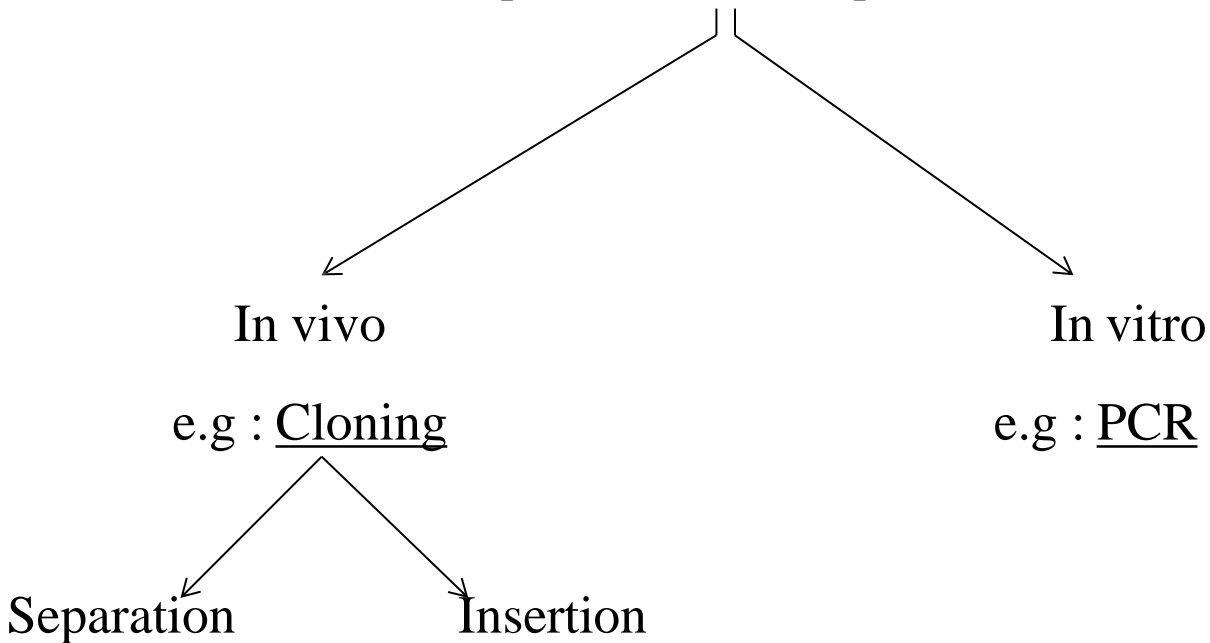


# Cloning

Amplification = Multiplication



## ▣ Cloning :

-Different process that can be used to produce genetically identical copies of biological entity .

-The copied material which has the same genetic makeup as the original , is referred to as a clone .

- Researchers have cloned a wide range of biological material ,including genes , cells , tissues and even entire organisms , such as a sheep .

◆ Aim of cloning : Multiple copies of specific sequence .

## ◆ used in ( cloned DNA Importance “Application” ) :

- Detailed analysis ( Downstream application ) .
- Work out the function of the gene .
- Look at how mutation may affect a gene function .
- Investigate a gene’s characteristics (Size , expression , tissue distribution )
- Make large concentration of the protein coded for the gene .

## ■ Cloning Types :-

- there are three different types of artificial cloning : Gene cloning , reproductive cloning and therapeutic cloning .

1- Gene cloning : produces copies of genes or segments of DNA .

2- Reproductive cloning : produces copies of the whole animals .

3- Therapeutic cloning : produces embryonic stem cells for experiments aimed at creating tissues to replace injured or diseased tissue .

## ■ Gene cloning mechanism :

- the procedure consists of :

- inserting gene from one organism ( foreign DNA ) into the genetic material of a carrier called a Vector .

- Vectors : include bacteria , Yeast cells , Viruses or plasmids

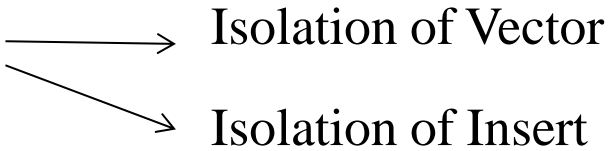
- Plasmids : are small DNA circles carried by bacterial .

- After the gene is inserted , the vector is placed in laboratory conditions that prompt it to multiply , resulting in the gene being copied many times over .



## 5- Growing

- the Transformed Recombinant bacteria will growing in a liquid media , then it will be filtrated .

6- **Isolation**  Isolation of Vector  
Isolation of Insert

## Q: What is the general DNA cloning protocol?

1The chosen of DNA is “ cut” from the source organism using restriction enzymes .

2The piece of DNA is “ pasted” into a vector and ends of the DNA are joined with the vector DNA by ligation .

3The vector is introduced into a host cell , often a bacterium or yeast , by a process called transformation .

- the Host cells copy the vector DNA along with their own DNA , creating multiple copies of the inserted DNA .

4- The Vector DNA is isolated or separated from the host cells’ DNA and purified .

-DNA that has been (cut) and (pasted) from an organism into a vector is called recombinant DNA , because of this DNA cloning is also called recombinant DNA technology .

