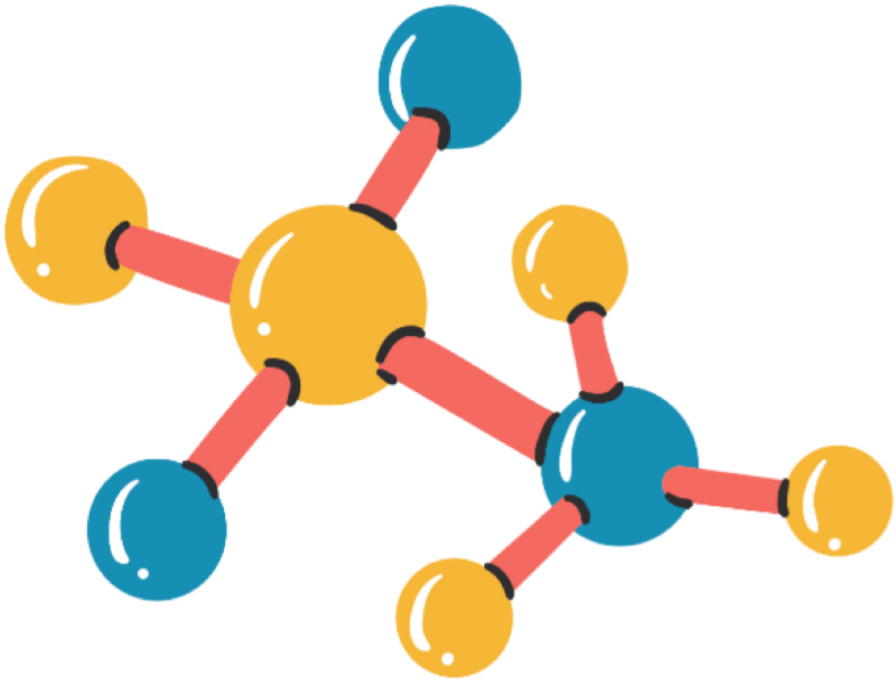
Sheet no. 5

Biochemistry



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Introduction:

This sheet is the first sheet of molecular techniques

. There are two techniques:

Recombinant DNA techniques and enzymatic techniques

*Here, we will talk about (recombinant DNA techniques)

What is DNA cloning?

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 bacteria, human cells grown in labs, animals, or even the human body as a whole.

*Suppose we have a DNA fragment, it can be a gene or any sequence in the human genome and we put it in a biological system like bacteria, yeast or a human cell may bethat used in the lab-cultured human cell ,so we put DNA inside these cells to make many copies of this DNA fragment .

*or this DNA fragment can be a gene that produces a protein, so we can insert this gene as well in the different type of cells and we can produce many copies of this geneor we can express a gene in itself into a protein inside in this cell.

It usually involves:
The formation of a recombinant DNA composed of a vector (a carrier of the gene or the DNA segment of interest; usually a bacterial plasmid) and a gene that encodes a protein or a non-coding RNA using restriction endonucleases.
Insertion into the cell(s).

Recombinant DNA is basically a piece of DNA that is made of DNA from two or more different sources like human DNA integrated into bacterial DNA, so we put this DNA in a vector -which is a carrier of this DNA fragment of interest an example is a bacterial plasmid- so we put it in a bacterial plasmid and use restriction endonuclease, so insert this recombinant DNA into cells and these cells are the factories they producecopies of this DNA fragment or express the gene into proteins.

*Endonuclease: is an enzyme that degrades DNA within molecule.

*The endonuclease breaks the phosphodiester bond between nucleotides within theDNA fragment.

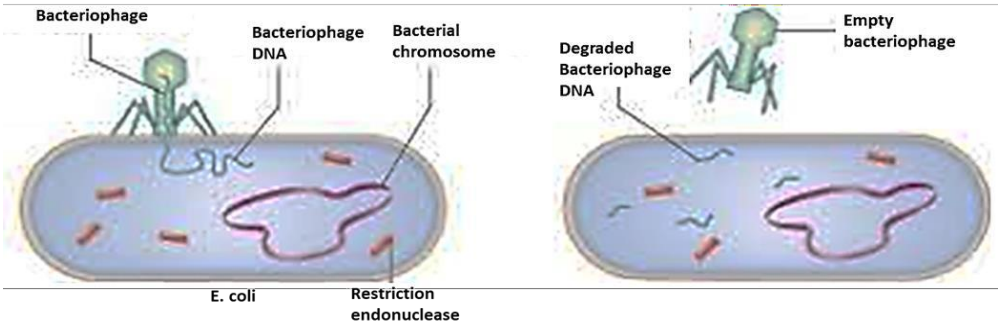
*Exonuclease: is an enzyme that degrades DNA and removes the nucleotide from either ends from 5 prime ends or from 3 prime end.

Restriction endonucleases: Bacterial enzymes that recognize and cut (break) the phosphodiester bond between nucleotides at specific sequences (4- to 8-bp restrictionsites) generating restriction fragments.

bacteria produce this enzyme toprotect themselves from bacteriophages.

**Bacteriophage: A virus that sits on the plasma membrane of the bacterial cells and inserts the DNA inside these cells, it controls the bacterial cells it controls the genetic system of this type of bacterial cells and what itdoes, it amplifies the cell and makes many copies of bacteriophages.

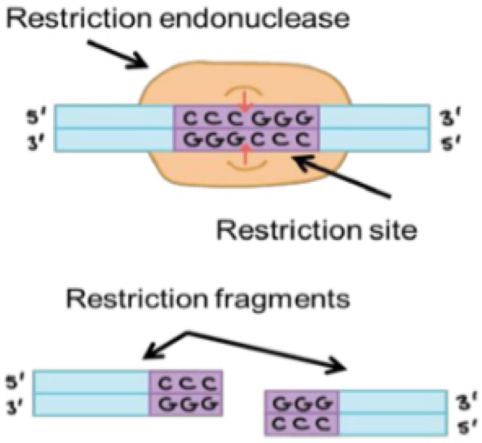
Eventually, bacterial cells become fold of these bacteriophages so they just exploredreleasing many bacteriophages and these bacteriophages then can infect other bacterial cells, so in order to protect themselves from these bacteriophages is that produce restriction endonucleases and they Cleave bacteriophage DNA.



endonuclease that cleave DNA by breaking phosphodiester bond and recognize a specific sequence of hundred restriction endonuclease to each bacterial

And these restriction endonucleases recognize certain sequences which known as restriction site and cleavage within the restriction site to produce restriction fragments so the pieces of bacteriophage DNA are called restriction fragments.

And these are called restriction endonucleases because : They are restricting the growth of bacteriophages.



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).

The restriction site that is recognized by restriction endonucleases are in general palindromic sequence, meaning that is read the same from either fragment

** so going from 5 prime to 3 prime this restriction endonuclease ECORI which produces by a bacteria known as E coli you can read the restriction site recognizes the sequence GAATTC if you read the other fragment in complementary strand from 5 prime to 3 prime, it reads exactly the sameGAATTC.

*HindIII is another restriction endonuclease which recognize in this sequence AAGCTT, if you readthe other complementary strand and from 5 prime to 3 prime as well it reads exactly the same.

*SmaI same exact thing

EcoRI	5'	GAATTC	3'
	3'	CTTAAG	5'
HindIII	5'	AAGCTT	3'
	3'	TTCGAA	5'
SmaI	5'	CCCGGG	3'
	3'	GGGCCC	5'

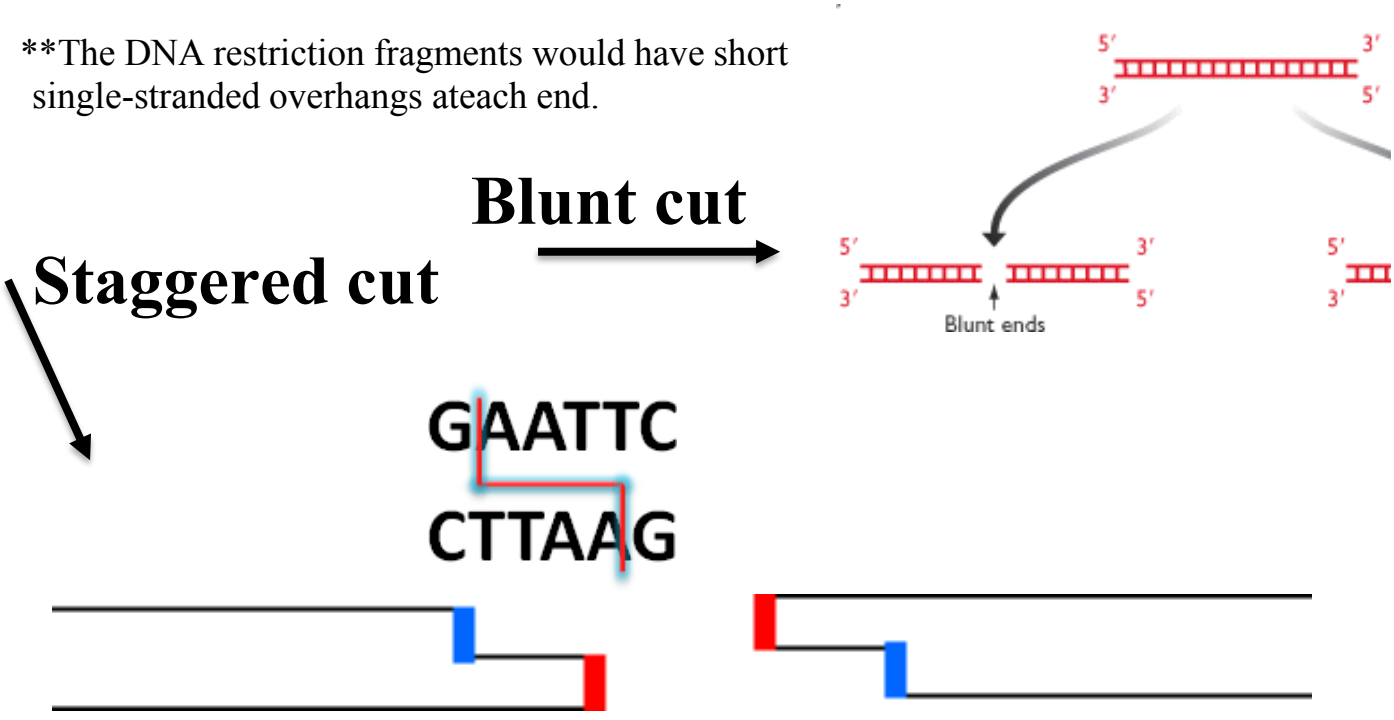
- *Types of cuts by restriction endonucleases:*

