BHARATHIDASAN UNIVERSITY

Tiruchirappalli- 620024,
Tamil Nadu, India

Programme : M.Sc., Biochemistry

Course Title : GENETIC ENGINEERING

Course Code: BC302CR

Unit – I

RESTRICTION AND MODIFICATIONS ENZYMES
CLONING VECTORS

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UNIT - I

RESTRICTION AND MODIFICATIONS ENZYMES.
CLONING VECTORS

RESTRICTION ENZYME :

- Restriction enzyme, also called **restriction endonuclease**, is a protein produced by bacteria that cleaves DNA at specific sites these sites are called **restrictions site**.
- Restriction enzyme are **molecular scissors.**
- The are 3000 restriction enzymes have been thoroughly investigated, and more than 600 of them are now commercially available and often employed in labs to modify and alter DNA.

RECOGNITION SITE:

- The DNA sequences recognized by restriction enzymes are called palindromes. Palindromes are the base sequences that read the same on the two strands but in opposite directions.
- For example, the palindrome appears
- 5′ GAATTC 3′ ,3′ CTTAAG 5′
- Restriction enzymes recognize a specific sequence of
- nucleotides, and produce a double-stranded cut in the DNA.
- These cuts are of two types:
- Blunt ends
- Sticky ends

HISTORY:

- The term restriction enzyme originated from the studies of phage λ.
- Restriction enzymes were first discovered in the 1960s by scientists Werner Arber and Hamilton Smith, who were studying the mechanisms that bacteria use to defend themselves against viral infections.
- In 1978, Daniel Nathans and Hamilton Smith were awarded the Nobel Prize in Physiology or Medicine for their discovery of restriction enzymes and their application in molecular biology.
- First restriction enzyme was isoltaed in 1970 by HindlllI.

NOMENCLATURE:

- Each enzyme is named after the bacterium from which it was isolated using a naming system based on **bacterial genus, species** and **strain.**
- For e.g **EcoRI**

Abbreviation	Meaning	Description
E	*Escherichia*	genus
co	*coli*	species
R	RY13	strain
I	First	order of identification in the species

TYPES:

- Restriction endonucleases are categorized into three groups. The are Type I, Type II, Type III.
- These types are categorization based on
- Their composition.
- Enzyme co-factor requirement.
- The nature of their target sequence.
- Position of their DNA cleavage site relative to the target sequence

TYPE I:

- Type I enzymes are complex, multi subunit, combination restriction-and-modification enzymes that cut DNA at random far from their recognition sequences .
- It requires both ATP and S-adenosyl-L-methionine cofactors to function.
- The are three different subunit.
- Example : EcoB

TYPE II:

- These are the most commonly available and used restriction enzymes
- They are composed of only one subunit.
- Their recognition sites are usually undivided and palindromic and 4-8 nucleotides in length,
- they recognize and cleave DNA at the same site.
- They do not use ATP for their activity.
- Example : EcoR I, Hind III

TYPE III:

- Type III restriction enzyme recognize two separate non-palindromic sequences that are inversely oriented.
- They cut DNA about 20-30 base pairs after the recognition site.
- These enzymes contain more than one subunit.
- It requires both Mg2+ ion and S-adenosyl-L- methionine cofactors.
- Example:EcoPI

APPLICATION:

- They are used in gene cloning and protein expression experiments.
- Restriction endonucleases are most frequently used as a technique in genetic engineering.
- Used in methods for DNA fingerprinting.
- They help in gene cloning, protein expression research, and the insertion of genes into plasmid vectors.
- DNA mapping.
- Gene sequencing.

MODIFICATIONS ENZYME:

- *Modification enzymes, also known as modifying enzymes, are enzymes that alter the chemical structure of molecules including proteins, lipids, and nucleic acids. Some common types of modifying enzymes include:*
- *Kinases: add a phosphate group to a protein or other molecule*
- *Phosphatases: remove a phosphate group from a protein or other molecule*
- *Proteases: cleave peptide bonds to break down proteins*
- *Lipases: cleave lipid molecules*

- Transferases: transfer a functional group from one molecule to another
- Methyltransferases: add a methyl group to a molecule
- Acetyltransferases: add an acetyl group to a molecule
- Modifying enzymes play crucial roles in various cellular processes, such as:
- Signal transduction pathways
- Gene expression and regulation
- Protein degradation and turnover
- Metabolic pathways
- DNA replication and repair

CLONING VECTOR:

- Cloning vector is used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and expressed.
- It is used amplify single molecule of DNA into many copes.
- The cloning vector is chosen according to the size and type of DNA to be cloned.
- Vectors may be plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs) and mammalian artificial chromosomes (MAC).

HISTORY :

- Scientists (Herbert Boyer, Keiichi Itakura and Arthur Riggs) working in Boyer's lab (University of California) recognized a general cloning vector with unique restriction sites for cloning in foreign DNA and the expression of antibiotic resistance genes for selection of transformed bacteria.
- In 1977, they described the first vector designed Boliver and Rodriguez for cloning purposes, pBR322-a plasmid.
- This vector was small, ~4 kb in size, and had two antibiotic resistance genes for selection.

CHARACTERISTICS:

- It should small in size.
- It must have an origin of replication.
- It must also be compatible with the host organism.
- It must possess a restriction site.
- Low molecular weight.
- Easily isolated & purified.
- Easily isolated into host cell.