## **Q: What is the standard DNA cloning steps ? ( 7 steps )**

- 1- Choice of host organism and cloning vector .
  - 2 preparation of vector DNA .
  - 3preparation of DNA to be cloned .
  - 4- creation of recombinant DNA .
  - 5 Introduction of recombinant DNA into host organism .
  - 6 Selection (Isolation) of organisms containing recombinant DNA
  - 7Screening for clones with desired DNA inserts and biological properties .
- 

### **1- Choice of host organism and cloning vector .**

- Host : Bacterium E.coli ( Escherichia coli )
- Vector : Plasmid
- E.coli and plasmid vectors are in common use: because they are technically sophisticated ( , versatile ) (تلماختسلا ددعم , روطتم) widely available , and offer rapid growth of recombinant organisms with minimal equipment.
- The vector almost always contains four DNA segments :-
  - 1An origin of DNA replication is necessary for the vector (recombinant sequence linked to it )
  - 2one or more unique restriction endonuclease recognition sites that serves as sites where foreign DNA may be introduced .
  - 3 a selectable genetic marker gene that can used to enable the survival of cells that have taken up vector sequences .
  - 4an additional gene that can be used for screening which cells contain foreign DNA .

## **2- preparation of vector DNA.**

- The cloning vector is treated with a restriction endonuclease to cleave the DNA at the site where foreign DNA will be inserted .
- Cleaving the vector DNA and foreign DNA with the same restriction enzymes .
- Most modern vectors contain a variety of convenient cleavage sites that are unique within the vector molecule ( so that the vector can only be cleaved at a single site )
- the cloning vector may be treated with an enzyme ( alkaline phosphatase ) that dephosphorylates the vector ends .
- Vector molecules with dephosphorylated ends are unable to replicate , and replication can only be restored if foreign DNA is integrated into the cleavage site .

## **3- preparation of DNA to be cloned.**

- DNA extracted from tissue sources or from extinct animals .
- DNA is purified using simpler methods to remove contamination proteins ( Extraction with phenol ) , RNA ( ribonuclease ) and smaller molecules ( precipitation or chromatography )
- PCR methods are often used for amplification of a specific DNA or RNA (RT-PCR) sequences prior to molecular cloning .
- the purified DNA is then treated with restriction enzyme to generate fragments with ends capable of being linked to those of the vector .

## **4- creation of recombinant DNA.**

- DNA prepared from the vector and foreign source are simply mixed together at appropriate concentrations and exposed to an enzyme ( DNA ligase ) ( Ligation process ).
- The resulting DNA Mixture containing randomly joined ends is then ready for introduction into the host organism .

## **5- Introduction of recombinant DNA into host organism.**

- The DNA mixture previously manipulated in vitro is moved back into a living cell ( the Host ) .
- there are 4 experimental methods :-

**1Transformation** :- when **microorganism** are able to take up and replicate DNA from their local environment .

**2 Transfection** :- in **mammalian cell** culture .

- Transformation & Transfection require preparation of the cell through a special growth regime and chemical treatment .

**3Electroporation** :- use high electrical voltage pulses to translocate DNA across the cell membrane .

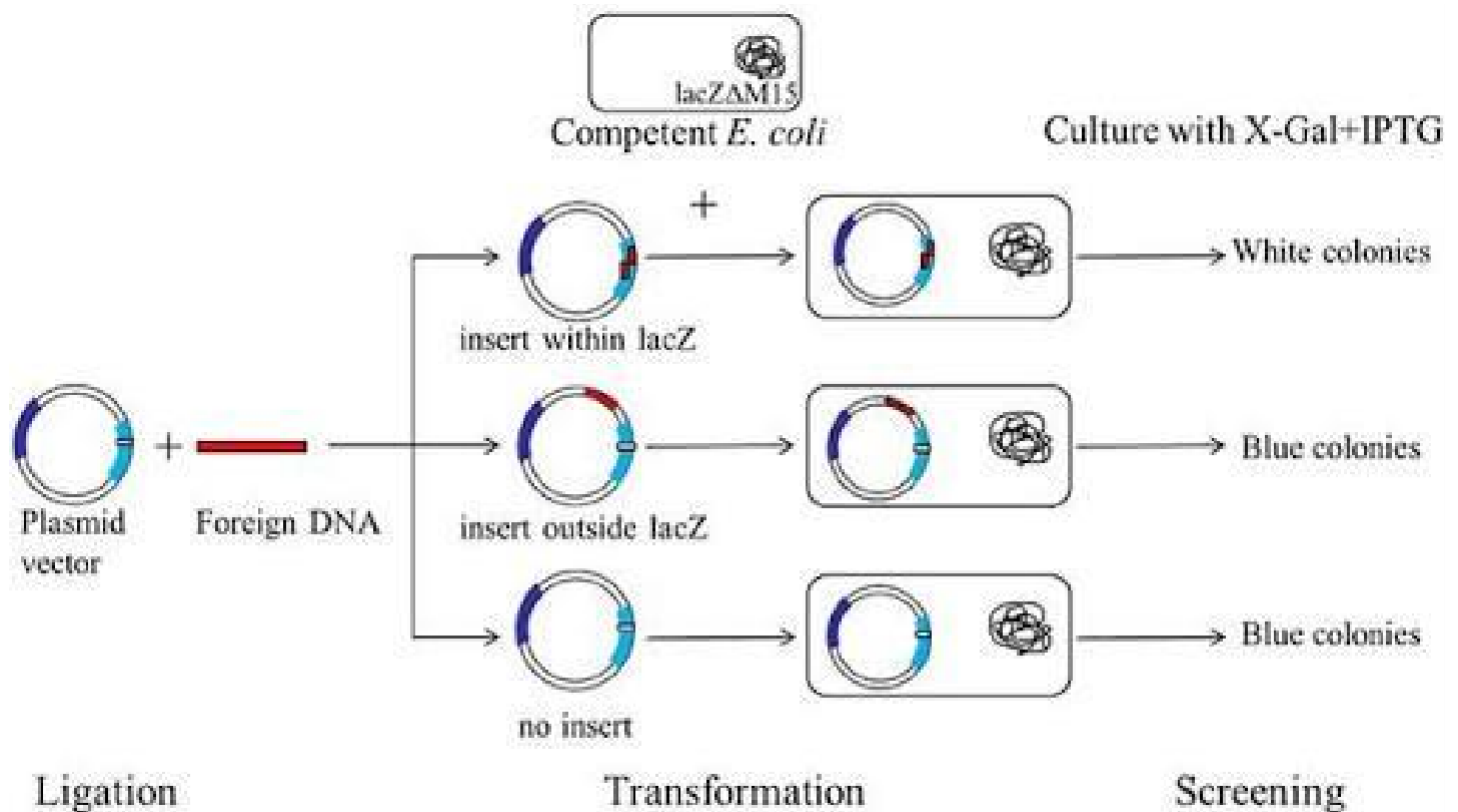
**4Transduction** :- involve the packaging of the DNA into virus-derived particles , and using the virus-like particles to introduce the encapsulated DNA into the cell through a process resembling viral infection .

