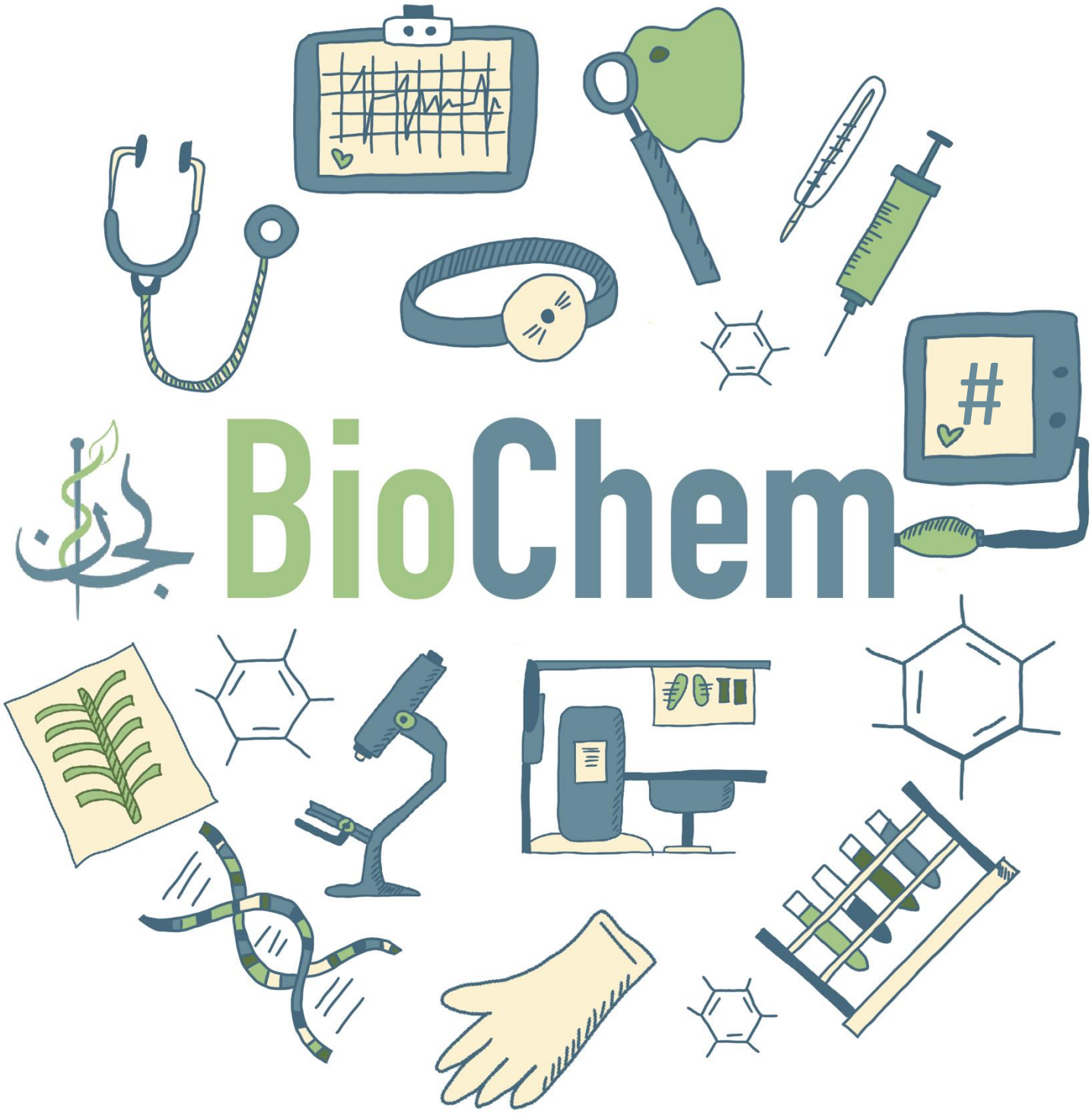

BioChem

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DNA CLONING

DNA cloning is a technique that allows for amplifying a DNA segment into many, many copies in a biological system like ^bacteria^ .

In another words if I say I want to clone DNA, it means that I want to make multiple copies of this DNA fragment.

DNA Cloning usually involves :

I. The formation of a recombinant DNA composed of a vector (a carrier; usually a bacterial plasmid) and protein-coding gene using restriction endonucleases usually it contains 2 or more pieces of DNA coming from different sources .
II. Insertion into bacteria.

Nucleases are enzymes that cleave nucleic acids (DNA, RNA) specifically we have:

1-**Endonucleases** that cut DNA in the middle of the molecule .

2-**Exonucleases** which cleave the DNA from the end(3 prime /5 prime) of the molecule.

Restriction Endonucleases: bacterial enzymes that recognize and cut (break/cleave) the phosphodiester bonds between nucleotides within DNA molecule at specific sequences (4-8 bp restriction sites) generating **restriction fragments**.

Why are they called restriction specifically :

1. Cause they are restricted where they cut .

2. they restrict the growth of bacterial phages .

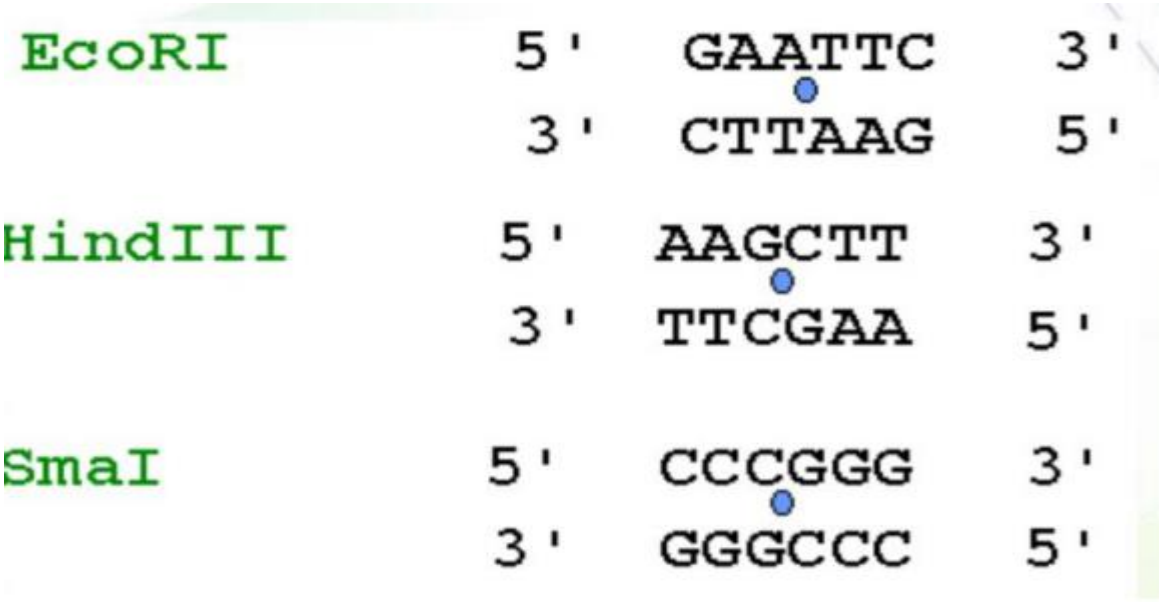
For example, in this figure restriction endonucleases recognized specific sequences and cut between C and G to generate restriction fragments.