1- Blunt: enzymes cut at the same position on both strands giving blunt-endedfragments.

// Blunt // تعني صريحة لدرجة الوقاحة تغيير جو بعد قراءة الجريدة اعلاه

2- Staggered (off-center) : enzymes cut the two DNA strands at different positions generating sticky or cohesive ends.

**The DNA restriction fragments would have short single-stranded overhangs ateach end.



*Extra explanation:

These restriction endonucleases, in general, can cleave DNA in two different ways either make blunt cut, means like sharp you have cleavage let's say between G and Aand this is known as staggered ends.

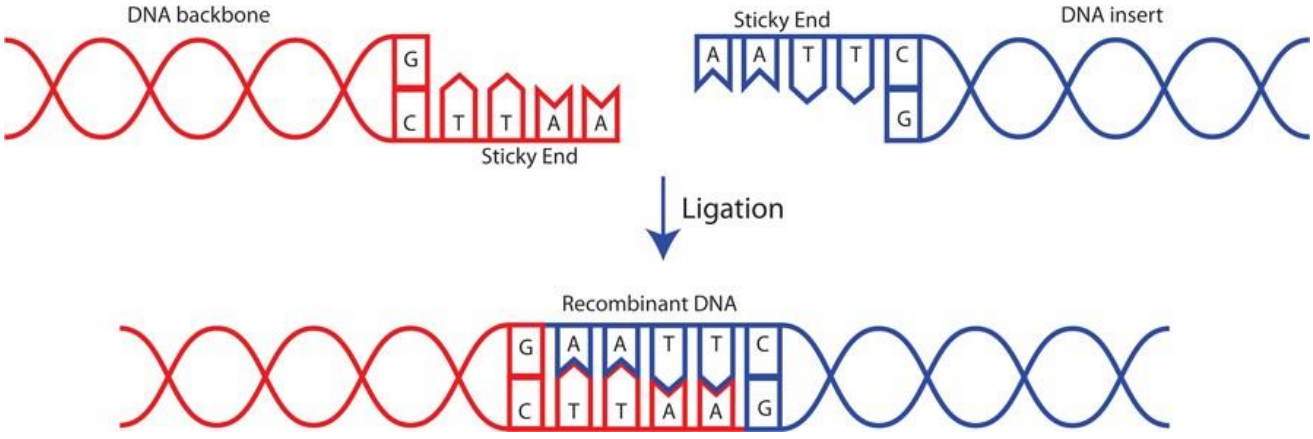
This is the restriction site of the endonuclease ECORI

Now ECORI recognizes GAATTC and makes a cut between G and A in both strandsand produces sticky-ended DNA fragment.

*The ends are free they don't have hydrogen bonds and are known as sticky- ended DNA fragments.Because they reform hydrogen bonds with each other and are known as the cohesiveend.

***Remember the cleavage occurs by breaking phosphodiester bonds not hydrogen bonds*.**

Hydrogen bonds are reversible so DNA fragments can come back with each other butare not stable they can dissociate from each other.

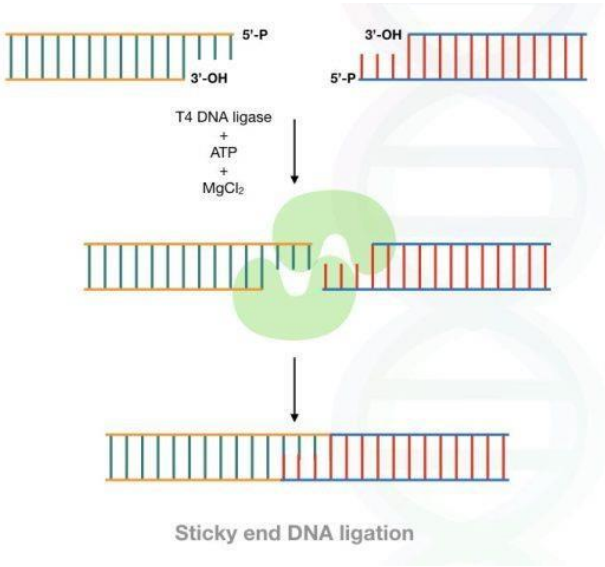


-Two strands from two different sources may be from bacteria ,a mouse or a humanand these fragments have GAATTC when you add ECORI you have cut between DNA on each one of them so each one will produce sticky-ended DNA fragments so they combine these two DNA fragments to each other the ends are complementary with each other regardless they are mouse-human or whatever so they have GAATTC . and have a cut between G and A and one of them and same thing with another one andhave sticky end with another fragment

-Two ends can meet, and they are complementary with each other by the formationof hydrogen bonds between strands if you add **ligase,** you have the formation of a phosphodiester bond between G and A on the 1st strand and G and A on the 2nd strandyou can recombine these two DNA fragments ((one fragment lets say human and anther fragment from mouse DNA fragment.

DNA ligase:

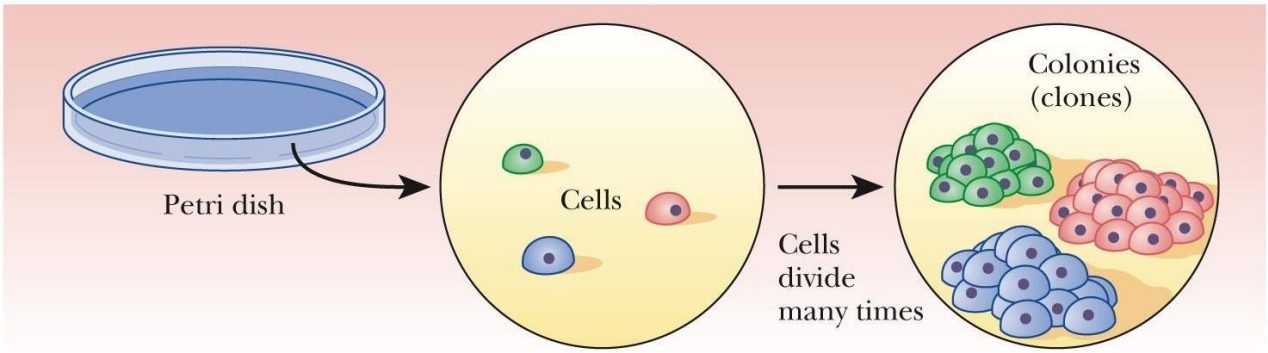
It covalently joins DNA ends (for example, restriction fragments) by catalyzing the ATP-dependent formation of phosphodiester bonds between the 3'-hydroxyl group ofone strand and the 5'-phosphate end of another strand.



If you add DNA ligase you have the formation of phosphodiester bonds between twostrands and you have again recombinant DNA .
**ligase is ATP dependent!!!

Cloning :

Cloning means that you make several copies of one thing.
A clone is a 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.

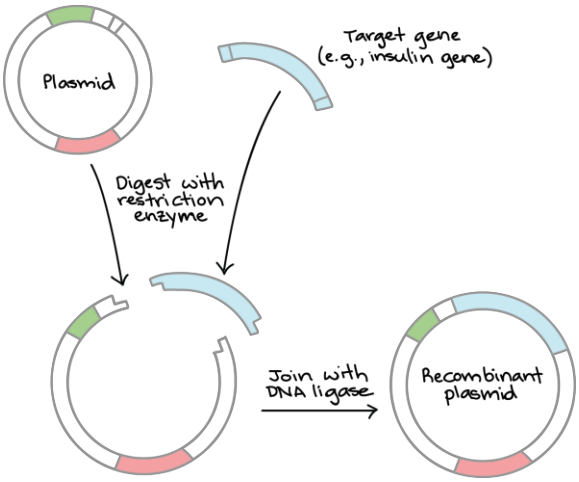


all the cells in each group originates from the same cell -so if i say Human cloning this mean I am making a copies of the same human individual having the same exact genetic background**.**

How do we clone a DNA molecule? 🤔
*a DNA fragment of interest is inserted into a DNA carrier (called a vector) that canbe replicated.