-Electroporation & Transduction are highly specialized methods , they may be the most efficient methods to move DNA into cells .

## 6- Selection (Isolation) of organisms containing recombinant DNA

- a small fraction of the cells will actually take up DNA .
- cells that have not taken up DNA are selectively killed, and only those cells that can actively replicate DNA containing the selectable marker gene encoded by the vector are able to survive .
- When bacterial cells are used as host organisms , the selectable marker is usually a gene that confers resistance to an antibiotic that would otherwise kill the cells , typically ampicillin .
- cells harboring (contain) the plasmid will survive when exposed to the antibiotic , while those that have failed to take up plasmid sequences will die .

## 7- Screening for clones with desired DNA inserts and biological





- **blue –white Screening system •**

Ex: PUC<sup>19</sup> vector

- The blue –white screen is a screening technique that allow for the rapid and detection of recombinant bacteria in vector –based molecular cloning experiments .
- DNA is then inserted into a host cell viable for transformation , which are then grown in the presence of X-gel .
- Cells transformed with vectors containing recombinant DNA will produce white colonies .
- Cells transformed with non-recombinant plasmid ( i.e only the vector ) grow into blue colonies .

That mean :-

- ◆foreign DNA is inserted into a sequence that encodes an essential part of beta-galactosidase ( lac z ) , an enzyme whose activity results in formation of blue –colored colony on the culture medium that is used for this work .
- ◆insertion of the foreign DNA into the beta-galactosidase coding sequence disables the function of the enzyme, so that containing transformed DNA remain colorless ( white ) .

- **Note :-**

- The total population of individual clones obtained in a molecular cloning experiment is often termed a **DNA library** . )

- DNA Library** : collecting of Recombinant Vector containing cloned sequence that cover all genome of specific organism .

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## **Application of molecular (DNA) cloning :-**

### **1- Genome organization and gene expression**

- Elucidation ) (of the complete DNA sequence of the genome of a very large number of species .
- Exploration of genetic diversity within individual species .
- molecular clones are used to generate probes that are used for examining how genes are expressed .
- cloned genes can also provide tools to examine the biological function and importance of individual genes , by allowing scientists to inactivate the genes , or make mutation using regional mutagenesis or site-directed mutagenesis .

### **2- production of recombinant proteins**

- obtaining the molecular clone of a gene can lead to the development of organisms that produce the protein product of the cloned genes , termed as **recombinant protein** .
- practically it is difficult to develop an organism that produces an active form of the recombinant protein in desirable quantities .
- ◆ many useful proteins are currently available as recombinant product :-
  - (1)Medically useful proteins whose can correct a defective or poorly expressed gene ( eg: recombinant VIII , a blood clotting factor deficient in some forms of hemophilia , and recombinant insulin , used to treat some forms of diabetes ) .
  - (2)assist in a life –threatening emergency ( e.g tissue plasminogen activator , used to treat strokes )

(3) Recombinant subunit vaccines , in which a purified protein can be used to immunize patients against infectious diseases , without exposing them to the infectious agent itself ( e.g hepatitis B vaccine )

(4) Recombinant proteins as standard material for diagnostic laboratory tests .

### 3- Transgenic Organism

- cloned genes may be inserted into organisms , generating transgenic organisms , also termed genetically modified organisms ( GMOs)

- a number of GMOs have been developed for commercial use :-

- animals and plants that produce pharmaceuticals or other compounds ( pharming ) .

- Herbicide-resistant crop plants .

- fluorescent tropical fish ( Glofish ) for home entertainment .

## 4- Gene Therapy

- Involve : supplying a functional gene to cells lacking that function .
- aim : correcting a genetic disorder or acquired disease .
- Gene therapy divided into :-

### 1- Alteration in **germ cell** ( sperm or egg )

- result in permanent genetic change for the whole organism and subsequent generations .

### 2- **Somatic cell** gene therapy ( analogous to an organ transplant )

-one or more specific tissues are targeted by direct treatment or by removal of the tissue , addition of the therapeutic gene in the lab. , and return of the treated cells to the patient .

- mostly for the treatment of cancers and blood , liver , and lung disorders .

