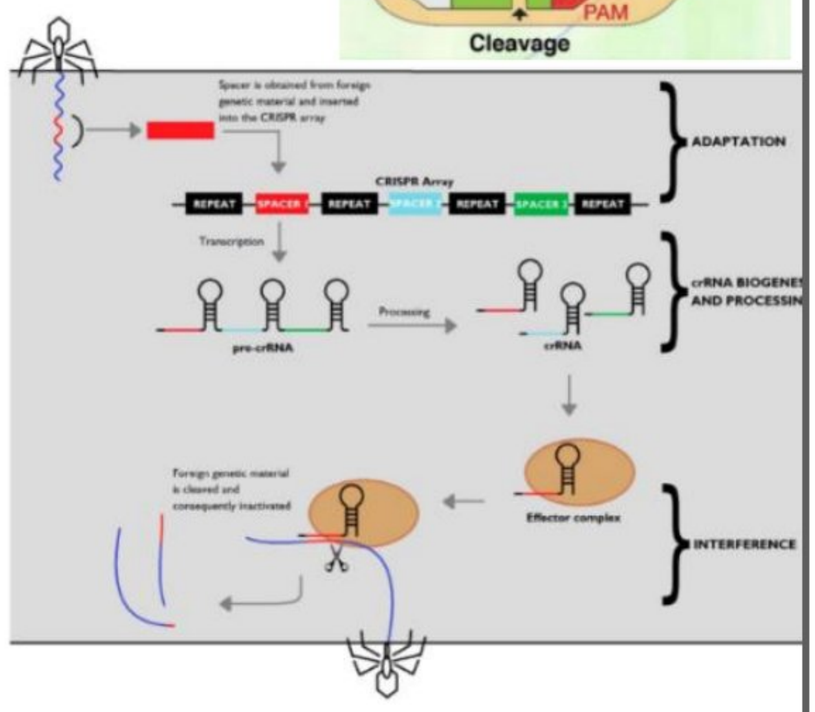
- Blue yeast colonies are picked and plasmids are isolated to identify the **unknown genes/proteins** that interact with the **known gene/protein** , then we identify this gene (using **sequencing**, **PCR**, **immunoblotting** …)

❖ CRISPR-CAS9 and gene editing

- **CRISPR:** clustered regularly interspaced short **palindromic repeats** exist as <u>bacterial genetic</u> system that constitutes the **immune**
- **Cas9 (Ribo-nuclease):** <u>RNA-guided nuclease</u> that can either **create single or double strand breaks**



- In the adjacent figure, a genetic system <u>exist in bacteria</u> of **palindromic repeats** (in black) separated by other viral sequences (colored)
- Cas9 is associated to a short RNA fragment known as **guide RNA** (gRNA) or **single guide RNA** (sgRNA) that <u>direct the nuclease into its target on DNA</u>
- **RNA** molecule is **complementary** to the target segment of the genome



- When a phage infects a bacterial cell it inserts its DNA into the cell, the cell degrades the phage DNA into smaller pieces and <u>integrates one of these fragments into the CRISPR</u>
  - ✓ The palindromic repeats are **hydrogen bounded** and form **stem-looped** structure
- The cell transcribes the **DNA** producing several **RNA molecules** (gRNA) ➔ These **RNA molecules** integrated into the **Cas9 nuclease** and guides it to the <u>same</u> phage DNA (when it infect the cell again) to degrade it.