Palindromic sequences

The sequences recognized by restriction endonucleases—their sites of action—read the same from left to right as they do from right to left (on the complementary strand)

They are read the exact same way from 5 prime to the three prime .

Examples of restriction endonucleases with palindromic sequences

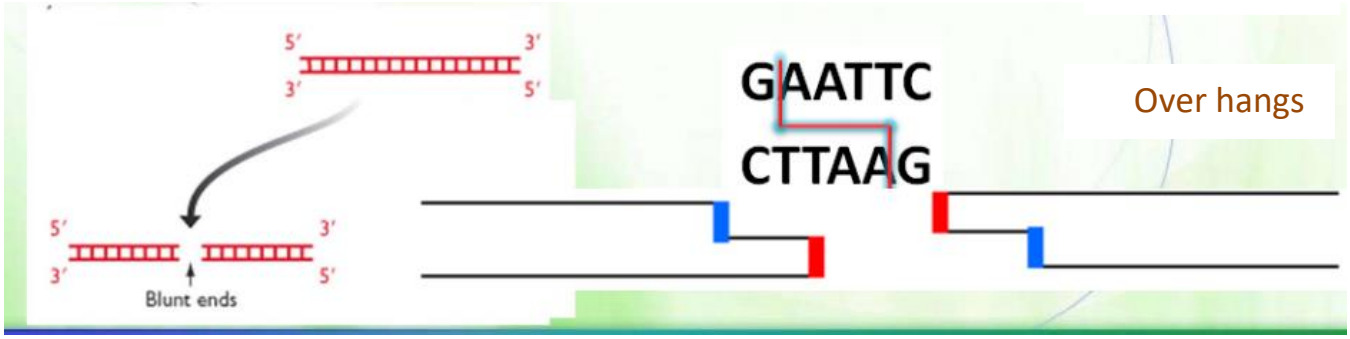


Restriction enzymes cut DNA in two different ways:

- Blunt: enzymes cut at the same position on both strands (at straight line) giving blunt ended fragments.
- Staggered (off-center): enzymes cut the dna strands at different positions generating sticky or cohesive ends .

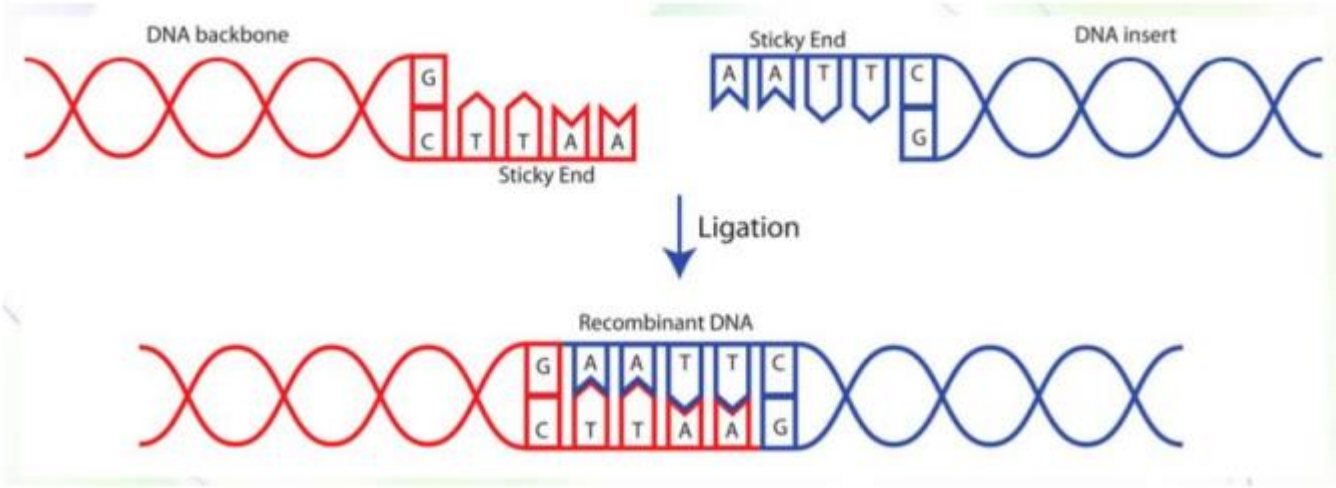
In the staggered The DNA fragments have short single-stranded overhangs at each end.

BLUNT ENDS **STAGGERED**



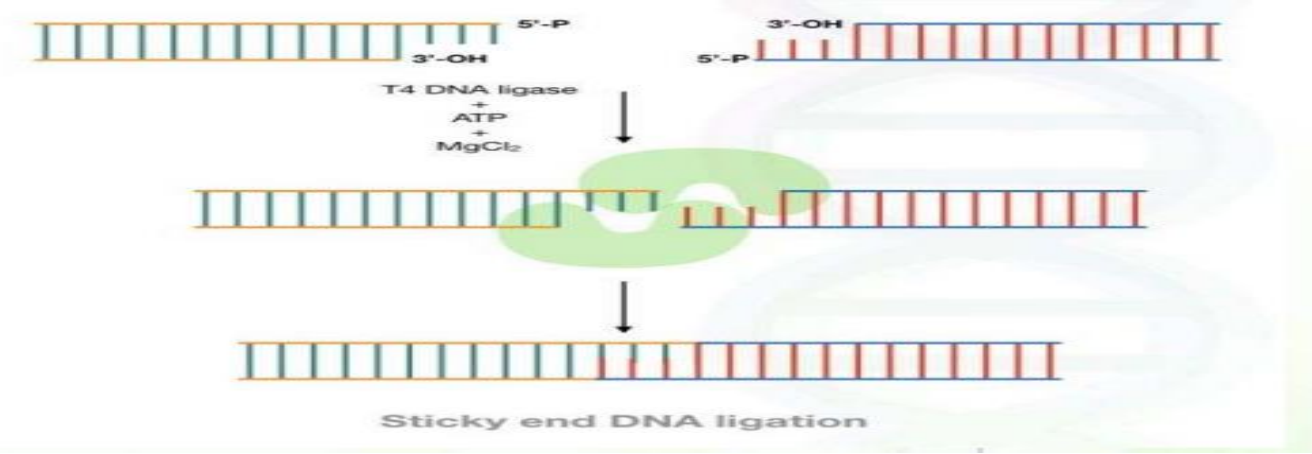
Note: the two kinds of cuts cannot be made by the same restriction enzyme. To make it clearer: There are restriction enzymes that can make blunt cuts and others that can make staggered cuts, but it's not possible for the same restriction enzyme to make both kinds of cuts