## Cloning Vectors

• **Cloning vector** :- a DNA molecule that carries foreign DNA into a host cell , replicates inside a bacterial ( or yeast ) cell and produces many copies of itself and the foreign DNA .

### • **Types of cloning Vectors :**

#### 1- **plasmid** )

cloning limit : 100-10000 bp or 0.1-10 kb

#### 2- **Phage** ( bacteriophage ) ( lambda $\lambda$ -phage )

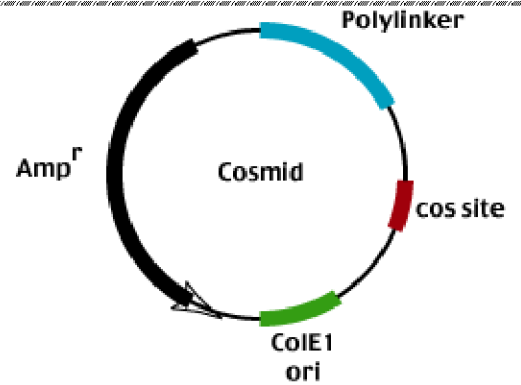
- linear DNA molecule .

-whose region can be replaced with foreign DNA without disrupting its life cycle .

- cloning limit : 8-20 kb .( ~ 25 kbp

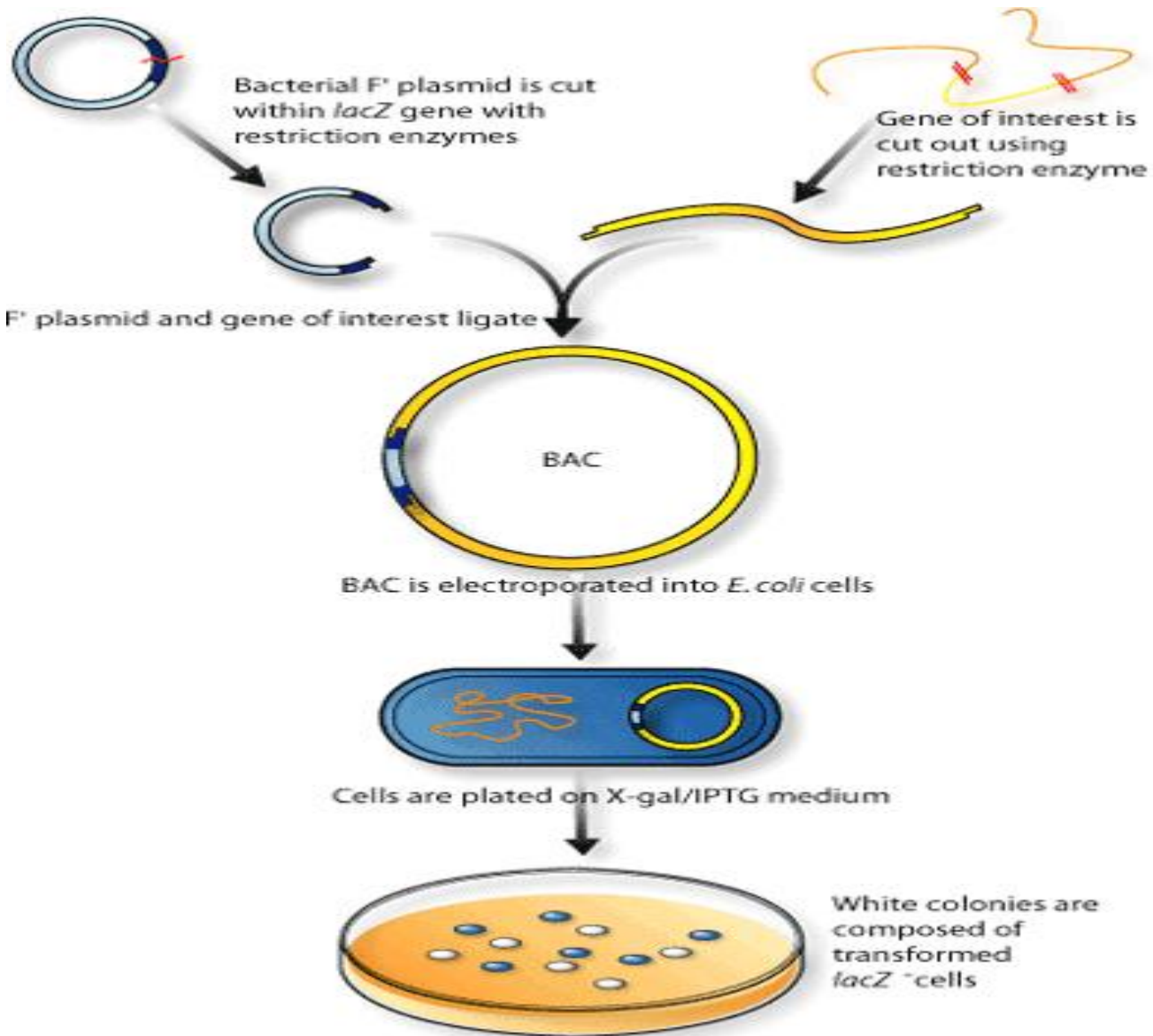
### 3- cosmid

- extra-chromosomal circular DNA molecule .
- combines features of plasmids and phage .
- cloning limit : 35-50 kb .