FEATURES OF A CLONING VECTOR

- **Origin of replication (ori):**
- **This makes autonomous replication in vector.**
- **Ori is a specífic sequence of nucleotide from where replication starts.**
- **When foreign DNA is linked to the sequence along with vector replication, foreign (desirable) DNA also starts replicating within host cell.**

- **Cloning Site:**
- **Cloning site is a place where the vector DNA can be digested and desired DNA can be inserted by the same restriction enzyme.**
- **It is a point of entry or analysis for genetic engineering work.**
- **Recently recombinant plasmids contain a multiple cloning site (MCS) which have many (up to 20) restriction sites.**
- **Selectable Marker**
- **Selectable marker is a gene that confers resistance to particular antibiotics or selective agent that would normally kill the host cell or prevent its growth.**

- A cloning vector contains a selectable marker, which confer on the host cell an ability to survive and proliferate in a selective growth medium containing the particular antibiotics.
- **Reporter Gene or Marker Gene.**
- Reporter genes are used in cloning vectors to facilitate the screening of successful clones by using features of these genes that allow successful clone to be easily identified.
- Such feature present in cloning vectors is used in **blue-white selection.**

NATURAL AND ARTIFICIAL PLASMID AS VECTOR:

- **Plasmids are small, circular DNA molecules that are commonly used as vectors in genetic engineering and molecular biology. While natural plasmids are found in bacteria and other microorganisms, artificial plasmids are created in the laboratory for specific purposes.**
- **Natural plasmids:**
- **Occur naturally in bacteria and other microorganisms**
- **Often carry genes that confer antibiotic resistance or other beneficial traits**
- **Can be easily isolated and manipulated in the laboratory**

ADVANTAGES:

- **Efficient replication: Natural plasmids have evolved to replicate efficiently in their host organisms.**
- **Stable maintenance: They are stably maintained in the host cells, even in the absence of selection pressure.**
- **Easy to manipulate: Natural plasmids are well-characterized and easy to modify.**
- **DISADVANTAGES**
- **Limited capacity: Natural plasmids have a limited capacity to carry foreign DNA.**
- **Unstable in certain hosts: They may be unstable in certain host organisms or under specific growth conditions.**

- **Artificial plasmids:**
- **Created in the laboratory using DNA manipulation techniques**
- **Designed to contain specific genes or regulatory elements**
- **Often used for gene expression, gene editing, and other biotechnological applications.**

ADVANTAGES:

- **Large insert capacity:** Artificial plasmids can accommodate large fragments of DNA.
- **High stability**: They are designed to be stable in a wide range of hosts and growth conditions.
- **Customizable**: Artificial plasmids can be engineered to contain specific features and regulatory elements.
- **DISADVANTAGES**:
- **Unstable in some hosts:** Artificial plasmids can be unstable in certain host organisms.
- **May require selection pressure:** They may require continuous selection pressure to maintain their presence in the host cells.

- **Both natural and artificial plasmids are used as vectors to:**
- **Clone genes**
- **Express recombinant proteins**
- **Introduce genes into host organisms**
- **Study gene regulation and function**

REFERENCE:

- https://www for geeks.org/restriction-enzymes.
- https://www.neb.com/tools-and-resources/feature-articles/foundations-of-mol cloning-past-present-and-future
- Molecular biotechnology By S.B Primrose .

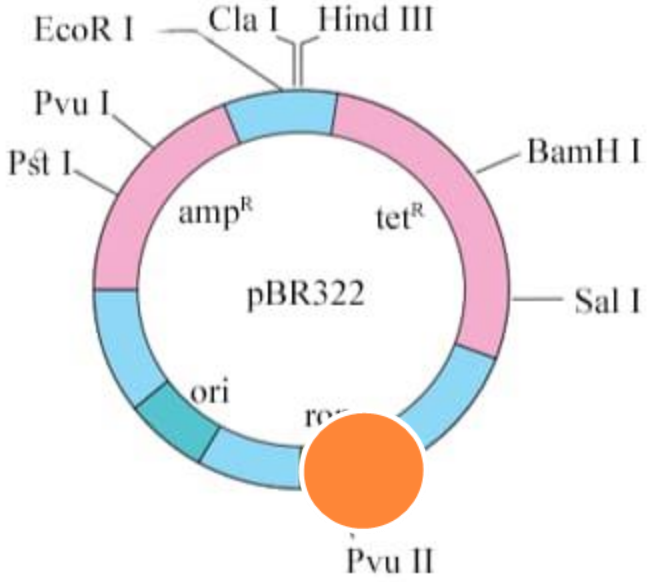
VECTORS USED FOR CLONING IN E.COLI , YEAST , HIGHER PLANTS AND ANIMAL CELLS

Introduction

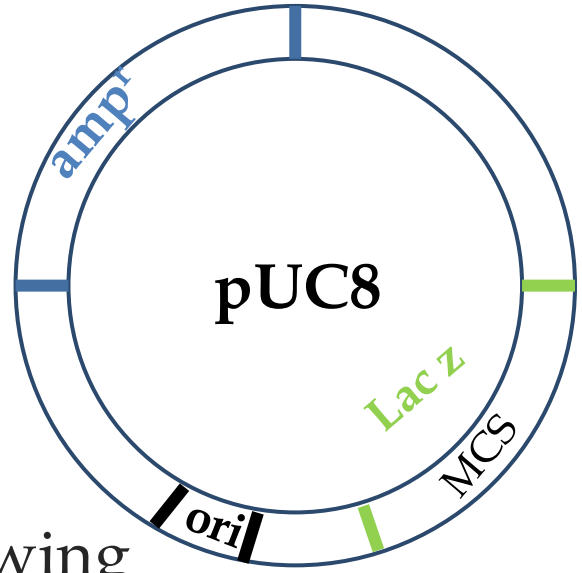
- Plasmids are small, circular, double stranded, extra chromosomal DNAs present in bacterial cells.
- They replicate independently.
- The plasmids are 1 kbp-200 kbp in size and have limited number of genes.
- The number of copies of a plasmid present in a cell is called copy number.
- The copy number of plasmids usually varies from 1 to 50.
- Vector are vehicle that act as a carrier for gene transfer.