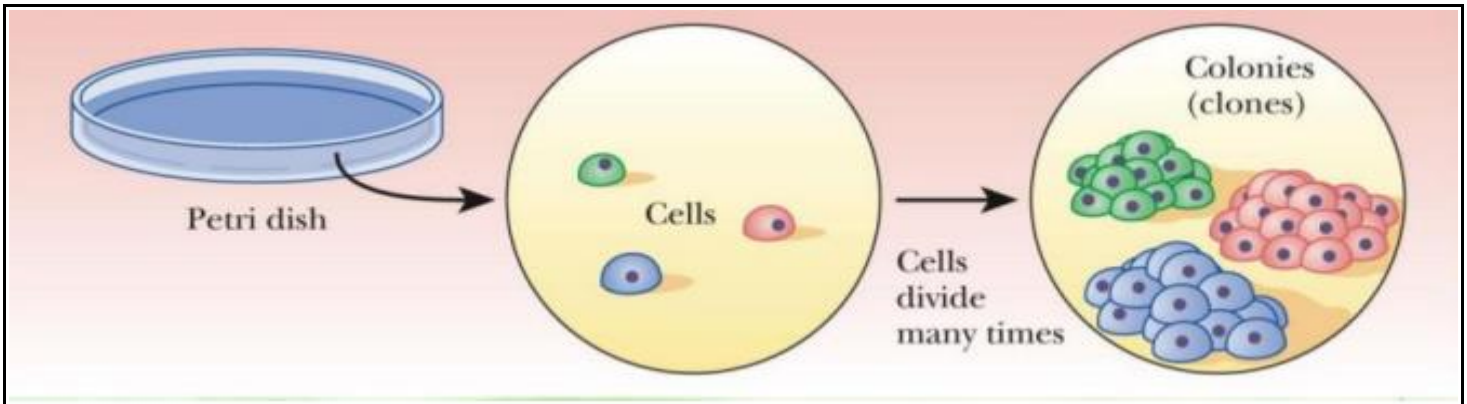
For example: we have a cut between G and A in the first strand and on the other strand the cut occurs between the G and A resulting in the formation of **"sticky ends"** we called them "sticky ends" BECAUSE the ends are complementary to each other which mean the ends can form hydrogen bonds between each other except that they are not stable **(why?)** because there are **no phosphodiester bonds** between nucleotides.



However hydrogen bonding are not enough SO we use **ligase** which covalently joins DNA ends (example, restriction fragments) by catalyzing the ATP-dependent formation of phosphodiester bonds between the 3 prime-hydroxyl group of one strand and the 5 prime phosphate end of another strand .

THIS process is called ligation (like gluing) of fragments with each other to become stable.





NOW lets talk more about cloning :

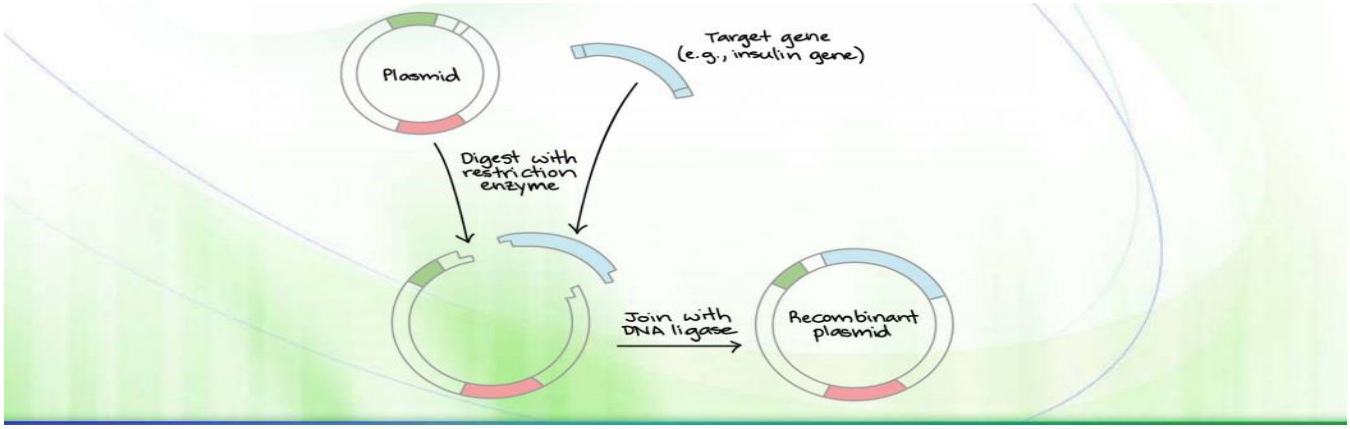
A CLONE is genetically identical population whether of organisms, cells, viruses, or DNA molecules.

Every member of the population is derived from a single cell, virus, or DNA molecule.

Cloning is really something natural in bacteria because we can have the bacterial cells in the plate (petri dish).

One single bacterial cell will eventually after relatively short time form a colony, each colony is originally cloned of one particular cell (these cells are identical because they have the same DNA sequence) .

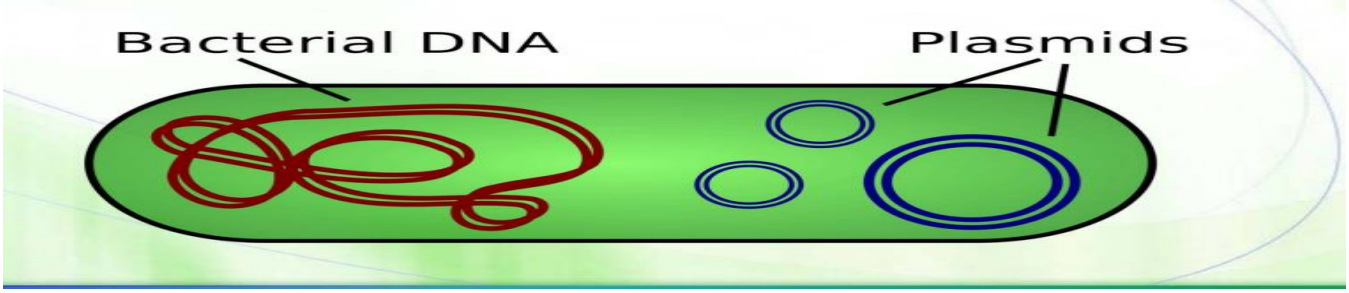
NOW lets talk about how we clone DNA molecule :



I. We Cut A single cut in the bacteria plasmid by specific endonuclease .
II. Then we cut in the target gene (DNA fragment) by the same previous endonuclease to make a complementary sticky ends .
III. we use ligase to make recombinant DNA .
IV. we insert recombinant DNA in the bacteria then bacterial cells make multiple copies of same plasmid.

If we want to remove the DNA fragment(target gene) from the plasmid?

We use the same endonuclease we used before to cut.



DNA PLASMID: is an excellent vector and it is extra circular chromosomal DNA pieces which is separate from the main circular bacterial DNA .