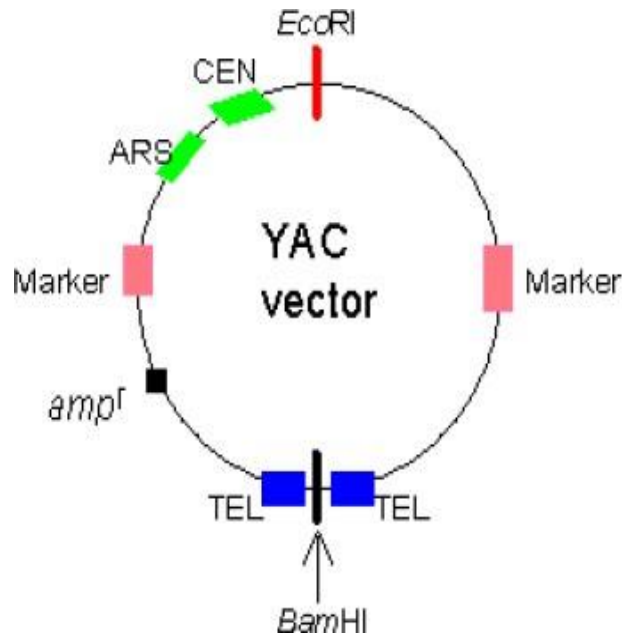


### 4- Bacterial Artificial chromosomes ( BAC)

- based in bacterial mini-F plasmids .
- cloning limits : 75-300 kb .



## 5- Yeast Artificial Chromosomes ( YAC )

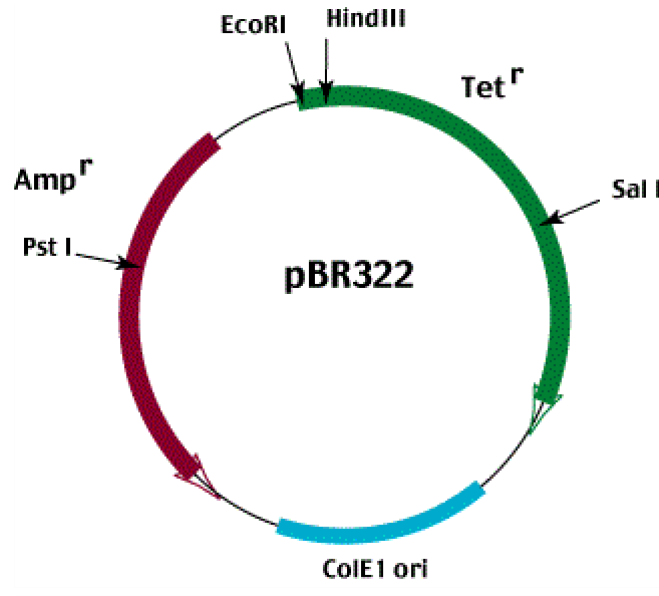


- Artificial chromosome that contains telomers , origin of replication , a yeast centromere , and selectable marker for identification in yeast cells.
- cloning limit : 100-1000 kb .

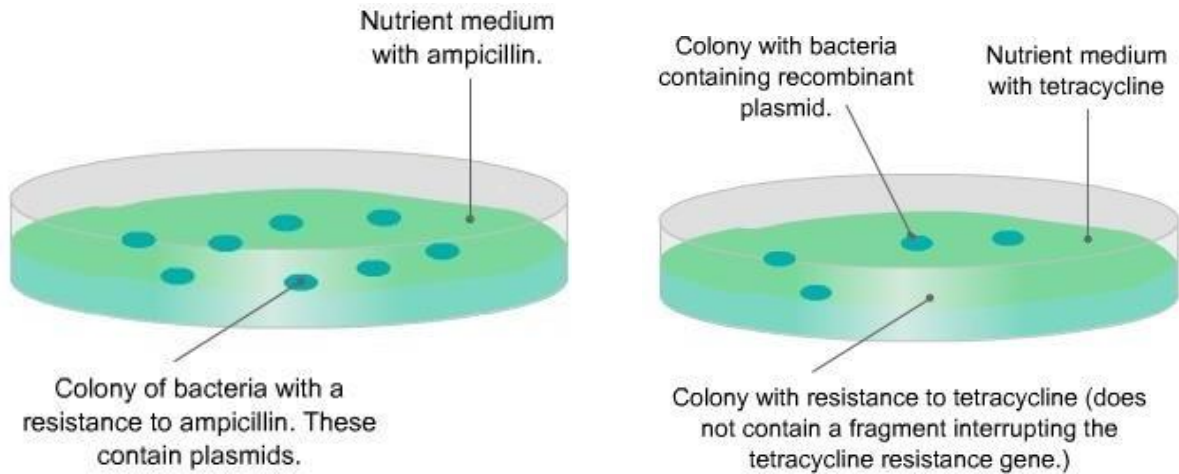
### ■ What is the properties of Plasmid ?

- 1- Extra-chromosomal circular DNA molecule .
- circular DNA more stable than linear DNA .
- 2- Small in size , so easy to separated .
- 3- Self replication : Autonomously replicates inside the bacterial cell .
- 4- Have multiple cloning site ( MCS)
- 5- Have gene enable screening ( selectable marker ; usually drug resistance ) .
- 6 have a known sequence .