Vectors used in e.coli

- **pBR322** is a artificial plasmid constructed by **Boliver** and **Rodriguez** in 1977.
- The copy number is 15-20 per cell.
- It consists of 4363 base pairs.
- It has two selectable gene markers – tetracycline resistance gene and ampicillin resistance gene.
- It has 9 unique restriction Site
- other than tet^r and amp^r.
- It carry large DNA segments of
- 5-10kbp.



- **pUC8** is an artificial plasmid vector. It is a cloning vector constructed from pBR322.
- It is constructed by **Messings** and his colleagues in **1983** at **University of California.**
- It consists of ori region, ampicillin resistance gene and lac z gene.
- It is 2676 bp in size.
- The lac z gene has a MCS which has
- recognition sites for 8 restriction
- enzymes.
- Foreign gene is inserted into the
- MCS and it inactivates the Lac Z gene.
- The recombinants are screened by growing the cells in a medium containing X-gal.

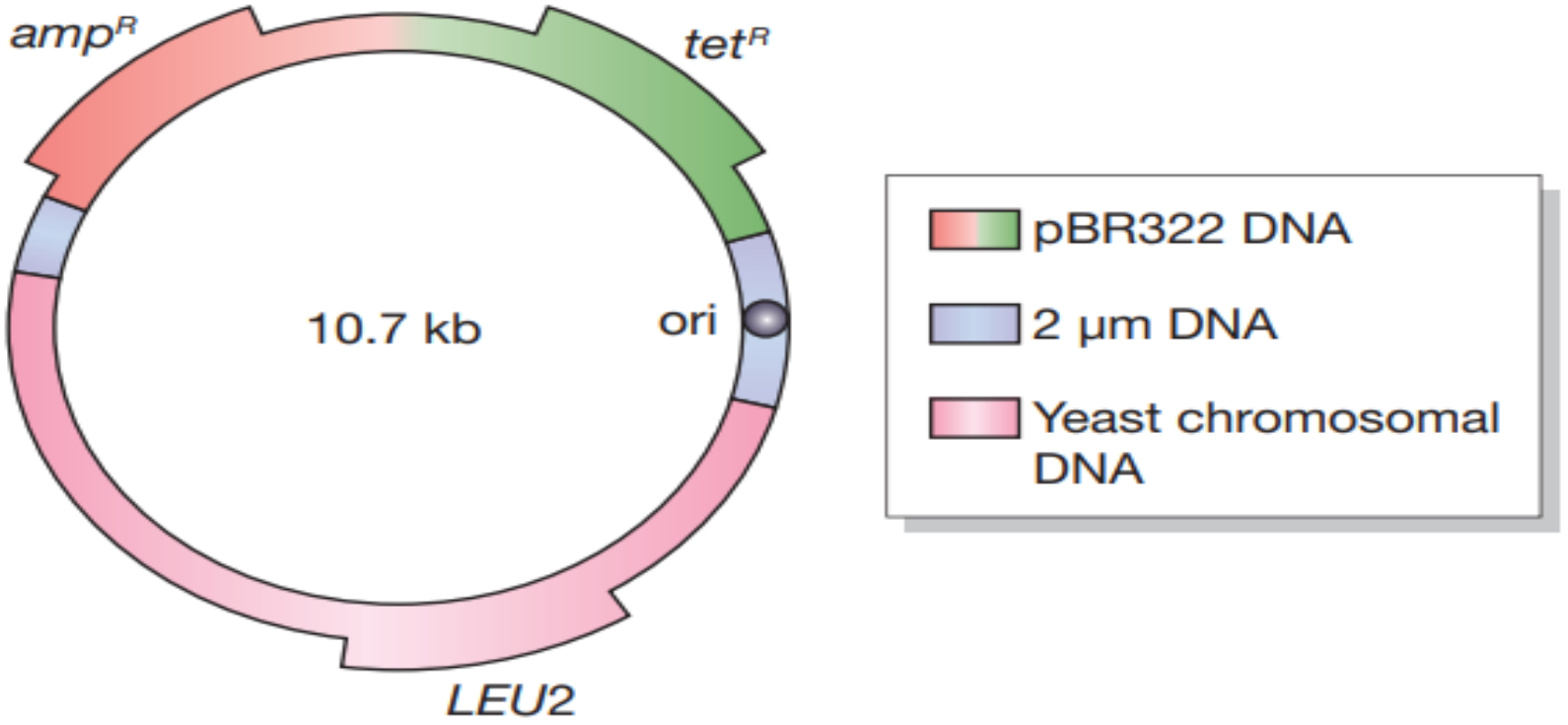


Vectors used in yeast

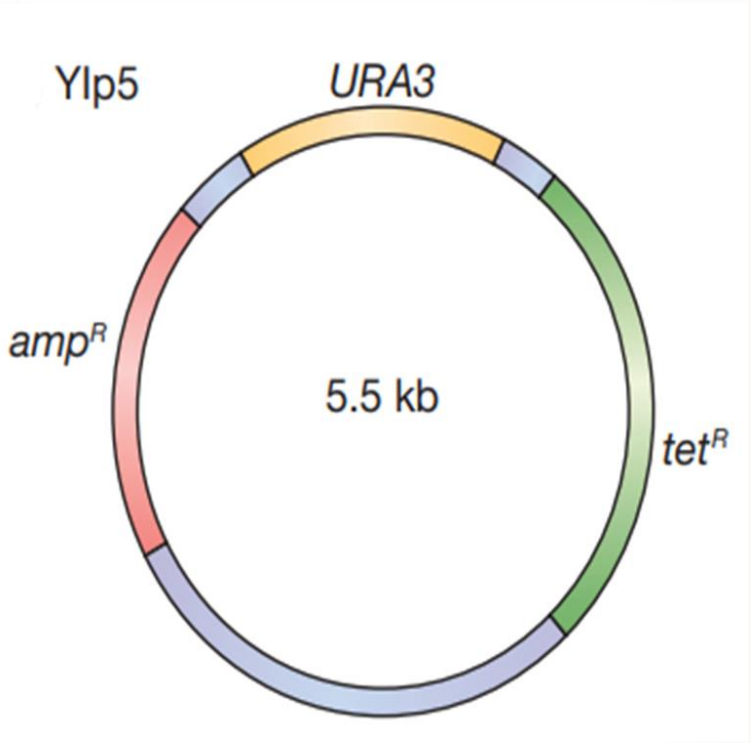
- The cloning vector for yeast are YEp, YIp, YRp, YCp, YAC.

- The YEp were constructed by beggs in 1978 from 2-micron of yeasts.
- This vector has tetracycle resistance (tc^r) and histidine synthesis genes has marker genes.
- The copy number of this vector is 25-175.
- The transformation frequency is high.

YEp


amp^R
tet^R
10.7 kb
ori
LEU2
pBR322 DNA
2 μm DNA
Yeast chromosomal DNA

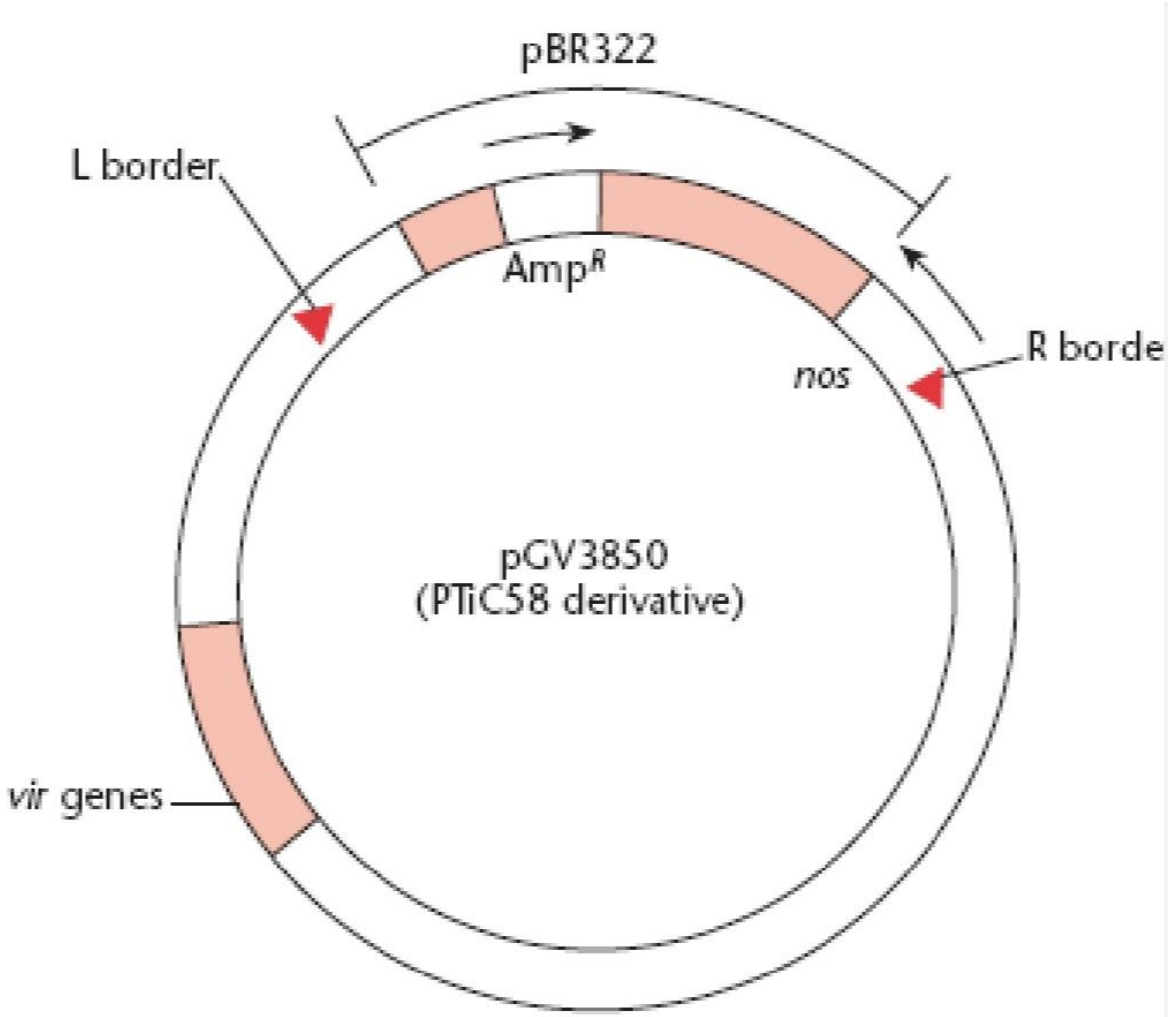
- The YIp was constructed by botstein and davis in 1982.
- It was constructed by inserting the yeast marker URA3 into coliE1 plasmid.
- And it also has leu gene.
- As a result the YIp integrated along with the chromosome.
- The URA3 inactivates the 5-fluoroorotic acid.
- The copy number is always one.
- Transformation frequency is low.
- Very useful to bring out genetic recombination studies with yeast.