Features of plasmids (most plasmids vectors contain at least three essential parts required for DNA cloning):

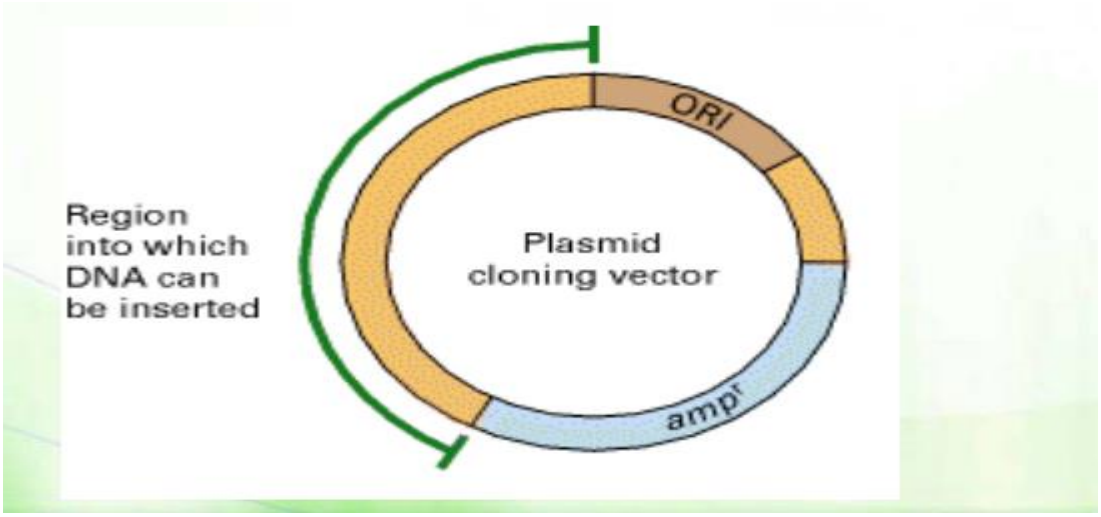
1.Can replicate independently of the bacterial chromosome and must have an origin of replication (ORI) which is a nucleotides sequence that DNA polymerase binds with and starts replication.

2. A foreign DNA fragment can be inserted (an insert) into it ^^in the site that is called cloning site where endonuclease claves^^ , this process is called recombinant DNA technology.

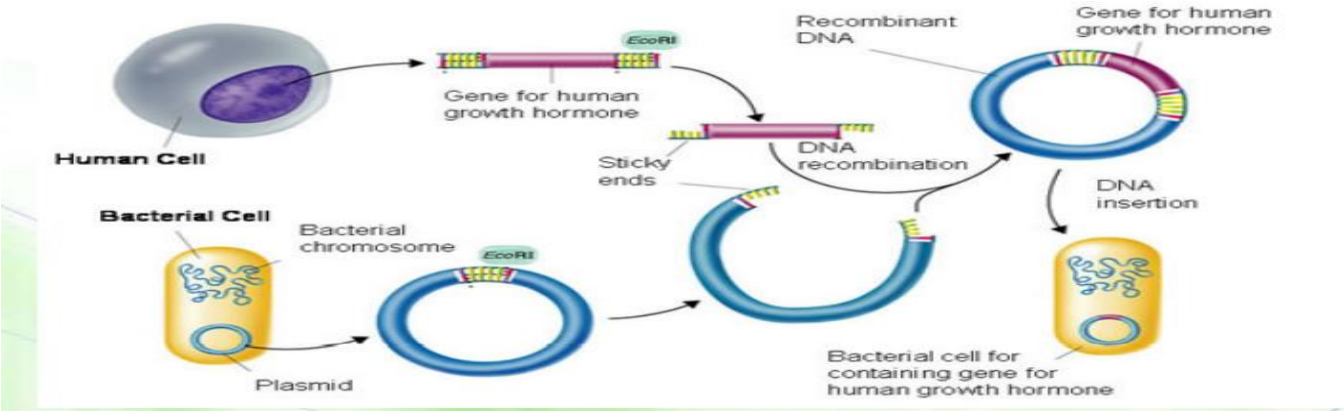
3. **(selectable marker)** Can be selected for /against by an internal drug ' resistance gene LIKE AMPr * **ampicillin** * in the figure .

* The bacteria that has a resistance gene in its plasmid can be distingushed from other bacteria that don't have when we use antibiotic

لما نستخدم مضاد حيوي البكتيريا الي عندها جين المقاومه رح تضل والي ما عندها رح تموت وهيك بنميز البكتيريا الي فيها البلازمايد الي بهمنا



Making The recombinant DNA



1.Take a piece of DNA from a human cell then cut this DNA by a restriction endonuclease

2.Cut a plasmid with the SAME restriction endonuclease **that will make just only one cut** because if it makes more than one cut it will become not cerculer and lose its shape

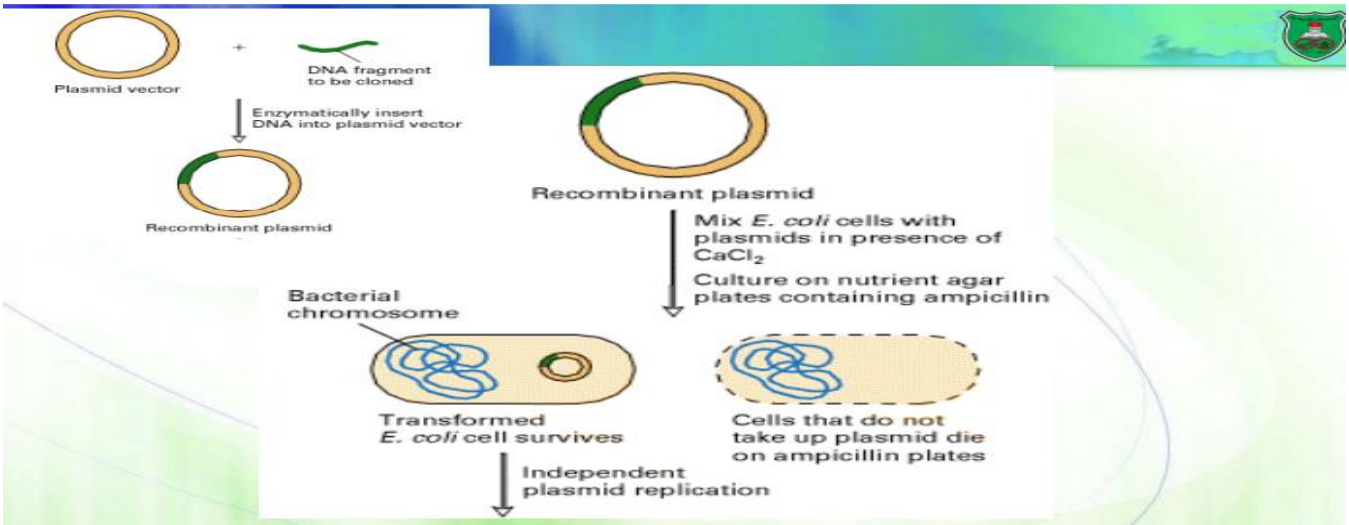
3.Combine human DNA with plasmid DNA to form plasmid DNA containing the human DNA fragment.