*The resulting DNA molecule is what is known as a recombinant DNA molecule.

*The procedure is known as recombinant DNA technology, which is part of geneticengineering



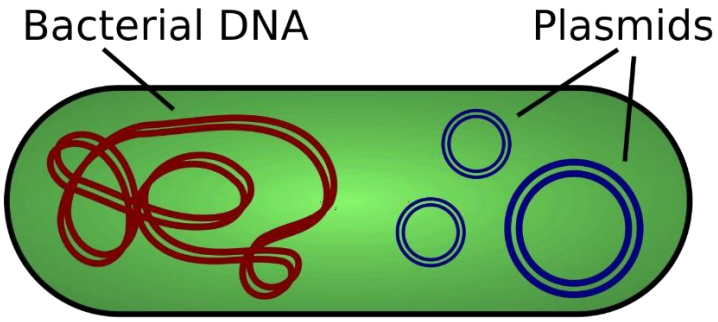
We need a vector we need a carrier and this carrier usually is the bacterial plasmid and plasmid is a single circular DNA that is fit bacterial cell and gives it advantagesand resists antibiotic drugs and so on.

This plasmid is cut by restriction endonuclease and forms sticky-ended DNA .

So the process is : We add the same restriction endoclease to the plasmid and the gene to produce a Complementary sticky ends , and to reform the phosphodiester bond we use ligase and form a recombinant plasmid (or recombinant DNA)

Using plasmids as vectors:

Bacterial plasmids are considered excellent vectors that are used for cloning (cloning vectors) or expression (expression vectors).
These are natural bacterial circular DNA that is not part of the main circular DNA chromosome of the bacterium.
A plasmid exists as a closed circle and replicates independently of the main bacterial genome.



The advantage of plasmids:

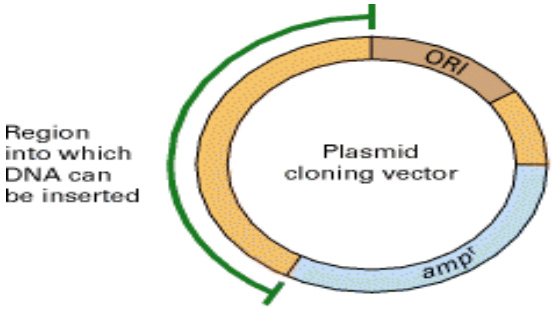
1)they can transfer from one bacterial cell to anther
2)they contain gene that benefits the bacterial cell that is how bacterial cell canclone as a community helping each other
3)they replicate independently of bacterial chromosome so bacterial cell can havemultiple copies of the same plasmid.

Features of plasmid cloning vectors:

Plasmid cloning vectors must have the following three components:
1) Their own origin of replication (Oric) that allows them to replicate independently ofthe bacterial chromosome.

- Side Note // **DNA sequence that allows initiation of DNA replication, so each plasmid has ORIis the place where the DNA replication starts. We use it two produce multiple copies of the plasmid**

2) A selectable gene such as an antibiotic resistance gene that allows for selectingfor/against the cells that have them.



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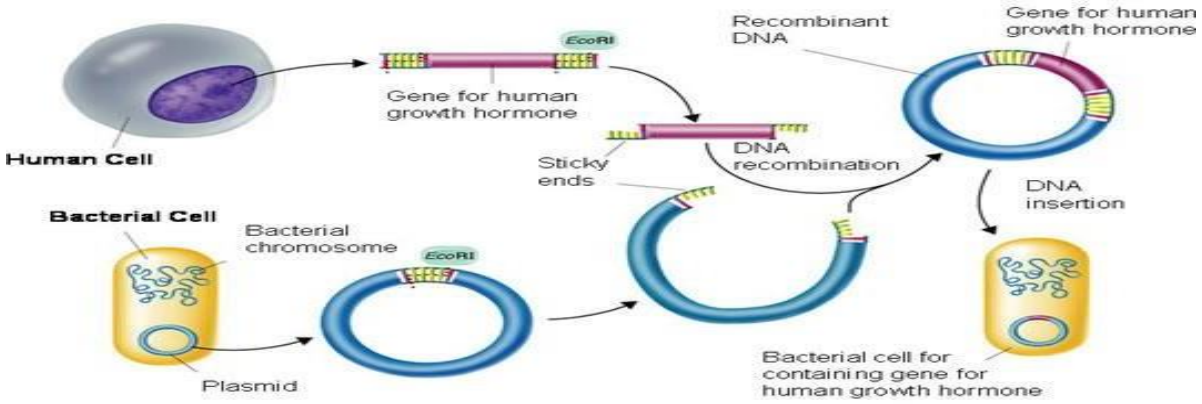
Side Note // Antibiotic resistance gene has to have a selective gene and bacterial cells have plasmid they will not die an example selective gene is an antibiotic resistance gene likegene allows becoming resistant of ampicillin so bacterial cells which have plasmid if we add ampicillin will survive in opposite bacterial cells which don't have the plasmid if we ad d ampicillin to them, they will die so we select the bacteria which has plasmid.

3) A restriction site that allows for insertion of the DNA segment of interest into the plasmid .

The Endonuclease make a single cut

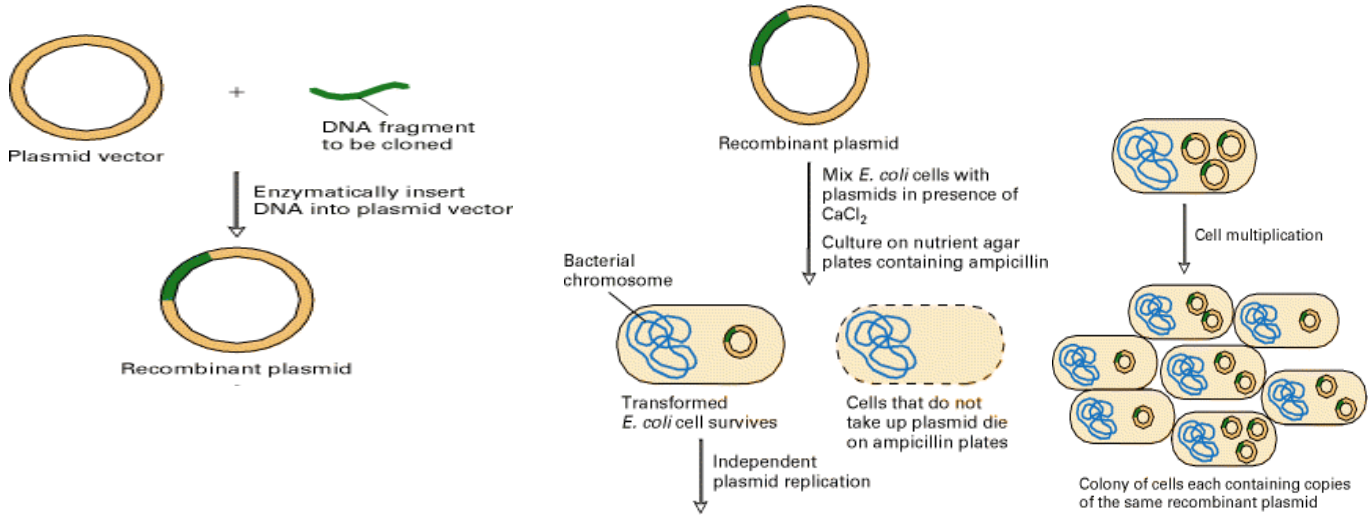
** If endonuclease makes two or three or more than on cut the plasmid loses its circular shape and become liner or flat.



**Both DNA fragments (the DNA to be cloned and a vector) are cut by the same restric tion endonuclease that makes DNA fragments with same sticky-ends hybridize(anneal) to each other, when mixed.
**A DNA ligase is added to "close" the plasmid.