Vectors used in higher plants

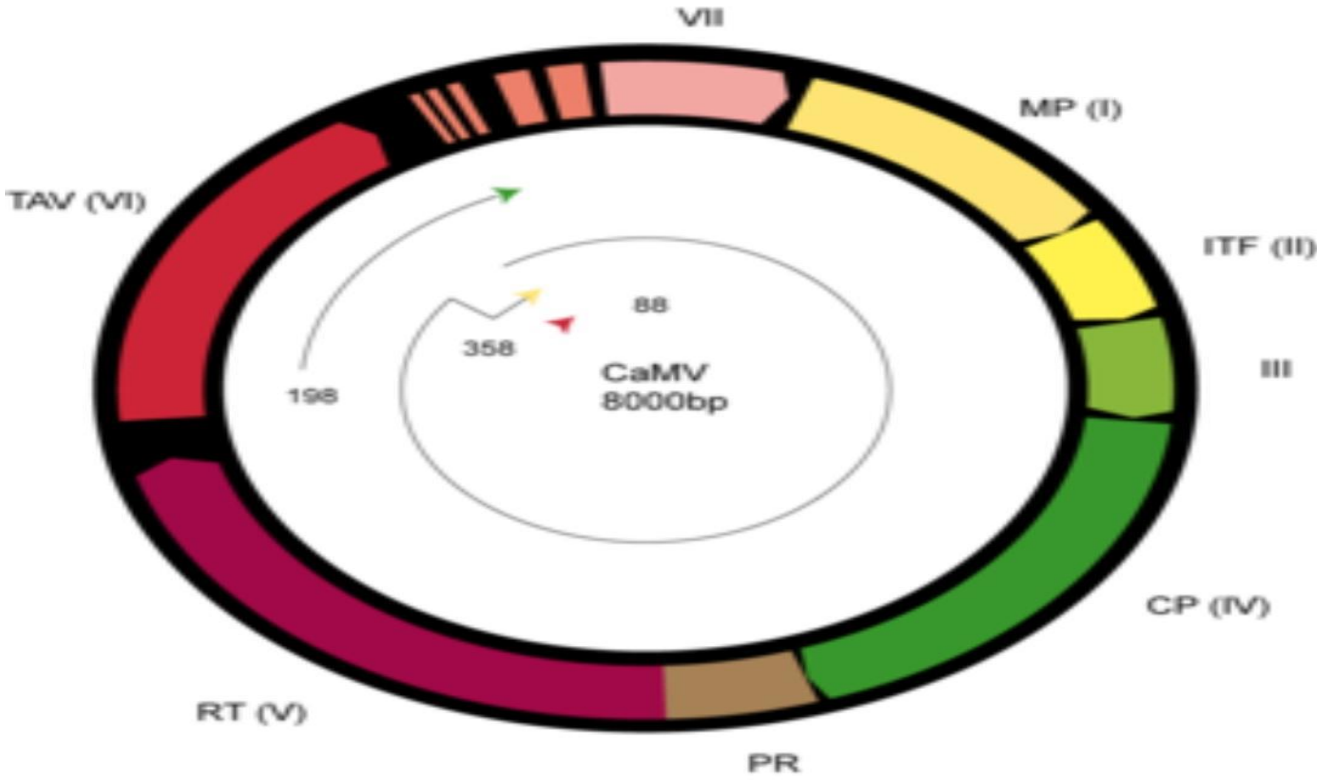
- There are two types of Ti derived plasmid namely **disarmed Ti plasmid** and **binary vectors.**

- **pGV3850** plasmid is a disarmed nopaline Ti plasmid constructed from pTiC58.
- The tumour inducing gene is substituted by a portion of gene from pBR322 in T-DNA.
- It has amp^r and Nos gene.
- As it has ***vir*** gene, it integrated into chromosomal DNA.


pBR322
L border
Amp^R
nos
R borde
pGV3850
(PTiC58 derivative)
vir genes

- **Cauliflower mosaic virus (CaMV)** is used as cloning vector by **Gronenborn** in 1981.
- It forms minichromosome in association with hosts histones.
- The minichromosome produces mRNAs and synthesis viral proteins.
- Meantime, it produce minus strand of DNA.
- Then the viral DNA is coated with protein and transferred to neighbouring cells.
- There is neither integration of viral dna to genomic dna of plant nor the cell is lysed.