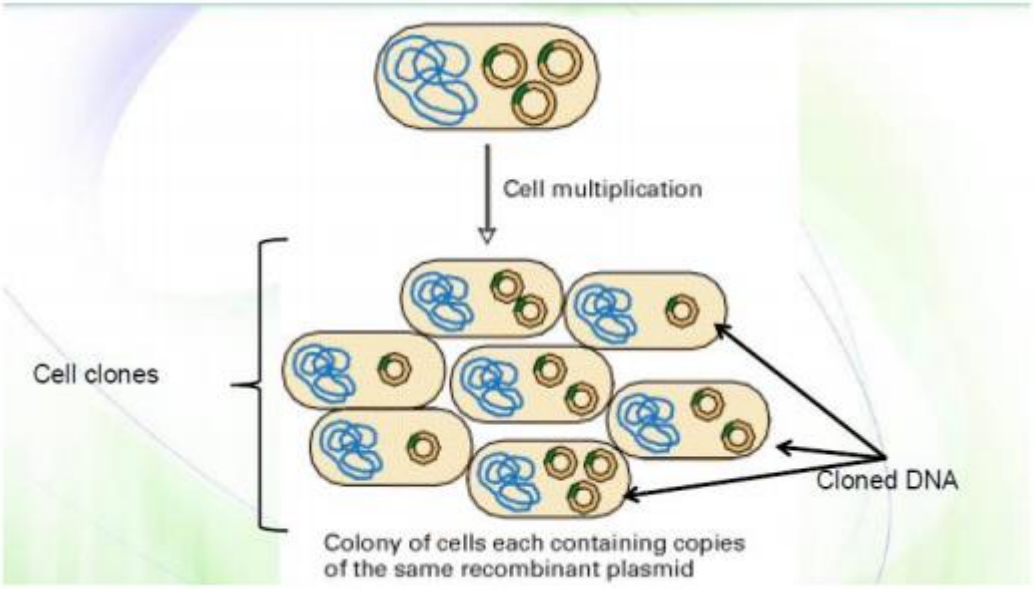
4.Add DNA ligase to make phosphodiester bonds creating a stable plasmid.

5.Finally, take this plasmid and put it back to the bacteria.

We use the same restriction endonuclease so we can have the same cohesive ends and they are complementary to each another (they hybridize with each other when mixed)

A bacterial cell can have at least one plasmid BUTTTT ONLY one chromosome **





We can take advantage of plasmid by not only cloning DNA fragment we can also make bacteria express a gene (in another words making m-RNA which can be transcribed and translated into protein and this is used in medicine).

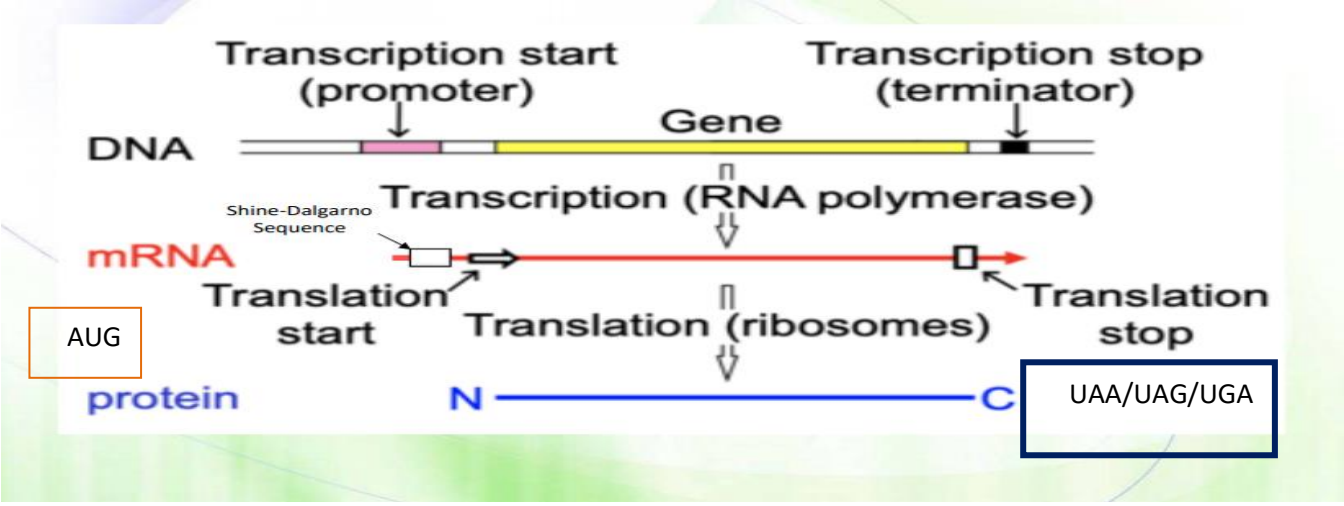
For example: insulin (for people with diabetes), growth hormone, plasminogen activator (which is used in blood clotting), erythropoietin.

Expression vectors contain additional sequences in order to make bacteria produce protein:

1.Promoter sequences (region where the RNA polymerase binds to in order to start transcription) located upstream of gene (before it).

2.Ribosomal binding sequences (Shine-Dalgarno [SD] sequences)

3.Transcription termination sequence The protein is expressed and then purified

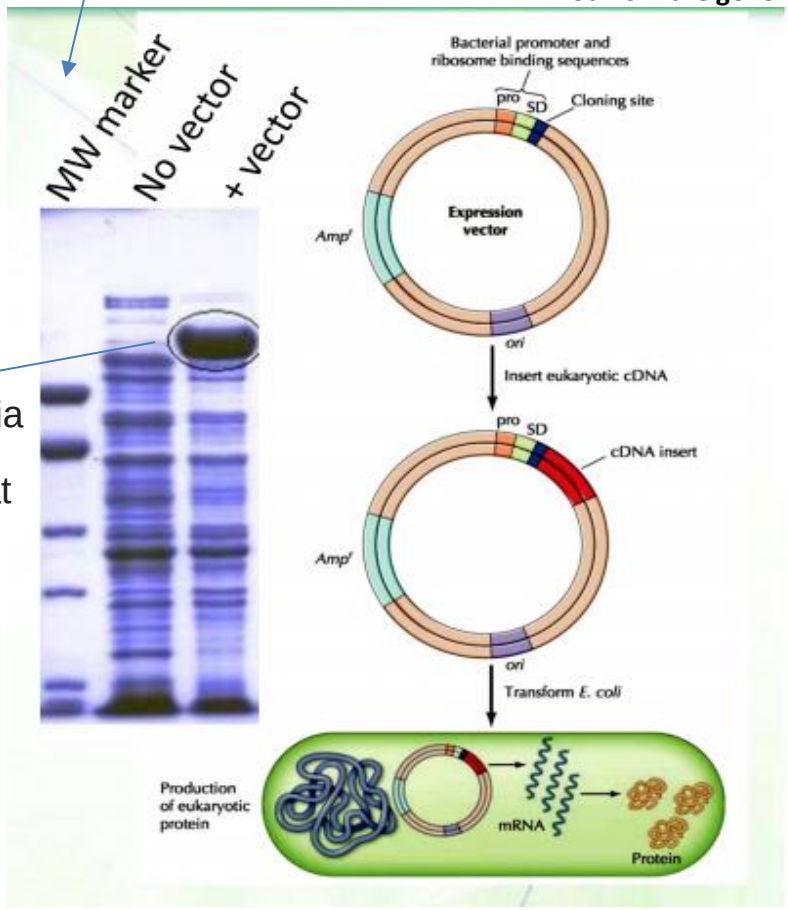


Pay attention promoter site are from plasmid /SD/CLONING not from the gene

THIS process is called **SDS-PAGE** is an electrophoresis method that allows protein separation by mass. Each band represent a protein .

This is the protein we let the bacteria Produces from the human gene that we inserted in the plasmid.

Cloning site : sequence where endonuclease recognize and cut .