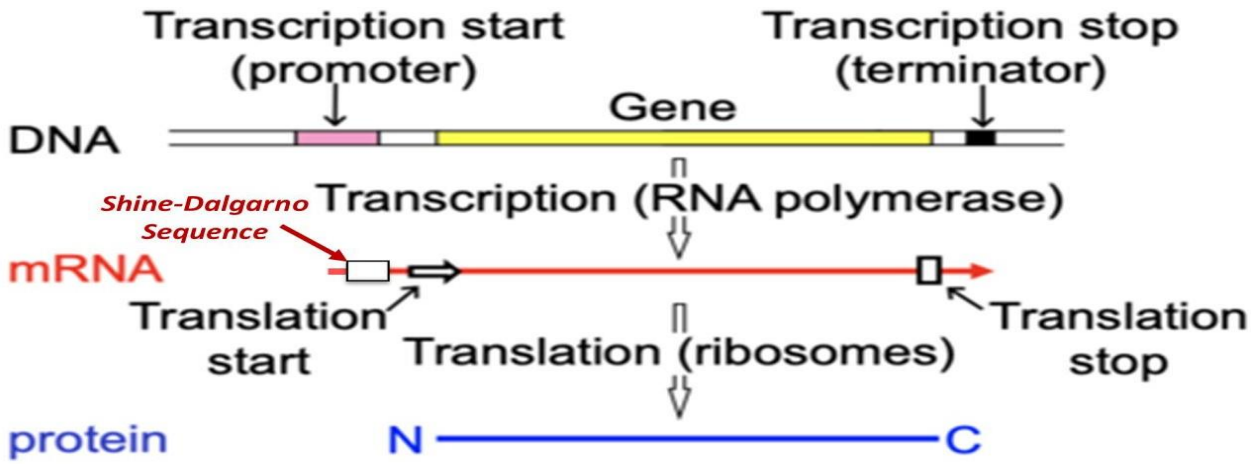
HOW WE MAKE A RECOMBINANT DNA ?

Here we take a human cell and take the DNA fragment that will amplify, we want toclone it and then we add restriction endonuclease producing sticky ends of these fragments and we take the plasmid from bacterial cell to produce sticky ends of plasmid and combine them with each other and we add a DNA ligase so we have nowrecombinant DNA and then we insert this plasmid to bacterial cell and these cells willmake many copies of the recombinant DNA. ☺



GENE EXPRESSION

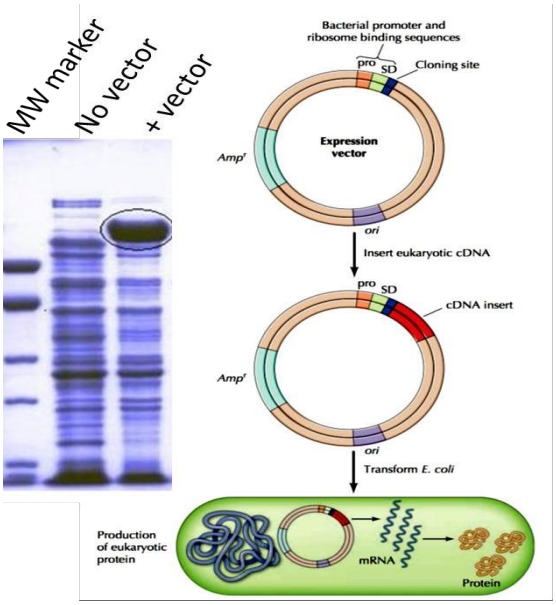
In order to express a gene you must have a promoter (a bending site for RNA polymerase) to initiate the transcription and produce mRNA. We also have to have a termination codon (UAG,UGA,UAA) and a translation start site (AUG). That how we get a polypeptide chain then it folds forming a 3d stricture -protein



DNA fragment as insulin gene for example in order to express this gene you must have a promoter which is a binding site of RNA polymerase and start transcription toproduce mRNA and have termination codon as well like (UAA, UAG, UGA) and translation start site AUG and production of the polypeptide that is folded in a three-dimension structure called protein.

in order to produce a lot of DNA fragments we use (cloning vectors) , but here we use (expression vectors) to produce a protein

Expression vectors:
**Expression vectors contain additional sequences:
1)Promoter sequences upstream of the gene to be inserted,
RNA polymerase binding site -transcription start site
2)Ribosomal binding sequences (Shine-Dalgarno [SD] sequences)
The translation take place here
,3)A transcription termination sequence.
To stop transcription

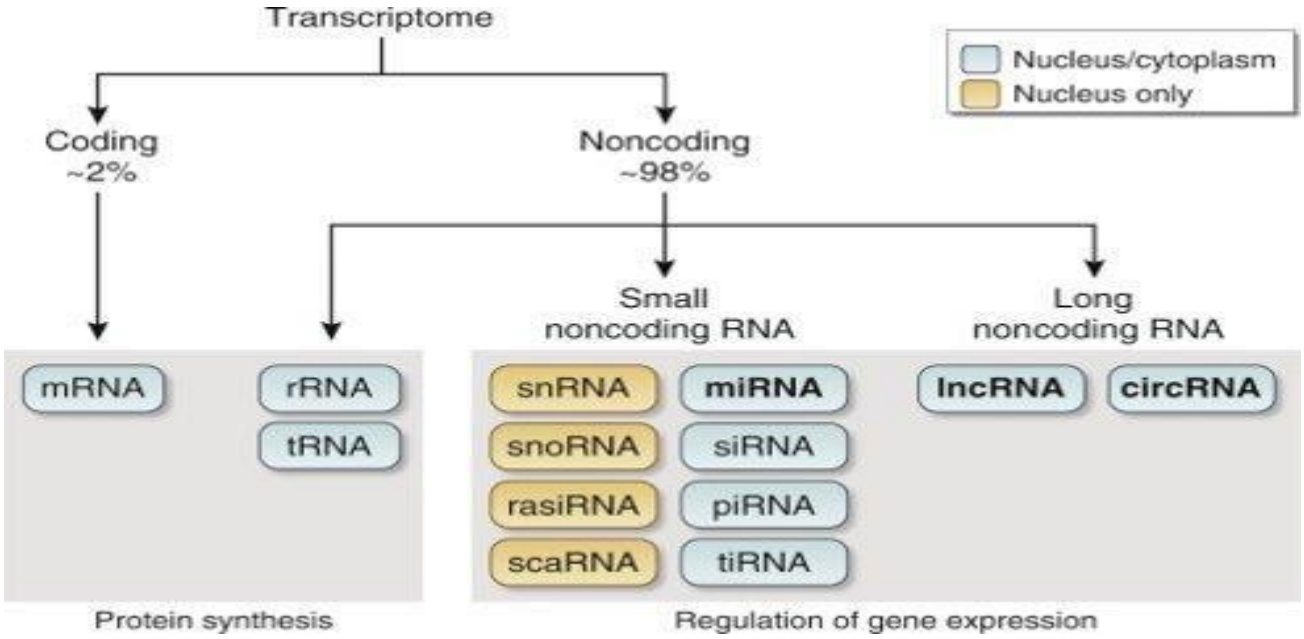


No vector : there is no expression of the proteins.
+ Vector : we can determine the protein of interest which is amplified from the intensity of the band.

NOTE: they are separated according the size

Because bacteria is a prokaryotic cell on the other hand humans have eukaryotic cells

1)There are many types of RNA molecules in human cells

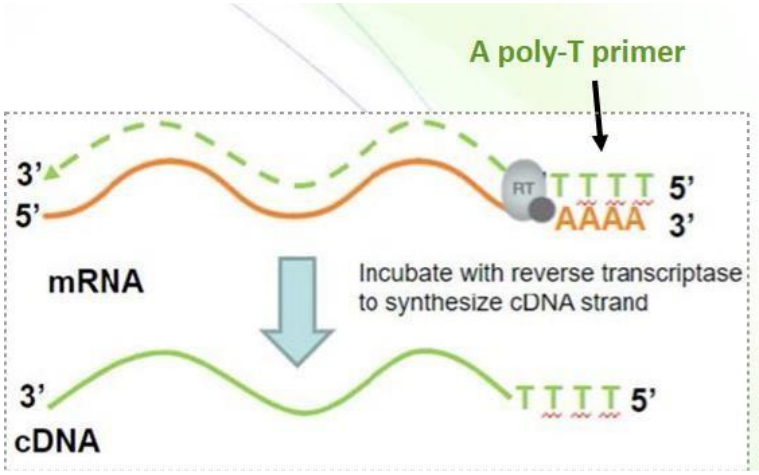


RNAs are either protein coding RNA molecules (mRNA) or noncodingRNA molecules that have important regulatory purposes inside cells (siRNA,rRNA,tRNA,…)

- the challenge is: How can we select for mRNAs from all these RNAs?
- the solution: by using #reverse transcriptase#: an enzyme that generates acomplementary DNA(cDNA) from mRNA using it as a template, it reverses transcription.

-- it is known that transcription is to convert DNA to RNA, here we have the reverse direction.
-- reverse transcriptase is viral in nature, it was discovered recently, it requires aprimer.
Quick revision: primer is a short RNA sequence added before replication startto allow DNA pol to work.
-- the primer that we care about is the primer that is added before reverse transcriptase starts, it doesn't matter if it is DNA or RNA.
-- reverse transcriptase primer has a poly T tail, so it binds to mRNA,which logically has a poly A tail.
And the reverse transcription happens: our lovely enzyme will make the 2nd complementary DNA cDNA.