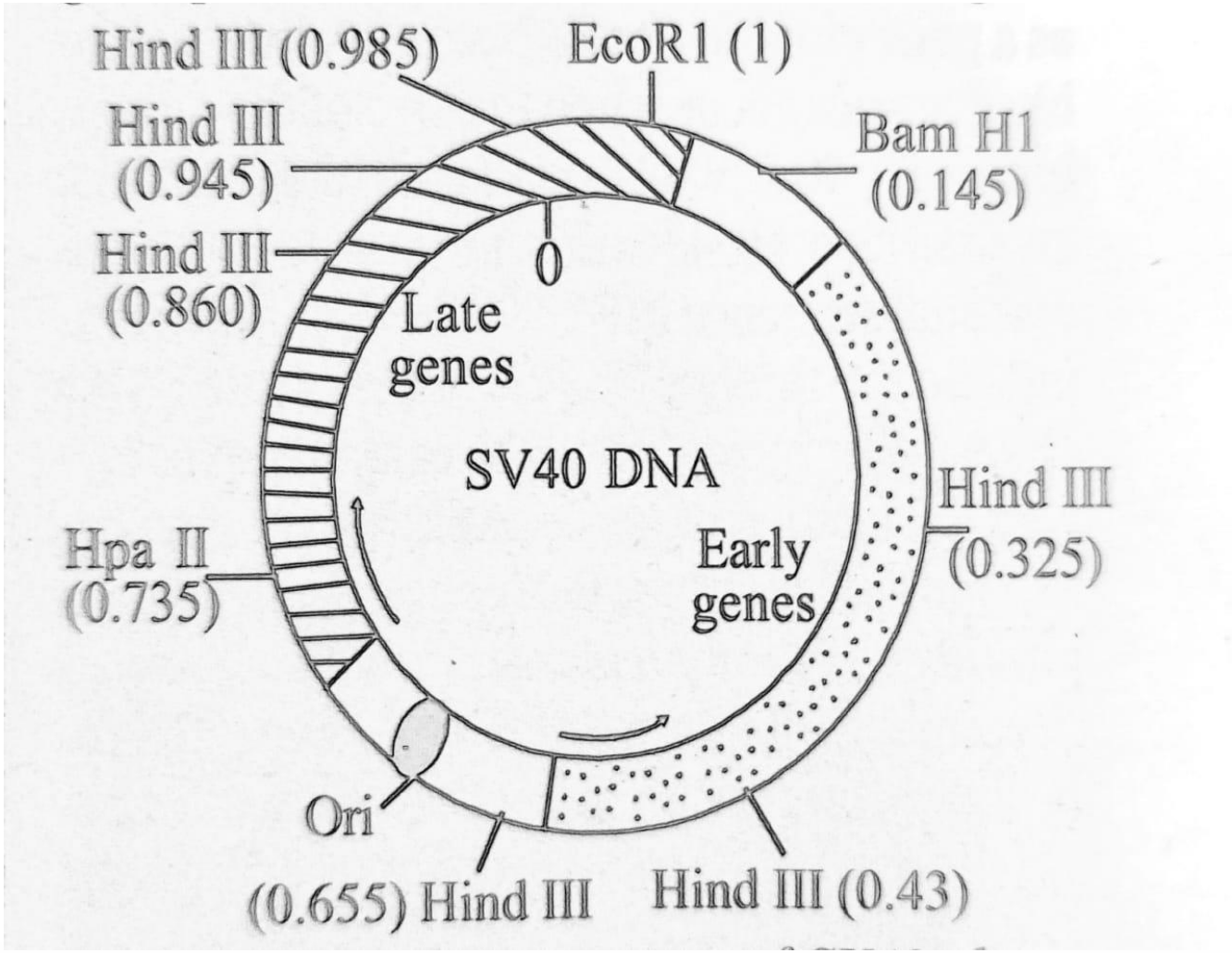
Cauliflower mosaic virus (CaMV)