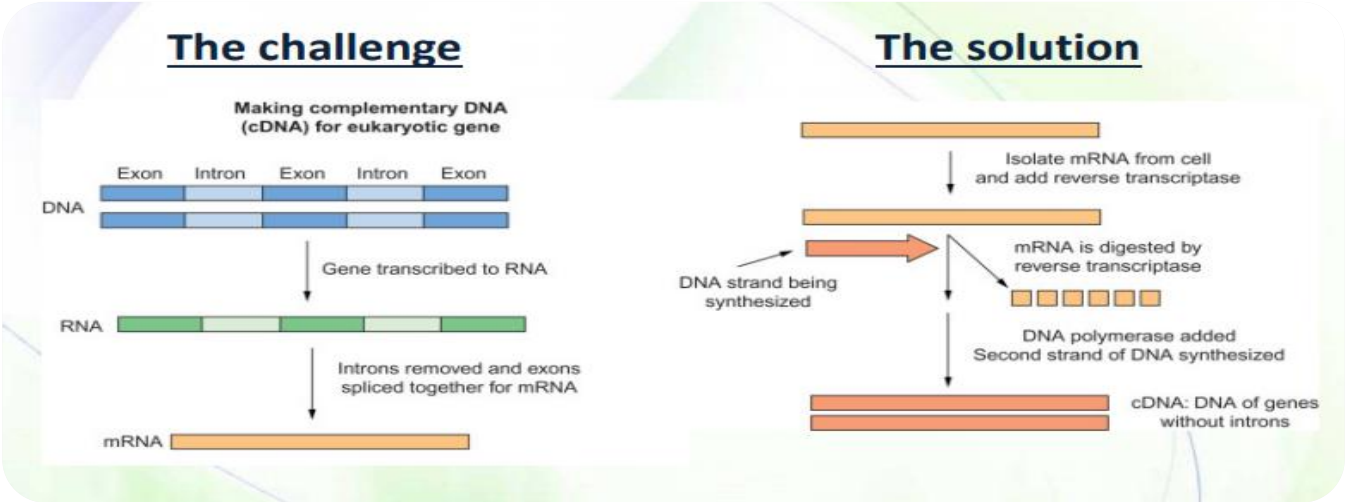


How do we deal with introns in human genes?

THE first challenge we face that we don't have a splicing techniques in bacteria so we cant get rid of introns so we need use a final product m-RNA then we use a reverse transcriptase to make a double stranded DNA that doesn't have introns

And can make final m-RNA from it **THE C-DNA that we will have we are going to put it in the plasmid .

بجيب ال ار ان ايه جاهز مجهز وبعمله دي ان ايه بالعكس وبعدين بحطو بالبلازميد ف البكتيريا



OTHER Challenges appear when producing human protein in bacteria:

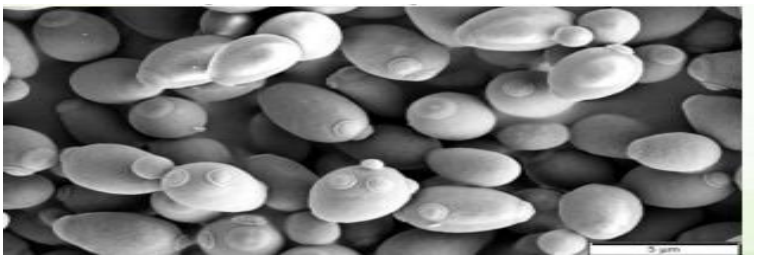
1. some human proteins have disulfide bonds (bonds between amino acids)but bacterial cells don't have these bonds (they don't have the enzymes to create them)

2.proteins in human cells can be modified by for example glycosylation (addition of sugar to proteins) so some human proteins are known as glycoproteins and this cant occur in bacterial cells

3.misfolding :folding that is formation of a protein with a functional structure may not happen in bacteria cause bacteria doesn't have folding assistance

4.degerdation because bacteria will recognize these proteins as foreign substances so they will degrade them .

Solution: use a eukaryotic system such as yeast because they are single cells and grow fast like bacteria.

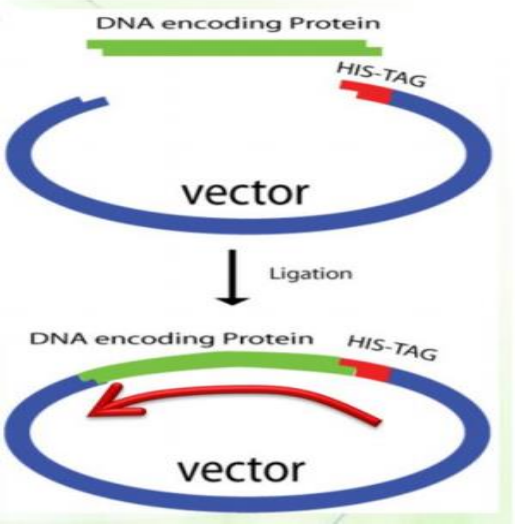


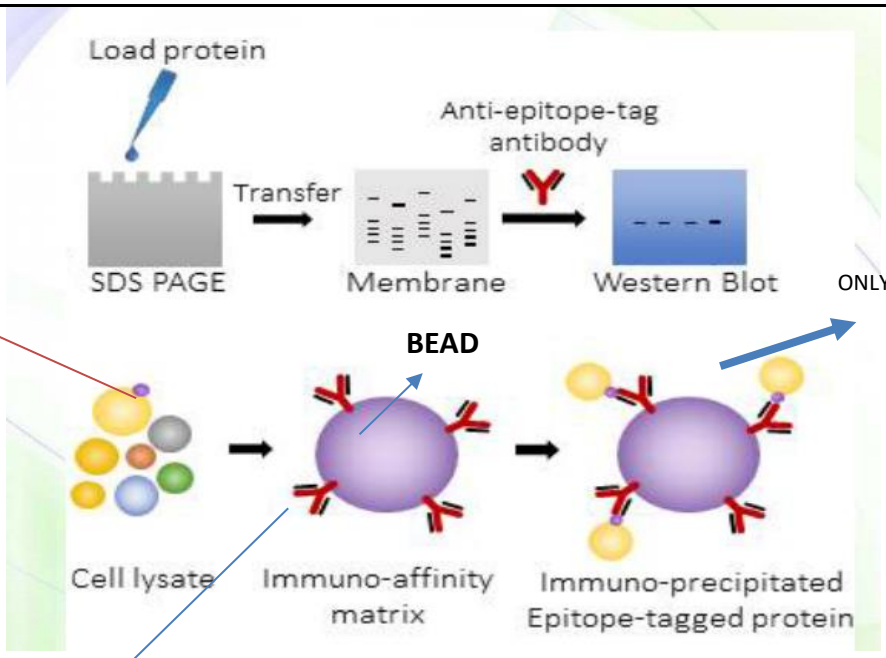
Protein tagging or creation of protein hybrids

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.

What is the important of tagging?

These tags allow for easy protein purification and detection We have small tags such as six histidines attached to a protein and some tags are large proteins (in this case we would have two proteins link to each other, proteins of enhancers and as well as another protein like GFP OR enzyme like GST





When we add the anti body it will bind with tag only not with protein in other words

The anti bodies are specific for tags not for proteins so we can detect and purificate the tagged proteins .