# Plasmid PR322

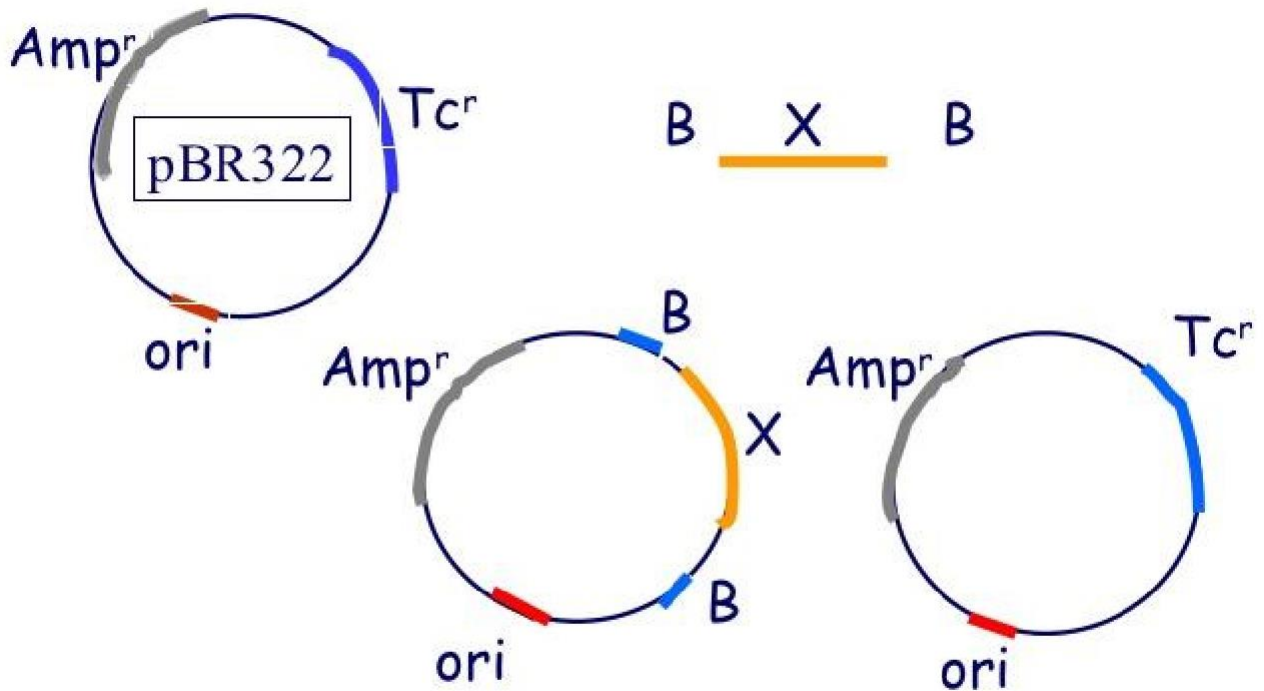


- **pBR322** is a plasmid and was one of the first widely used E.coli cloning vectors ... ( First Discovered )
- The **p** stands for "**plasmid**" and **BR** for "**Bolivar**" and "**Rodriguez.**" ءاملع
- **pBR322** is 4.7 Kilo base pairs in length and has two antibiotic resistance genes
  - the ampicillin resistance (Amp<sup>R</sup>) gene
  - and the gene tet<sup>R</sup> encoding the tetracycline resistance (Tet<sup>R</sup>) .
- It contains the origin of replication
- **pBR322** have one Type
- **Screening** depend on two Antibiotic Resistance ( 2 AB )
  - (Amp<sup>R</sup>) gene used to Discriminate between Transformed and Non-Transformed bacteria
  - (Tet<sup>R</sup>) gene used to Discriminate between Recombinant DNA in a bacteria and that with non-Recombinant DNA .



## Twin antibiotic resistance screening

-Screening by insertional inactivation of a resistance gene



Ampicillin resistant?      *yes*

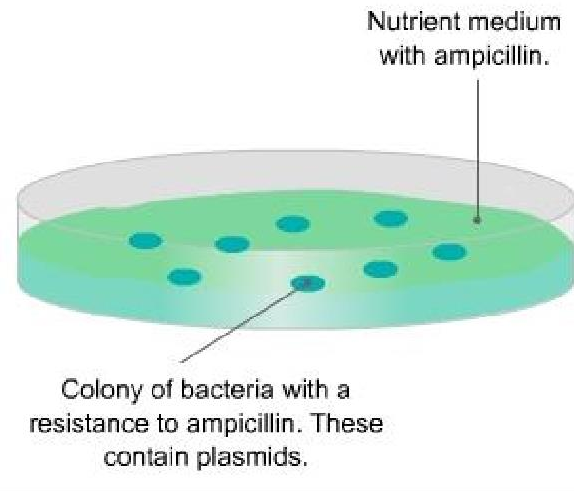
Tetracycline resistant?      *No*

*yes*

*yes*



## In Ampicillin Agar Plate



### 1- Non-transformed Bacteria :-

- Cannot grow on ampicillin agar plate

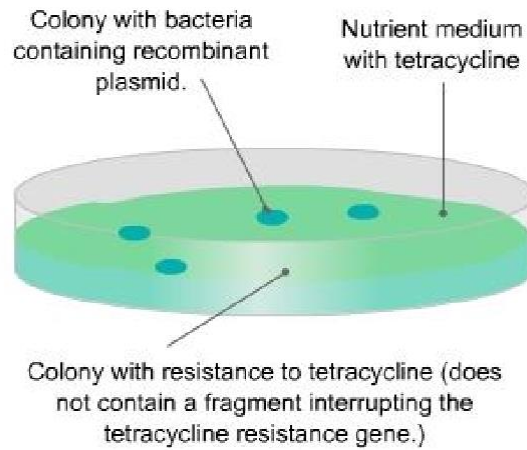
### 2 Trasformed Bacteria :-

Only transformed Colonies can grow in ampicillin medium , because it have plasmid with ( $Amp^R$ ) gene .