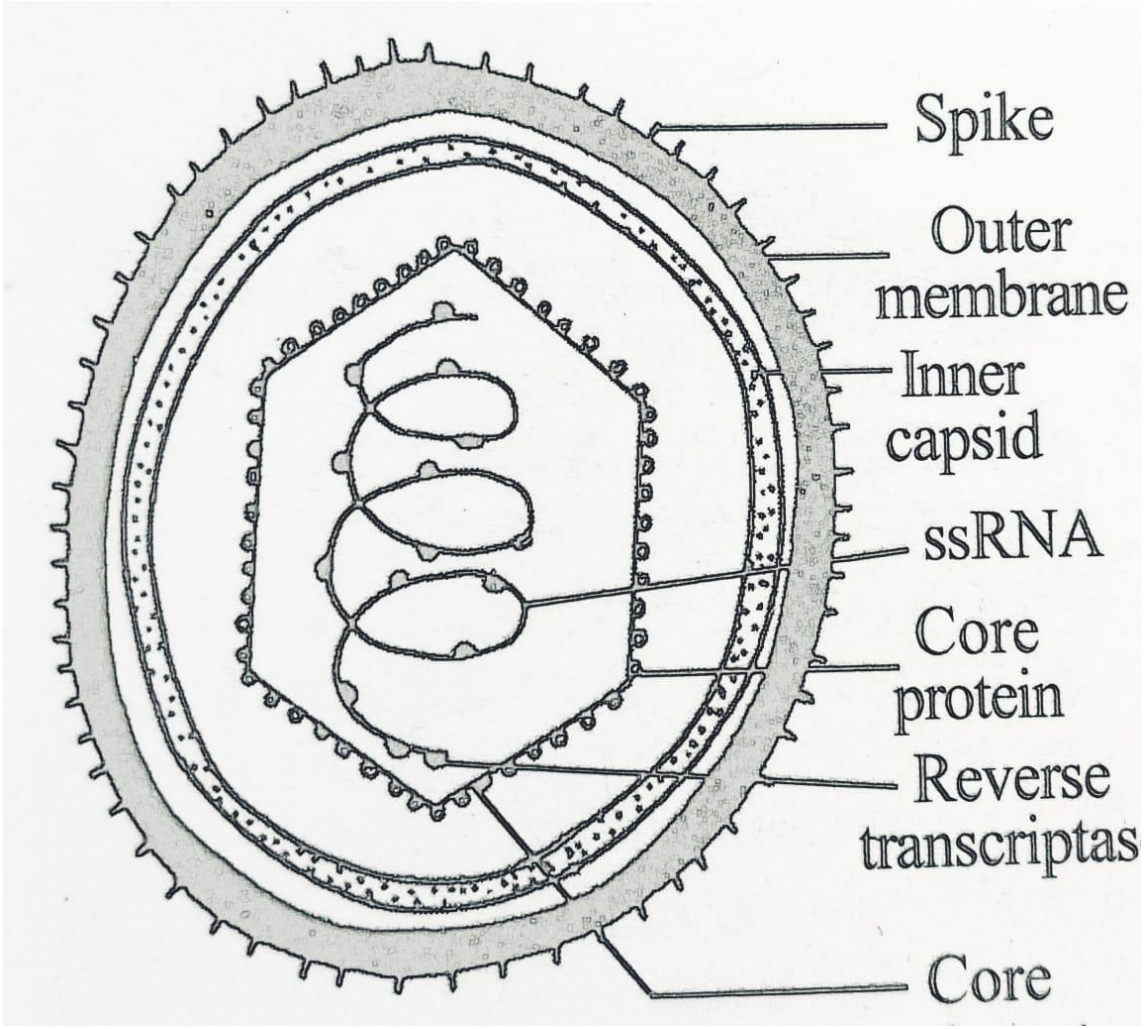
Vectors used in animal cells

- **Simian virus 40 (sv40)** has not directly used as gene cloning vector.
- Deletion of gene makes the vector replication defective one.
- It is provided by a helper plasmid.
- About 2.5 kb long DNA can be cloned.
- In 1976,**Goff** and **Berg** constructed first sv40 late gene replacement vector **SVGT-1.**
- They construct it by using HpaII and BamH1 as restriction enzyme.

Simian virus 40 (sv40)



- **Retroviruses** are used as a cloning vector because the infected cells never die for indefinite period.
- **Murine leukaemia virus (MuLV)** is the most used retrovirus for gene transfer.
- Less than 8kb gene can be transferred.
- It is provided with helper virus.
- The foreign gene inserted along with NEO gene as a gene marker.
- After construction of rDNA it is induced to mouse cell by using calcium phosphate mediated infection.
- The helper virus cannot be replicated.
- But the protein coat for rDNA provided RNA is synthesized and recombinant MuLV is formed.


Spike
Outer membrane
Inner capsid
ssRNA
Core protein
Reverse transcriptas
Core

CHARACTERISTICS OF EXPRESSION VECTORS. CONSTRUCTION OF DNA LIBRARIES- GENOMIC AND CDNA LIBRARIES. SCREENING OF RECOMBINANTS.

CHARACTERISTICS OF EXPRESSION VECTOR

- Expression vector :

A Vector used for a expression of a cloned DNA fragment in a host cell is called as expression vector

These vectors are frequently engineered to contain regulatory sequences that act as promoter and /or enhancer regions and lead to efficient transcription of the insert gene

Expression vectors are used for molecular biology techniques such as site-directed mutagenesis

The goal of well- designated expression vectors is the production of large amounts of stable messenger RNA and in extension proteins

Characteristics of expression vector

The chracteristics of expression vector is similar to cloning vector

Also has some important characteristics

The expression vector should contain :

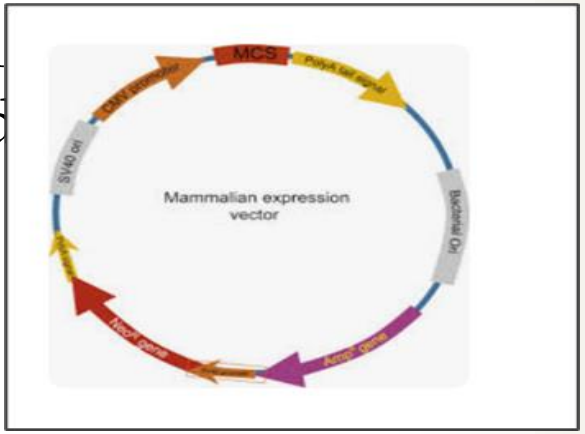
1) strong promoter

* So they can get large number of proteins

2) Transcriptional terminator

This regulatory sequence signals the end of transcription. RNA polymerase detaches from the DNA strand once it encounters the terminator, thus, the transcription ends.