All RNA molecules don't have a poly T primer and ability to transcribed by reverse transcriptase except mRNA,
We need it to express proteins, so expressing proteins wont happen with rRNA for example by a mistake, cause it doesn't have poly A tail 😊

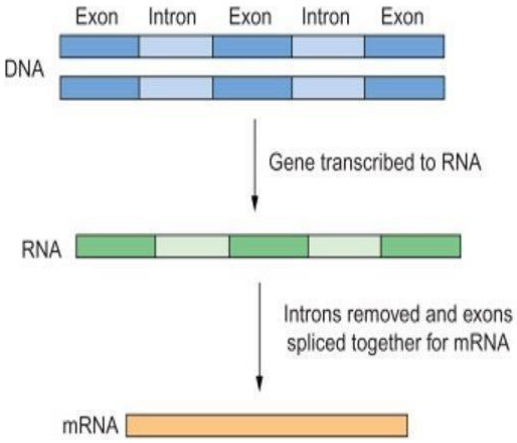


2) the 2nd challenge: genes contain introns(the removed parts) and exons(the translated part)

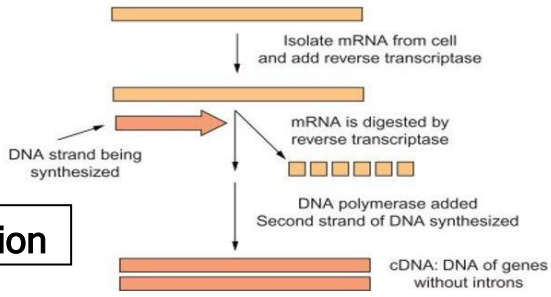
It is known that bacterial cells couldn't do splicing and getting rid of introns, sothe solution is to:

Get rid of introns Of mRNA then using reverse transcriptase to get cDNA before inserting it in plasmid

Add reverse transcriptase to produce double stranded cDNA that is presented without introns.



The intronic challenge

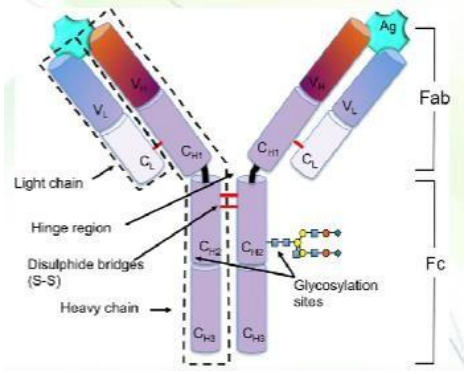


The revers sollution

3)3rd challenge: it is hard to express many human proteins in bacteria,because:

a) No internal disulfide bonds in bacteria : 

disulfide bonds are found in antibodies:



Antibodies
Are Y shaped, have 4 polypeptide chainsconnected by disulfide bonds
2 light chains and 2 heavy
also, within heavy chains there are disulfide bonds

If disulfide bonds are lost, the structure will be affected and may becomenonfunctional.

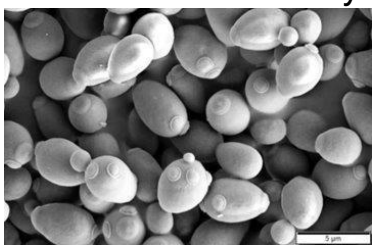
b)No post-translational modification (example: glycosylation)in bacteria:bacteria aren't able to modify proteins, they cant glycosylate proteins

glycosylation: is adding sugar on proteins

c)Protein misfolding:if 3 dimensional structure isn't formed properly, it leads to protein degradation(there are no CHAPERONES)

d) Protein degradation: the misfolding proteins wont be recognized andrefolded so it will be degraded.

Solution: use a eukaryotic system such as yeast or a human cell.



Yeast is a single cell organism, it is eukaryotic, has many proteins similar to human proteins, has a nuclear membrane
Yeast can fold, glycosylate and modify protein rather than bacteria

Protein tagging and creation of protein hybrids

_After expressing proteins in bacteria, yeast or human, how can weidentify, purify or isolate our protein from other proteins ?

BY TAGGING PROTEINS

Means by adding labels, tags on proteins creating*proteins hybrid*

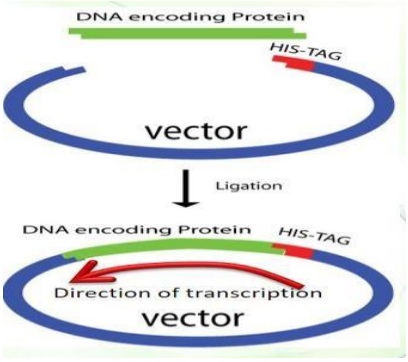
To understand the meaning of a tag better, look at your t-shirt, it has a label that informs you about it. 😊

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.

*How can we tag a protein?

1) use a vector contains a tag as a part of it,,, a tag is a small piece of DNAencodes amino acid sequence.
2) insert DNA fragments of the gene of interest.
3) the tag will become a part of the inserted gene
4) express the gene inside bacterial cells, and the tag is synthesized along with protein becomes a part of it.



A tagged, labeled protein : it allows to identify, detect and isolate protein

We will talk about 4 post- protein tagging techniques detection or purification by:

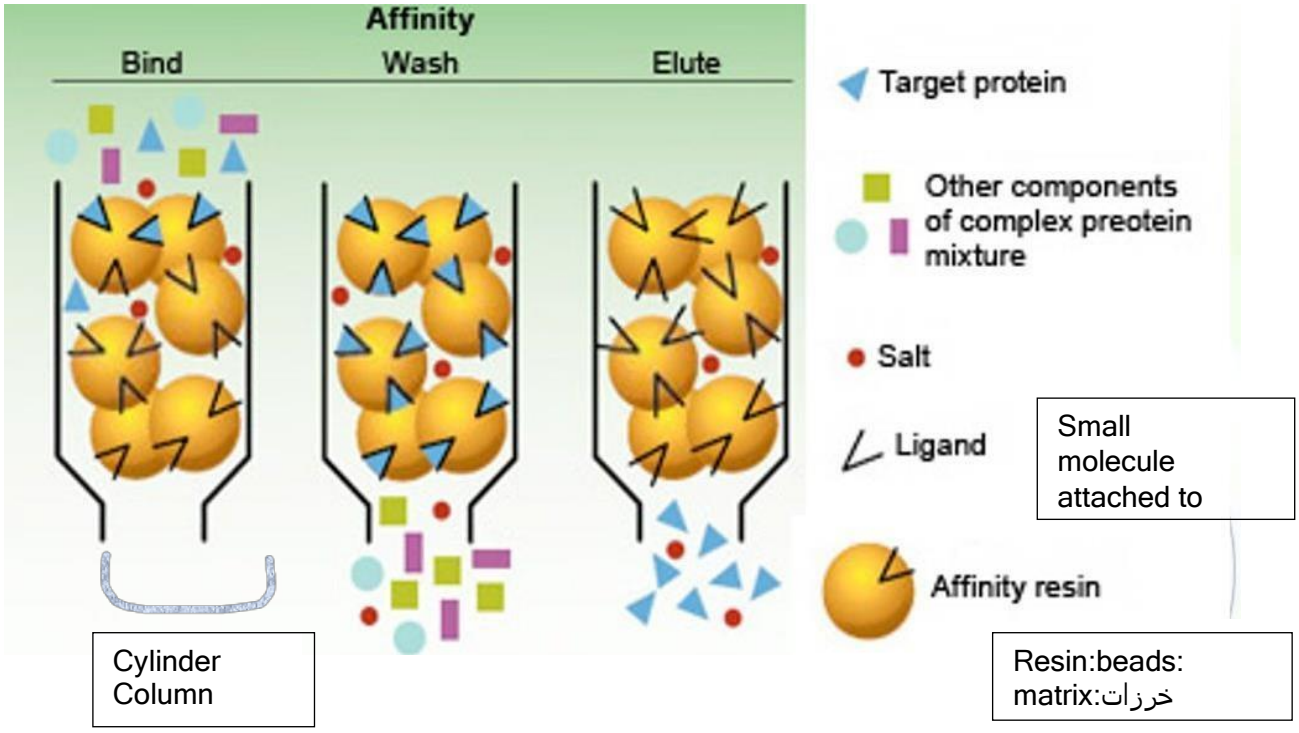
1) Affinity chromatography(purify protein)
2) Immunoprecipitation(purify protein
3) SDS page, GEL electrophoresis (detect protein)
4) Immunoblotting(detect)

نتّفق قبل ما انبلّش
Detect:
يعني أكشف مكان وجود البروتين
بدون ما أطلعه من مكانه
Purify :isolate: extract:
أستخلص البروتين من مكانه

1) AFFINITY CHROMATOGRAPHY

Affinity: strong interaction

Chromatography: a technique for the separation of a mixture by passing itin solution or suspension through a medium in which the components move at different rates.