### 3 Transformed Bacteria with recombinant DNA (have the Foreign DNA) :-

- carrying our gene of interest in ( $Tet^R$ ) gene position so it will inactivate it .
- Transformed recombinant can grow only in ampicillin medium and cannot grow on tetracycline medium due to insertional inactivation .
- So recombinant colonies can be easily selected from the Agar plate .

## In Tetracycline agar plate



### 1- Non-transformed Bacteria :-

- cannot grow on Tetracycline medium .

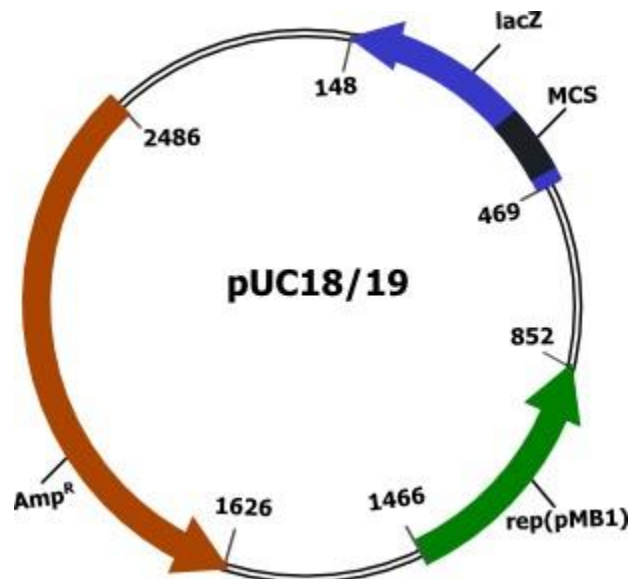
### 2- Transformed Bacteria :-

- Only transformed colonies can grow in tetracycline medium .

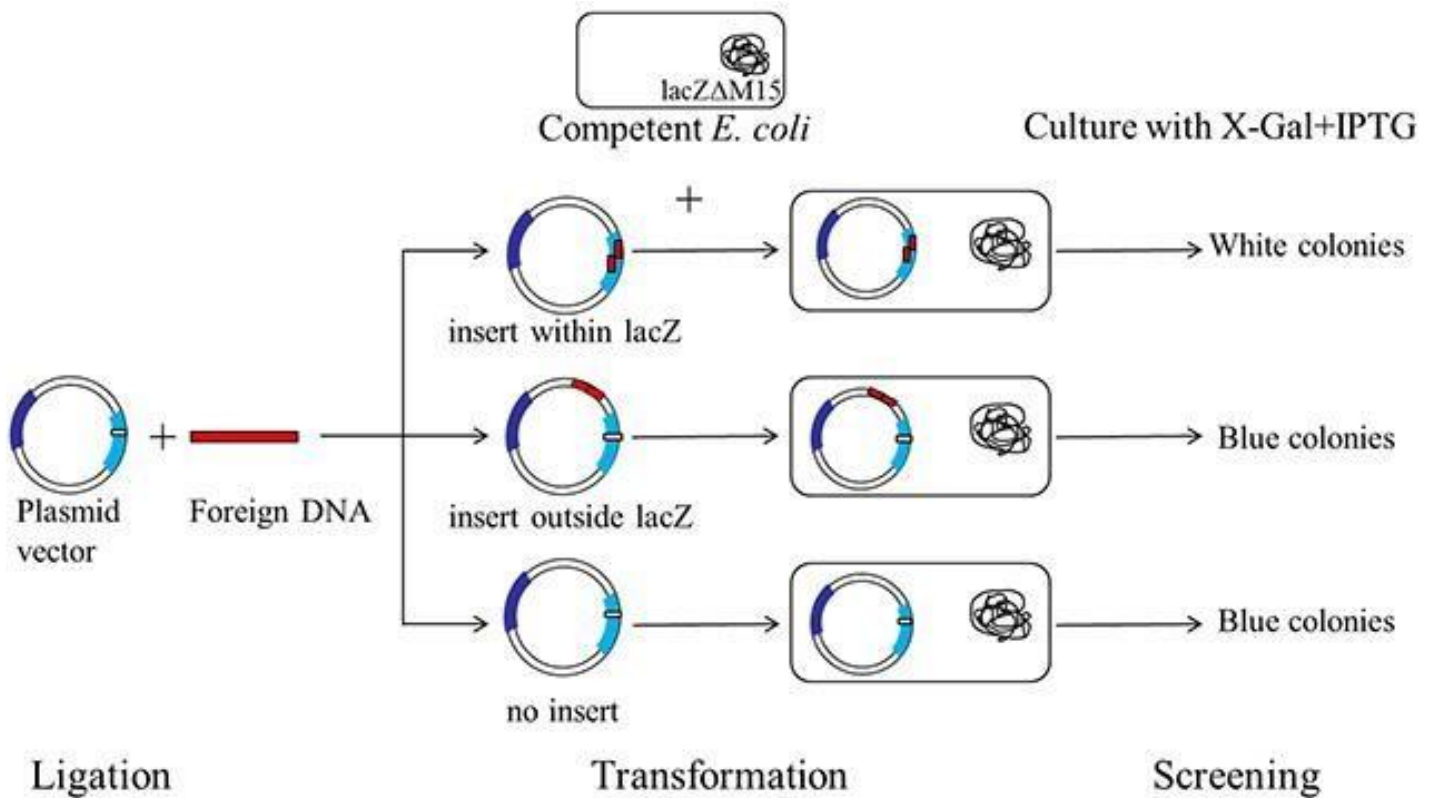
### 3- Transformed Bacteria with recombinant DNA :-

- carrying our gene of interest in ( $Tet^R$ ) gene position so it will inactivate it .
- Transformed recombinant cannot grow on tetracycline medium due to insertional inactivation .
- Non-recombinant can grow on tetracycline medium due to presence of ( $Tet^R$ ) gene that used in survival of the bacteria in the Tetracycline agar medium .