Note : tags don't effect on protein function at all or its structure

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

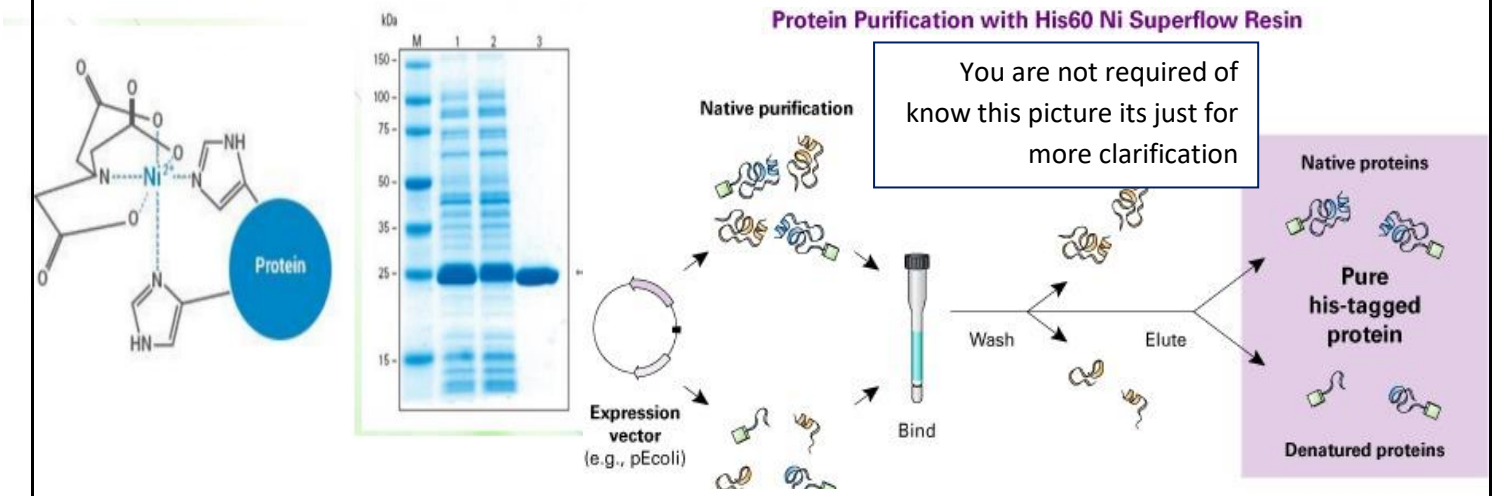
** **the doc focus on the red ones**

His tag:

(THERE are no natural protein that have his_tag , only the proteins we put in)

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

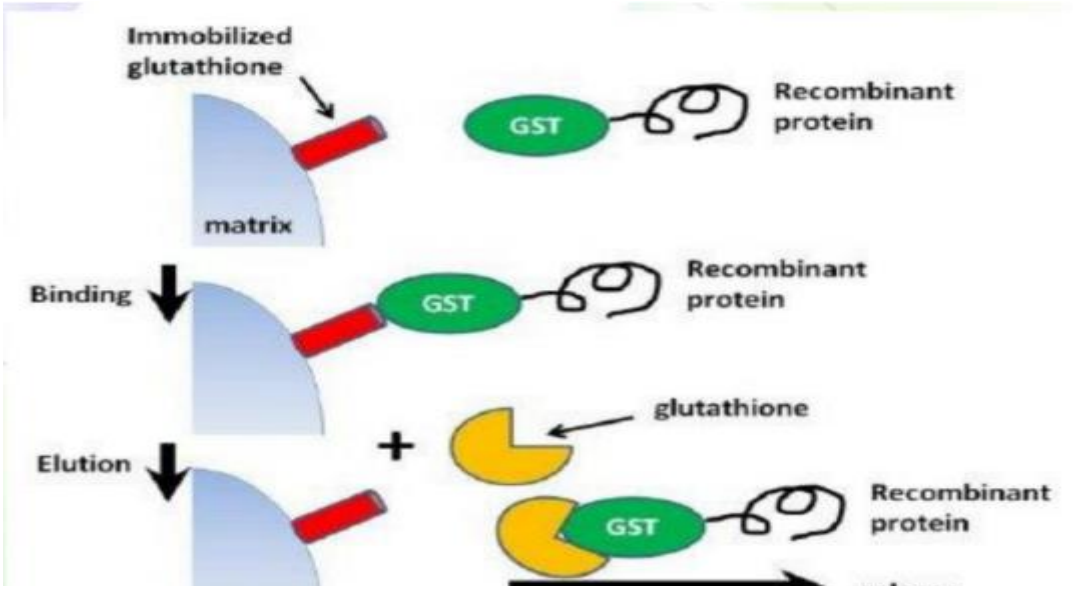
The importance of six-histidines is that they can bind to a metal like nickel to isolate it from other proteins that don't bind to nickel as we will show in the figure below:



Purification of GST tagged proteins:

Protein hybrid: a protein that consists of multiple different proteins.

Glutathione S Transferase (GST) is an enzyme that binds to a specific substrate which is glutathione (a tripeptide).



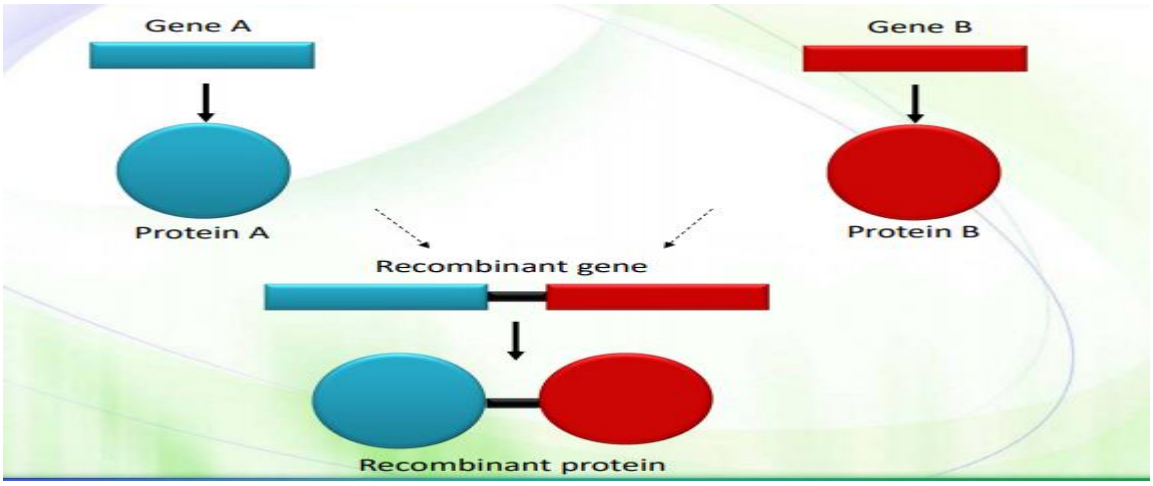
We can design beads which attached to glutathione residues, and when our sample passes through the beads, only GST-tagged proteins will attach to the beads, other proteins are washed away.

Then, we can release the GST-tagged Proteins by adding free glutathione so that the GST-tagged proteins bind to it and move along with it and are then collected.