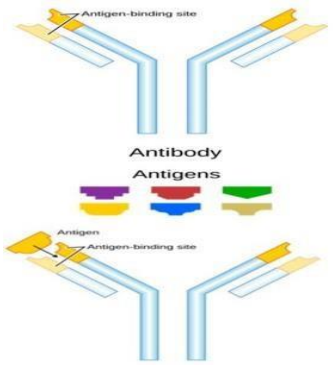
1) put beads in the cylinder
2) pass all proteins in the cylinder
3) the proteins that have affinity will binds with beeds and other proteinswill pass out
4) release or isolate the protein of interest from the column (purification)

https://www.youtube.com/watch?v=8_7cdfNO7OY

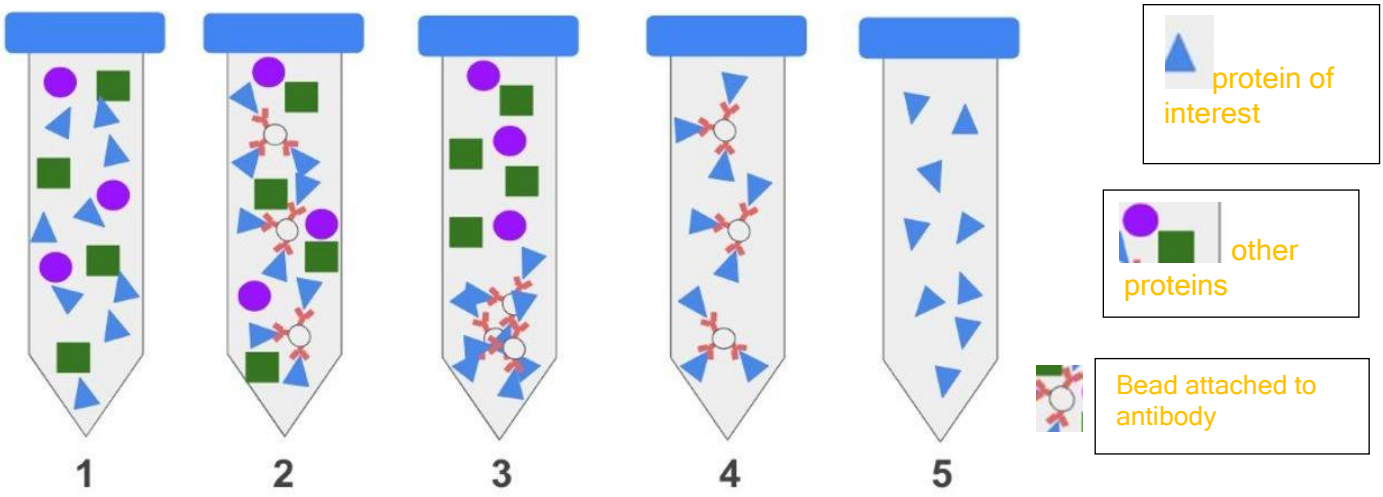
2) Immunoprecipitation=immunoglobulin

Immuno: using antibodies(they are extracted from some animals after injection)

Antibodies are very specific to one type of protein, it doesn't bind to other



Precipitation : going down ☹



Here, we have alarge collection of proteins

We add beads with antibodies

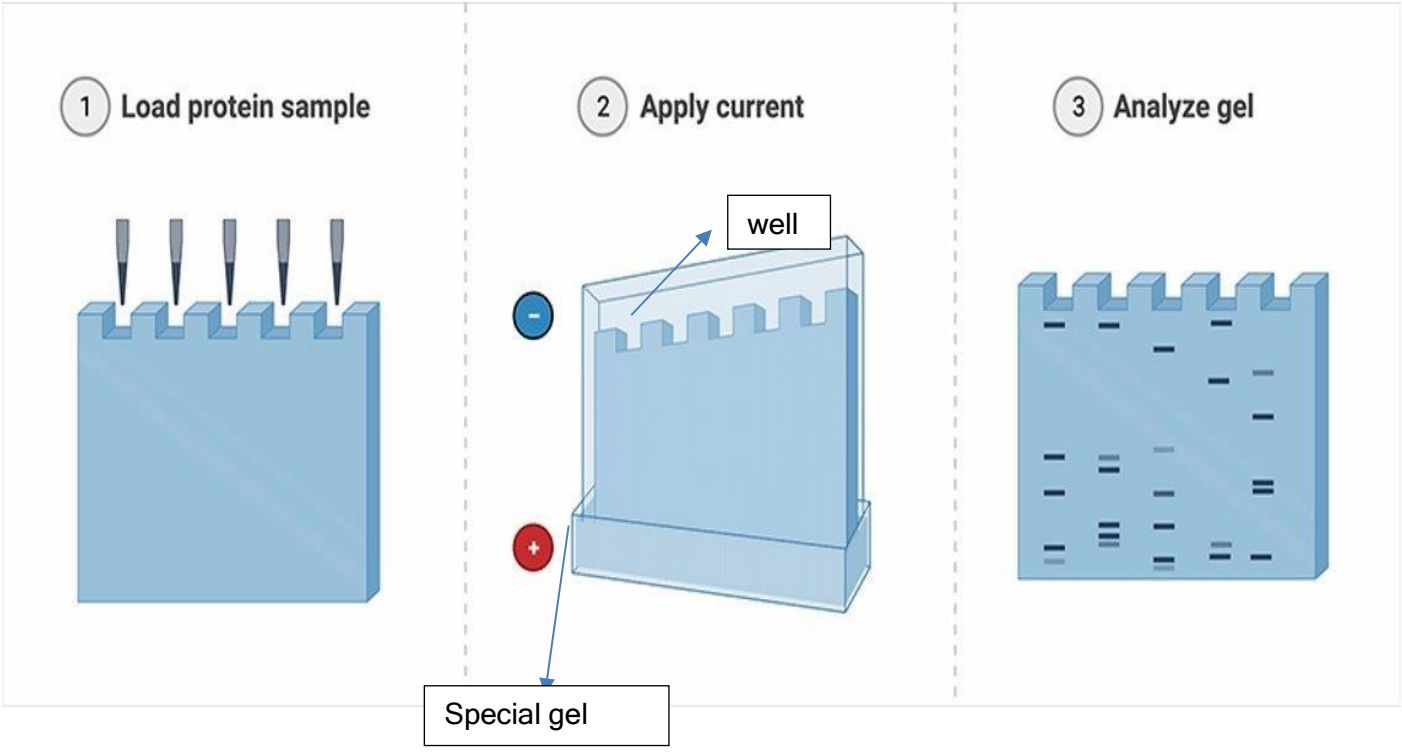
Antibodies are specific, they will bind to our protein only
The collection of bead, antibody and our protein is heavy, so they are going to precipitate than other proteins will be removed and our protein will be released from antibodies

https://www.youtube.com/watch?v=41T1Az_EsrE

3) SDS page- protein electrophoresis SDS-PAGE

(sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

Like in DNA fragments that were separated according to size, we could do the same with proteins



1)apply the sample into wells 2)apply the electricity

3) Proteins migrating in the gel like bands 4)getting different bands according to size

https://www.youtube.com/watch?v=MILiO1XnuqQ

4)immunoblotting (western blotting)

Like southern blotting in DNA

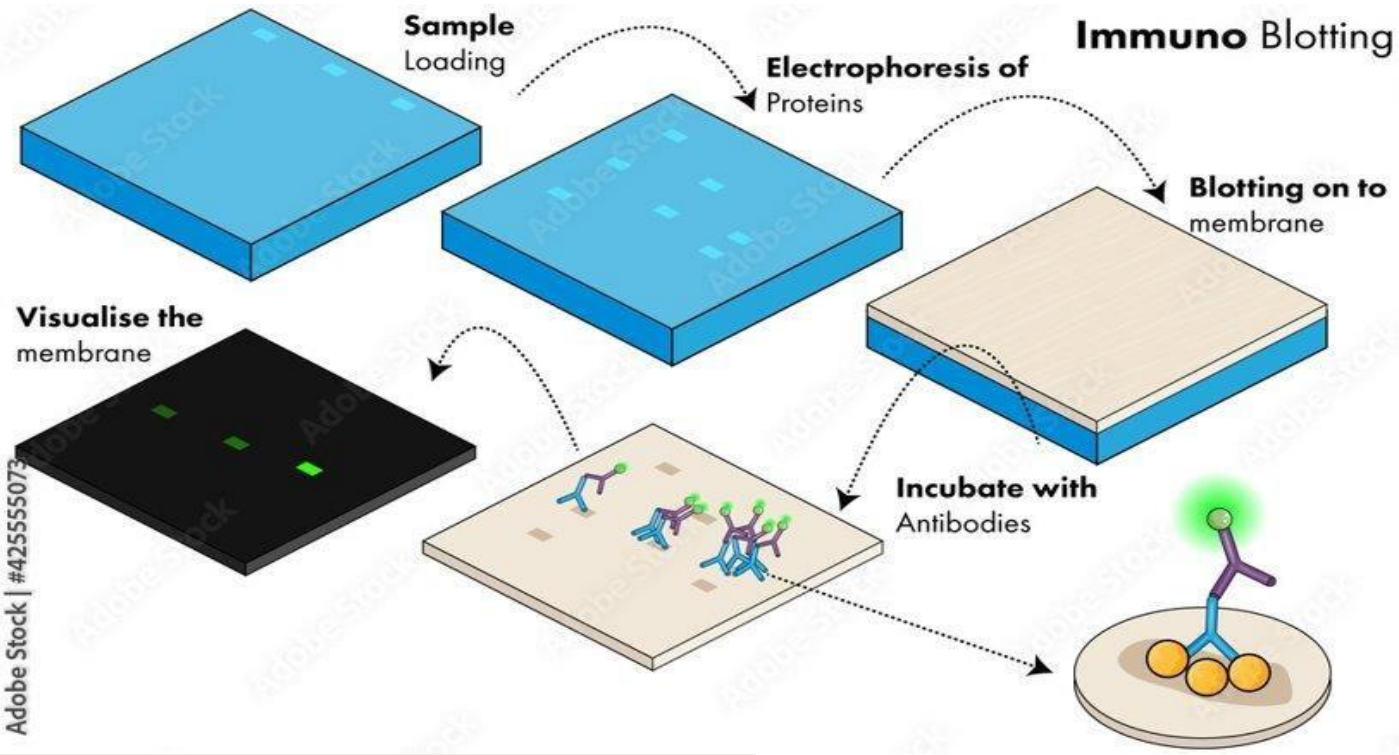
**we can do a step further after electrophoresis