- **DNA repair mechanisms in human cells**

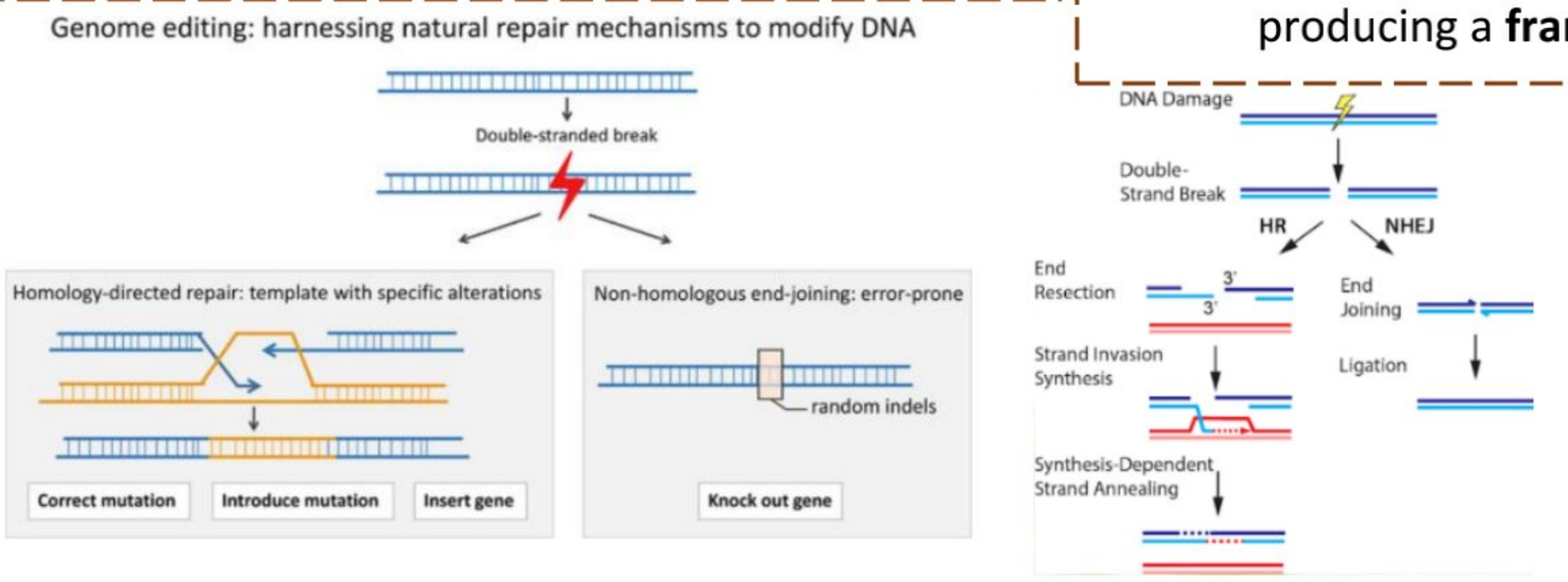
We have 2 repair systems when DNA has breaks:

Homology directed repair (HR)

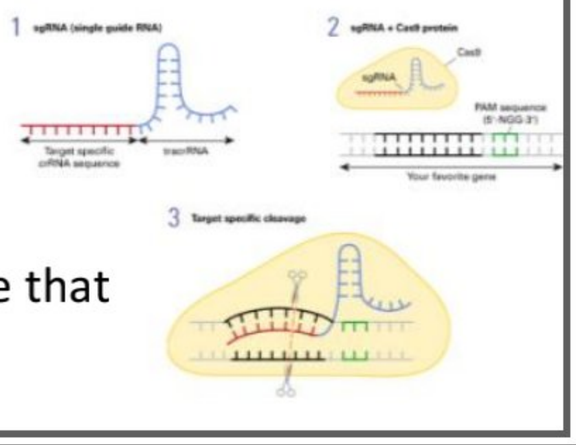
- When we have a **DNA** break ➔ the cell benefits from the present of the homologous DNA (**diploid cells**)
- We use a part of the <u>homologous chromosome as a template</u> to fill the gaps (breaks) in the damaged DNA

Non-homology & joining repair (NHJE)

- It **does not** depend on the presence of the homologous DNA)
- This system of repair produce ➔ **glued DNA molecule** ➔ that creating **indels** ➔ creating **defective gene (mutated or nonfunctional)**
  - ✓ **Indel** insertion or deletion of nucleotides producing a **frameshift** mutation