# PUC



- pUC19 is one of a series of plasmid cloning vectors ( The 2<sup>nd</sup> to Discovered )
- The “p” stands for "**plasmid**" and “UC” for the University of California .
- It is a circular double stranded DNA and has 2.3 Kilo base pairs
- PUC have two types :- PUC 18 & PUC 19
- It has one (**amp<sup>R</sup>**) gene (ampicillin resistance gene), and an N-terminal fragment of  $\beta$ -galactosidase (**lacZ**) gene (coloring gene)
- have only one Antibiotic ( 1 AB ) resistance .
- Lac Z gene :- after expression give  $\beta$ -galactosidase that convert X-gal from white to Blue colony .



- For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate.
- If  $\beta$ -galactosidase is produced, X-gal is hydrolyzed and produce an insoluble blue pigment.
- The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white.
- The desired recombinant colonies can be easily picked and cultured.
- Note :-
  - ( $\text{Amp}^{\text{R}}$ ) gene used to Discriminate between Transformed and Non-Transformed bacteria
  - (Lac Z) gene used to Discriminate between Recombinant DNA in a bacteria and that with non-Recombinant DNA .

# In Ampicillin Agar Plate

## 1- Non-transformed Bacteria :-

- Cannot grow on ampicillin agar plate
- 2- Trasformed Bacteria : - ( without Foreign insert )
- have (Amp<sup>R</sup>) gene and (Lac Z) gene.
- Only transformed Colonies can grow in ampicillin medium , because it have plasmid with (Amp<sup>R</sup>) gene .

## 3- Transformed Bacteria with recombinant DNA (have the Foreign DNA) :-

- carrying our gene of interest in Lac Z gene position so it will inactivate its function .
- Transformed recombinant can grow only in ampicillin medium .

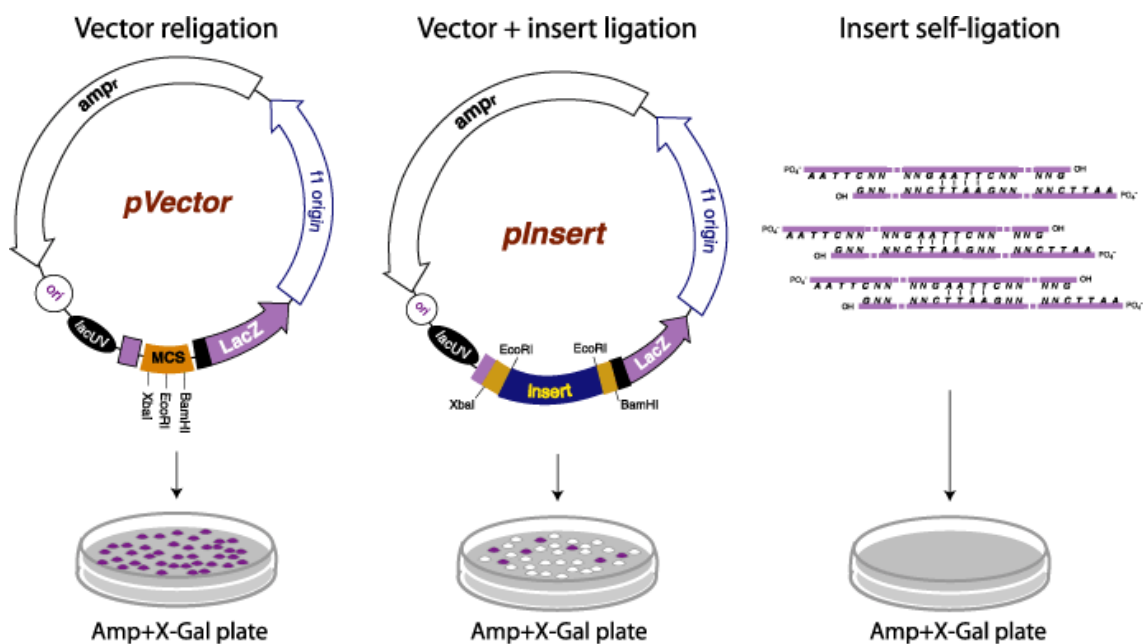
# In ( X-gal ) Lac Z Agar plate

## 1- Trasformed Bacteria : - ( without Foreign insert )

- have (Amp<sup>R</sup>) gene and Lac Z gene
- Only transformed Colonies can grow in ampicillin medium , because it have plasmid with (Amp<sup>R</sup>) gene .
- it also have Lac Z gene that after expression give  $\beta$ -galactosidase that convert X-gal from white to Blue colony .

## 2- Transformed Bacteria with recombinant DNA (have the Foreign DNA) :-

- carrying our gene of interest in Lac Z gene position so it will inactivate its function .
- The colonies formed by non-recombinant cells, appear blue in color while the recombinant ones appear white.
- The desired recombinant colonies can be easily picked and cultured.



The lecture is Done ☺ Q.A♥