1) we have proteins separated according to size in the gel

2) transferring the proteins to membrane(special paper)

3) adding antibodies attached to signals(like probe having fluorescence, radioactive signals)

4) the antibodies bind with the specific protein releasing a signal(could be acolor, florescence or luminescence, indicating its Prescence in the sample.



https://www.youtube.com/watch?v=EAKSr4Eclyw

Major protein tags

There are many tags with different purposes: (we will discuss 3 of them)

1)GFP(green florescent protein):

Allows to detect the protein of interest by florescence or antibody

2)GST: glutathione S transferase :

Purify the protein using antibodies, the antibody will bind to transferase exists inthe protein of interest.

3)poly HIS: is a small tag of 6 HISTIDINE AMINO ACID to allow purifying the proteinor detecting it using an antibody.

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

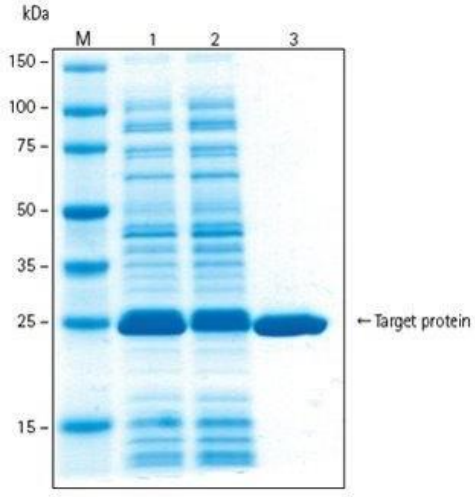
Only Red ones are required

Only detection

Detection and purification

Detection and purification

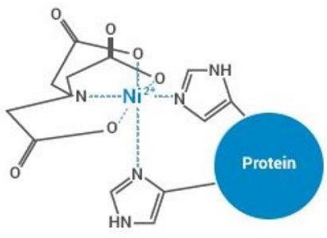
1) **His tag :** The addition of six histidines to a protein would allow for purification using beads with bound nickel ions.



Notes about the figure beside:
Here are highly expressed proteins in bacterial cells
Undergo gel electrophoresis
**the first two samples are total protein content in the cell including protein of interest.
**the highly intense band is the target protein.Its intensity indicates high expression
((1&2→before purification
3→after purification))

*How was it purified? By using affinity chromatography column (see page 14 again 😬)

The column has beads having Ni+2 on their surfaces, pol HIS tag like Ni+2 ♡so the six HIS will bind to Ni+2, causing any other protein to pass through column, and only beads with nickel binding to protein of interest through 6-HIS are found in the column.Then we can extract our protein.

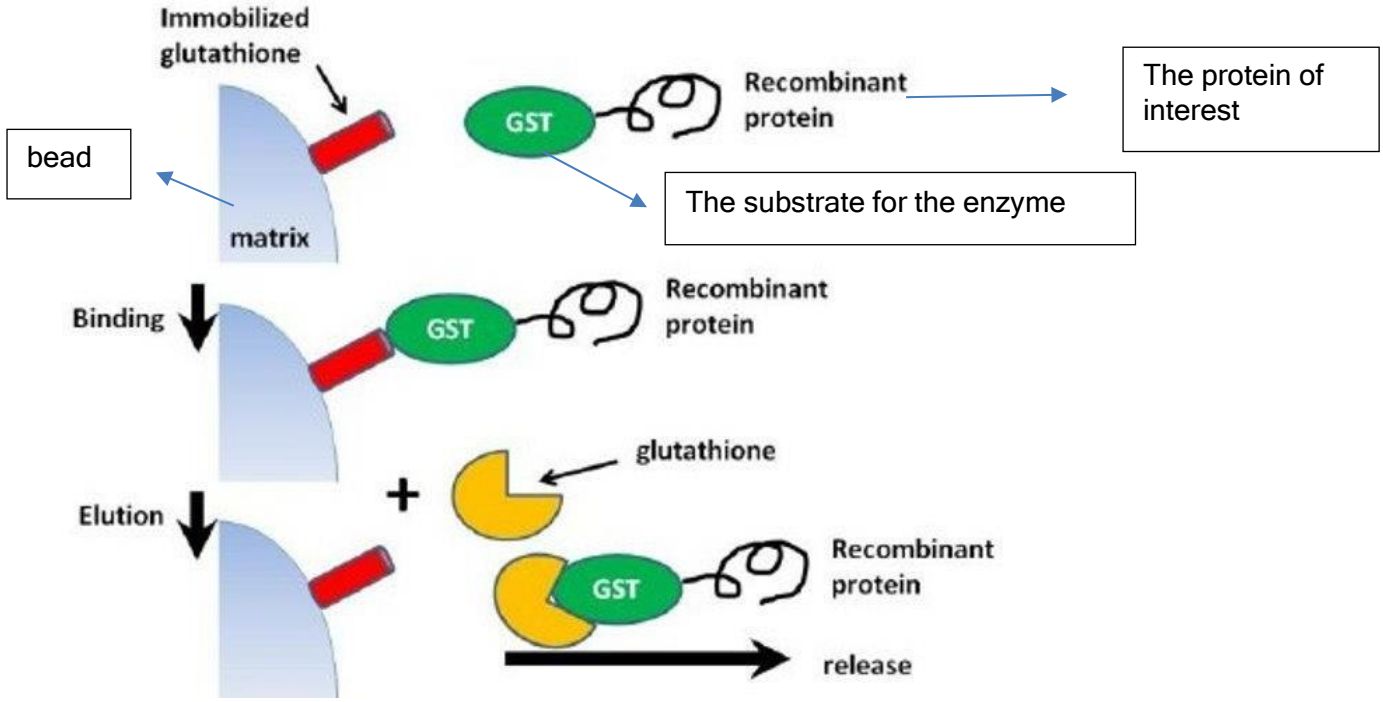


ما في بروتين بالوضع الطبيعي عنده
6 HIS
بالتالي هاد
Tagged protein

*so the steps are:
Clone the gene, insert it in the plasmid, get a recombinant plasmid, insert it in the bacterial cells ,express protein, purify protein(by affinity chromatography) and finally analyse (by electrophoresis)

Clone → Express → Purify → Analyze

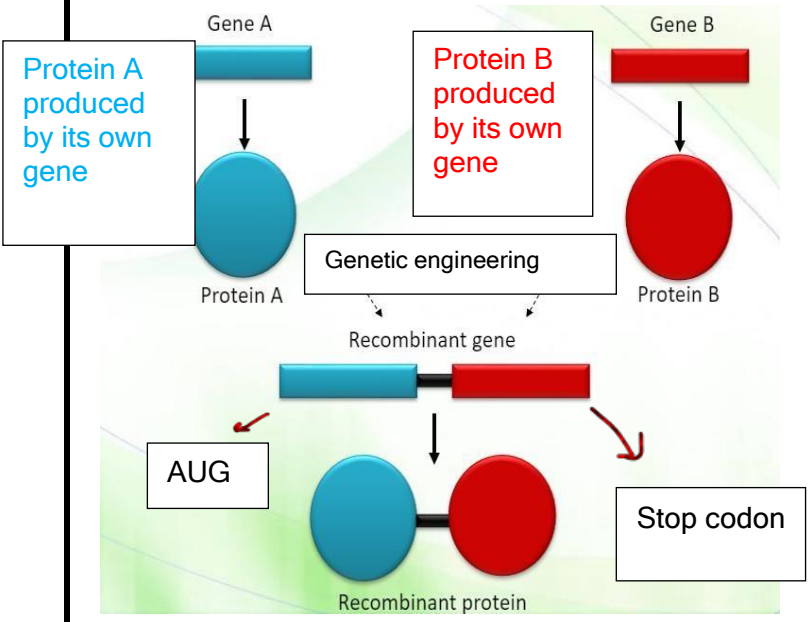
2) Purification of GST-tagged proteins



As the previous, we use affinity chromatography colomn, all proteinswill pass down and our protein will bind to bead as in the figure above:
1)binding:the recominant protein is attached to the substrate transferase that will bind to immobilized glutasthione matrix
2)elution: releasing our protein: by adding mobile free glutathion matrix, so it binds to recombinant protein through transferase, causing its purification.