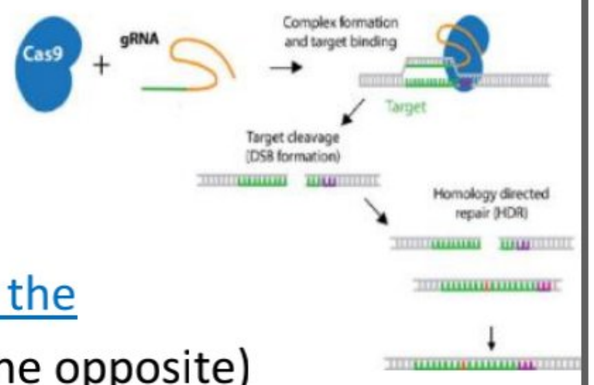


- By the recombinant DNA technology (cloning a gene into a plasmid) → **Cas9 gene** & **RNA molecule** that would integrated into **Cas9 protein** ➔ can be introduced into human cells
- Cas9 protein <u>is directed</u> (by **gRNA**) to a <u>region we want</u> in human chromosome that is **complementary to this RNA** → HDR system replaces the damaged DNA

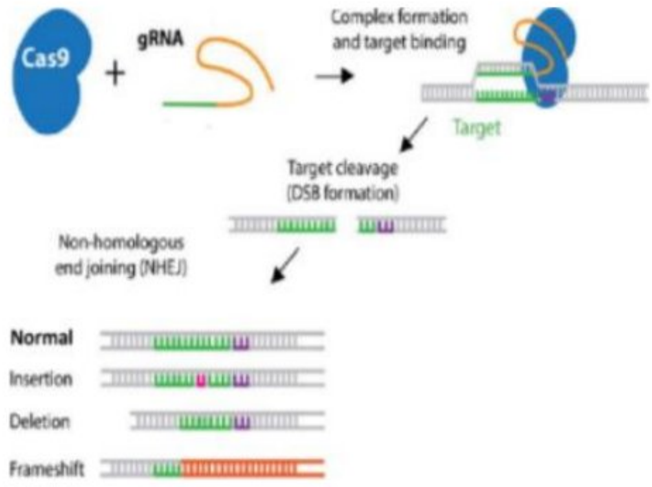


- **<u>We can use Cas9 protein in gene editing:</u>**

  - **Replacing a defective gene with the normal one:**
    - Introducing **Cas9 gene** + prober **gRNA** (Directing Cas9 to the region we want to replace)
    - Also we introduce the DNA to be inserted (the **normal DNA**)
    - Cas9 cut ➔ activation of the Homology directed repair system (it will use the DNA we introduced) ➔ a mutated gene is replaced by a normal one (or the opposite)



  - **Mutating a normal gene to be defective to study what will happen**
    - Introducing **Cas9 gene** + prober **gRNA** + **a defective DNA**
    - Cas9 cut the normal gene out and defective one is inserted (by HDR) to the chromosome ➔ then we can study what this gene will affect or what the importance of the normal gene



OR

    - We introduce **Cas9 gene** + prober **gRNA** **only**
    - Non-homology & joining repair system ➔ the cells will take the DNA and glue it ➔ creation of **indels mutations** ➔ leads to **a frameshift mutation** ➔ damaging the gene

- **<u>We can use Cas9 protein in other creative uses:</u>**

  - **We can use the Cas9 as a guider:**
    - By **associating this protein to Cas9** & its gRNA → and **mutating Cas9** so it bind a certain sequence without cutting it ➔ so it can guide the protein associated to it to the region where in functions

  - **Can used to visualize where the plasmid is taken to in the cell**
    - By associating Cas9 gene with GFP → giving a signal (light) → so we can visualize the where the plasmid that contain this gene is taken to

- **<u>Other Cas9 enzymes :-</u>**
  - **Cas12a**: a smaller enzyme that produce staggered cut rather than blunt cut
  - **Cas13a**: a specific enzyme for RNA nuclease

- Scientists are trying to edit our DNA & fingerprint or to change it using Cas9 system, cut certain region either to fix them or to stop genes, and make a pill of the DNA contain this system
- Scientists are also trying to do a genetic modifying of the human embryos by using Cas9 system
  - One of the experiment of modifying DNA disturbing a CCR5 gene which is important for the entry of HIV virus into our cells and the intelligence

- **Bioterrorism**
  - Certain things that can be done including modifying human genome; modifying human microbiome, modifying pathogenic bacterial genome and so on → causing dangerous conditions