Production of a recombinant protein

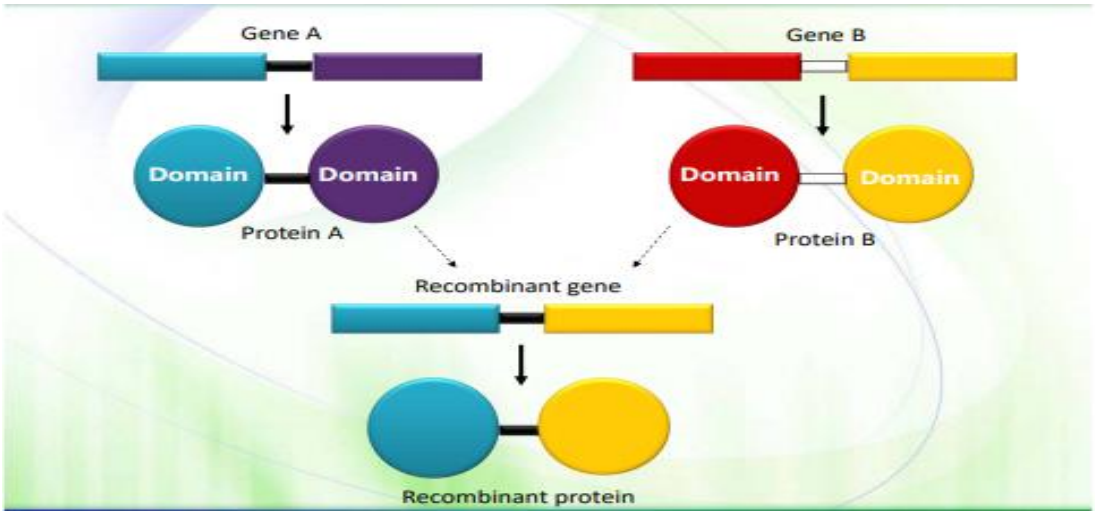
WE can make recombinant protein in two different ways : the first one we can mix 2 gens OR mix 2 domains from different proteins .

- We put gene A (known protein) and gene B (that I want to study further) together next to each other so when they are transcribed and translated they will produce one large protein linked to each other this is known genetic engineering.



- Another example is that we can take one domain from a two domain protein and another domain from a different two domain protein and put them together to make a recombinant protein (this protein will have two domains each from a different protein)

Note that both genes have just one only terminating site and one sd sequence one promoter .



Green Fluorescent Protein (GFP)

One of strong examples about separate function of domains in one protein is GFP .

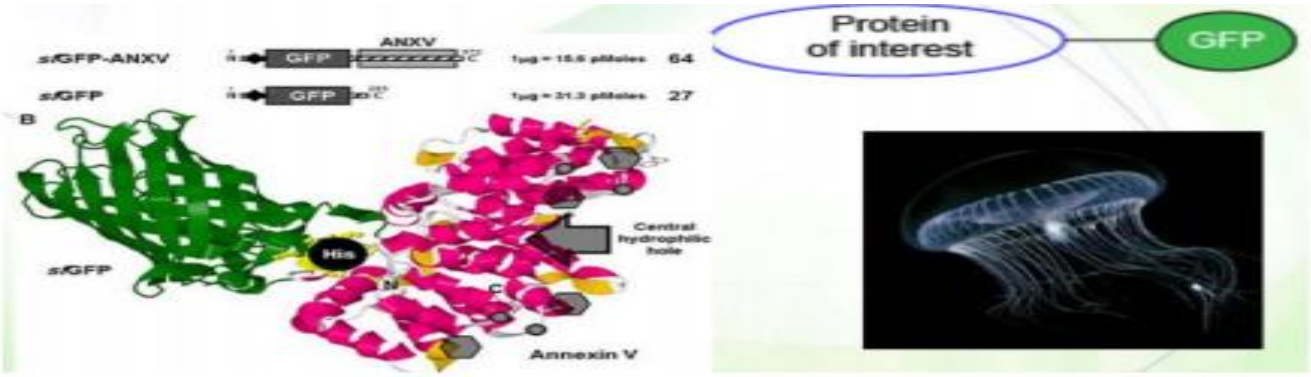
IN OTHER WORDS :The GFP portion of the GFP-tagged protein folds independently of the protein of interest, but it is attached to it. (The same concept as domains). Both proteins maintain their function.

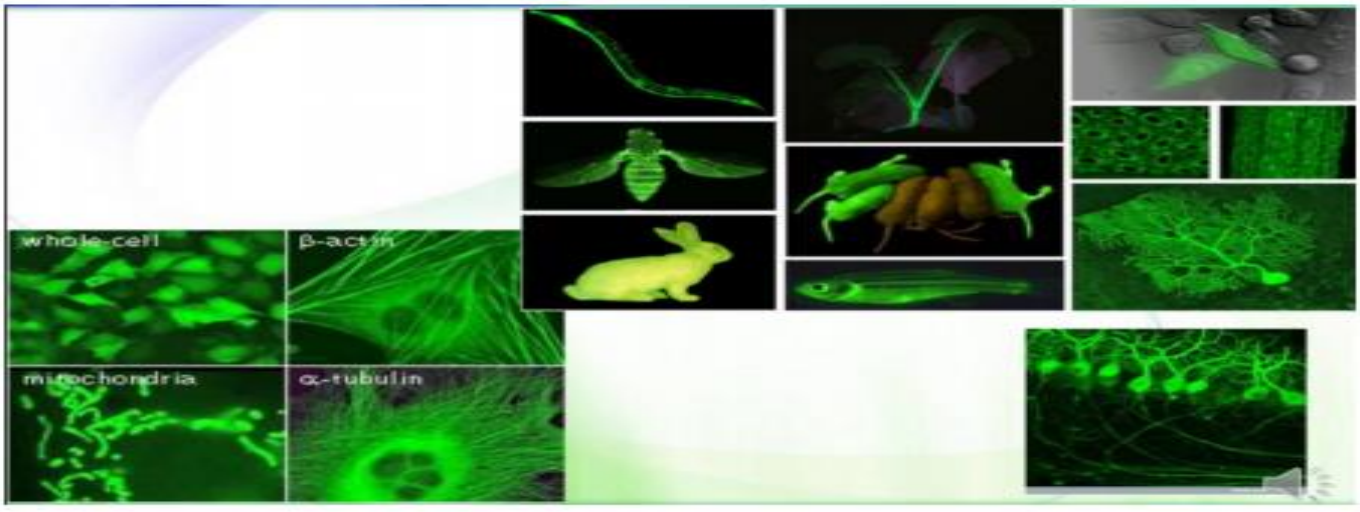
IMPORTANT:_Green Fluorescent Protein (GFP) allows for protein detection rather than f or purification purposes.

GFP (Green Fluorescent Protein): it is a protein that comes from jellyfish (these living creatures give a fluorescent color due to production of GFP) and scientists took advantage of this protein in tagging. They were also able to separate proteins with other colors later on.

(GFP is a fluorescent protein which gives a green color, so GFP-tagged proteins would obtain a green color which helps in labelling and detection. Examples of proteins that can be labelled with GFP: actin, tubulin, mitochondrial protein.

Whole cells can also be labelled (like neurons, which allows us to see how they are connected to each other and study neural networks) Whole animals can be labelled as well (their health is not harmed by the process) .





In the military we always say we don't rise to the level of our expectations, **we** fall to the level of our training. - David Goggins

THE END

WISH YOU ALL SUCCESS