Genetic Enrineering

1)production of a recombination protein



Here, there are 2 separated proteins from their own genes undergo genetic engineering, they need to modify their ends and beginnings(promoter and termination site), then we get a recombinant gene , these two genes will be expressed all together, all at the same time, so all polypeptides are produced at once.
Gene---→mRNA---→ polypeptide
(transcription) (translation)
Giving the recombinant protein

2) Production of a recombinant protein…The power ofdomains

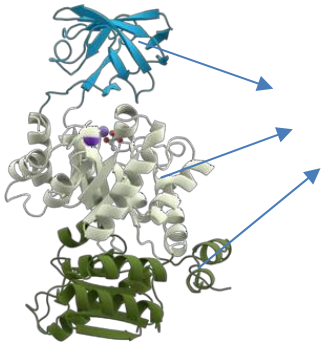
*now, how can we produce one protein made of different parts of proteins(domains)

**what is a domain?

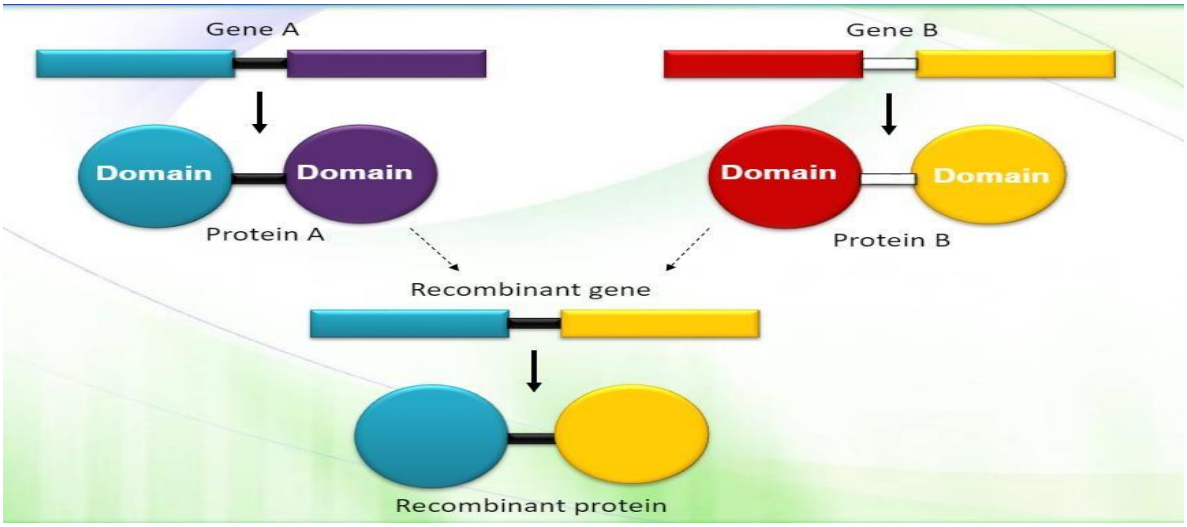
A **protein domain** is a compact region (or part) of the protein's polypeptide chain that:

1. has a **defined three-dimensional** structure,
2. is **self-stabilizing,**
3. folds **independently** from the rest.
4. can be disconnected from the protein and, yet, maintains its structure and function.

*Proteins can have several domains.



3 domains work independently
If any domain is cut, it doesn't affect others



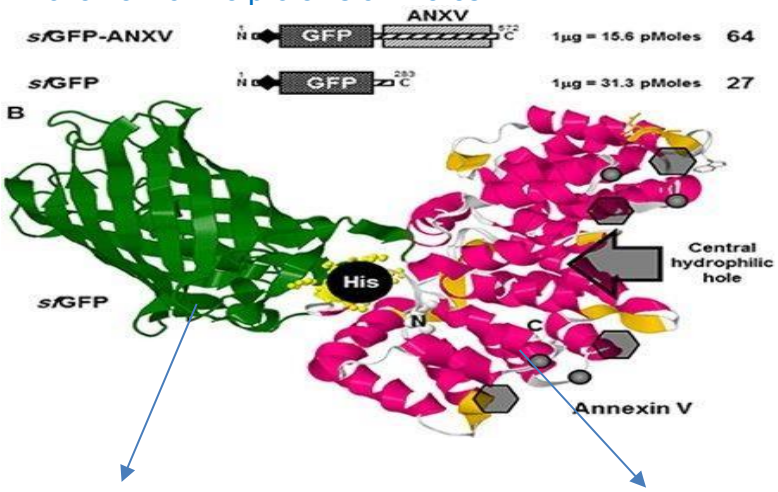
So, we can combine different domains from different proteins…In the figure above:
Gene A expresses protein A with 2 domains and the same for gene B
BY GENETIC ENGINEERING, we make recombinant gene by taking a domain from gene A and other domain from gene B without modifying ends and beginnings cause they are already exist, afterthat the recombinant gene will be transcribed and translated giving recombinant protein with differentdomains from different proteins.Every domain maintains its original structure and function.
**we can do genetic engineering with 2 ways in bacterial,yeast and even human cells.

Lets continue with the 3rd type of tags:

3) GFP-tagged proteins 

Green Fluorescent Protein (GFP) allows for protein detection rather thanfor purification purposes.
*GFP: a protein gives florescence naturally-by itself- , it is produced fromjelly fish

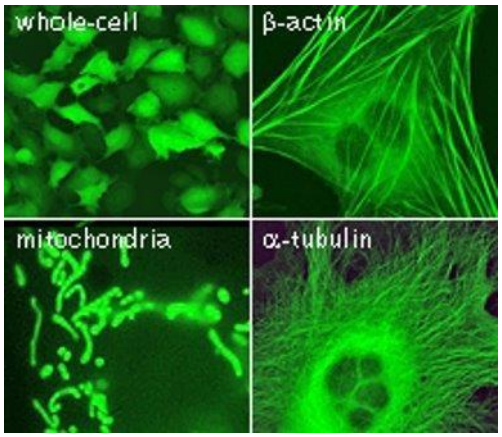
GFP gene is connected to our gene of interest
Producing GFP protein connected to the protein of interest, so our proteins of interesnt don't floresce themselves, they do because they bind to GFP, helping us to detect the place and movement of the proteins of interest



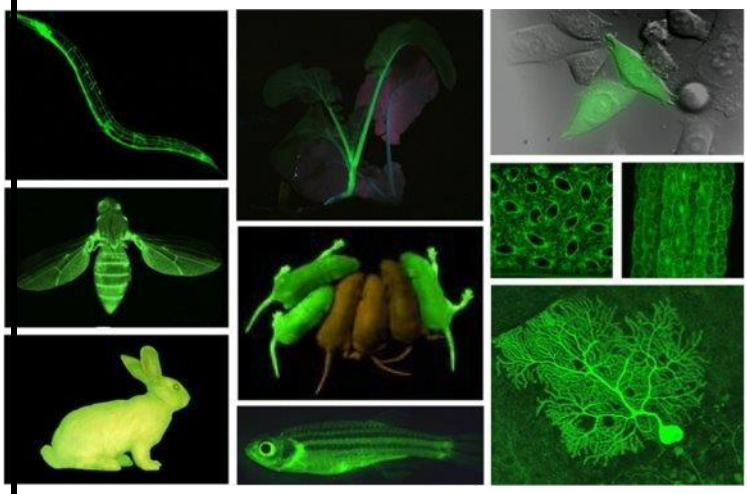
GFP

Annexin
The protein that binds to GFP in jelly fish before extraction

**WORLD OF POSSIBILITIES:
We have used GFP to study:



1) whole cell
2) actin, microfilament…
3)recombinant mitochondrial protein



All the organism here is fluorescing
As a result of expressing GFP

Neurones
We can see how they connect

**PAST PAPERS:

- You have studied the possible interaction between two proteins, dumbless and smartful. Dubmless has two domains X and Y. Smartful has two domains: A and B. You used the yeast two-hybrid system approach expressing different domain/protein combinations. You generated the following results (dumbless + smartful = blue colonies; A + X = blue colonies; A + Y = blue colonies; B + X = white colonies; B + y = white colonies). What is your interpretation?

A. Domain B interacts with both domains X and Y
b. Domain A interacts with both domains X and Y
c. The two proteins do not really interact with each other
d. Domain B interacts with X but not Y
e. Domain A interacts with X but not Y

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B

PAST PAPER QUESTIONS !

- The advantage of creating cDNA from human genome is:
  a. Lack of introns
  b. Lack of untranslated regions
  c. Lack of promoters
  d. Lack of iron binding elements
  e. Non of the choices is correct

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A

PAST PAPER QUESTIONS !

- This particular advantage of plasmids makes them favorable vectors for the production of large amounts of a recombinant human protein in bacteria
  a. They carry antibiotic-resistance genes
  b. The promoter they contain is human
  c. They are small
  d. They can be replicated in bacterial cells
  e. They are bacterial in nature

D

PAST PAPER QUESTIONS !

- The following sequence you do not expect not to be recognized by a restriction endonuclease:
  a. AGCT
  b. ATATAT
  c. GCAGCA
  d. GGATCC
  e. CTTAAG

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C