- MCS(multi cloning site)
- An MCS is found in a variety of vectors, including clon vectors to increase the number of copies of target DNA, and expression vectors to create a protein product.
- In expression vectors, the MCS is located downstream of promoter



- RBS (Ribosomal Binding site)
- A segment of the 5' (upstream) part of an mRNA molecule that binds to the ribosome to position the message correctly for the initiation of translation
- The RBS controls the accuracy and efficiency with which the translation of mRNA begins.
- Inducible expression system
- Inducible Expression Vectors allow for spatial and temporal control of gene expression in mammalian cells.
- This system is adopted from bacteria which are tetracycline resistant.

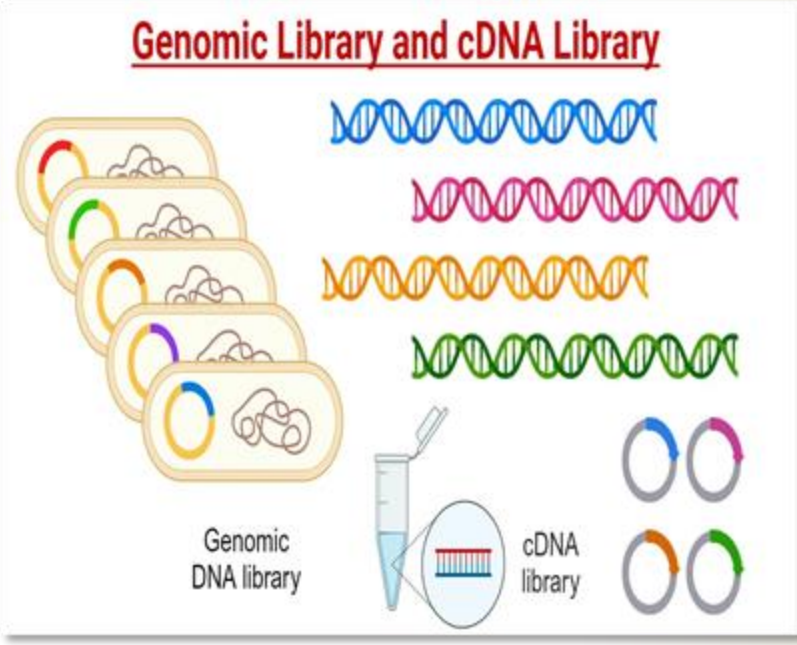
- DNA Library or Gene Library is simply the collection of DNA fragments cloned into vectors and stored within host organism

- They contain either the entire genome of a particular organism or the genes that are expressed at a given time.

Types of DNA Library

- Genomic information can be obtained by two primary methods.
- Based on this, DNA libraries are divided into two types: genomic and cDNA library.

Genomic Library



- A genomic DNA library is a collection of DNA fragments that represent all genetic information of an organism.
- This includes both coding and noncoding regions of the DNA.
- Genomic libraries are suitable for a wide range of applications, including genome mapping and comparative genomics.
- It allows the study of regulatory elements and noncoding sequences that are important in gene expression and regulation.

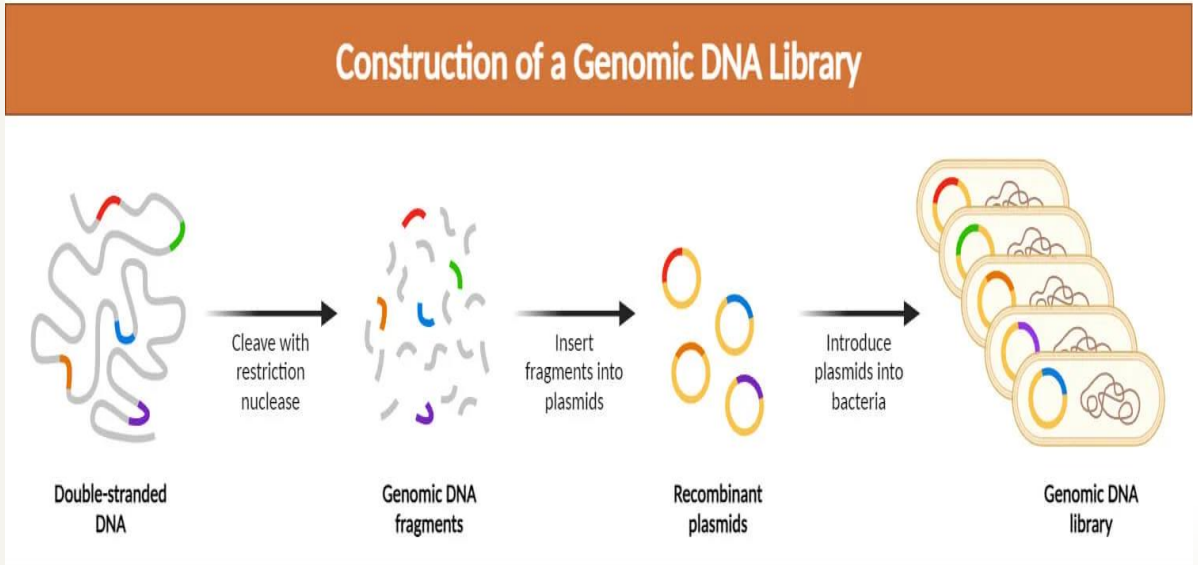
cDNA Library

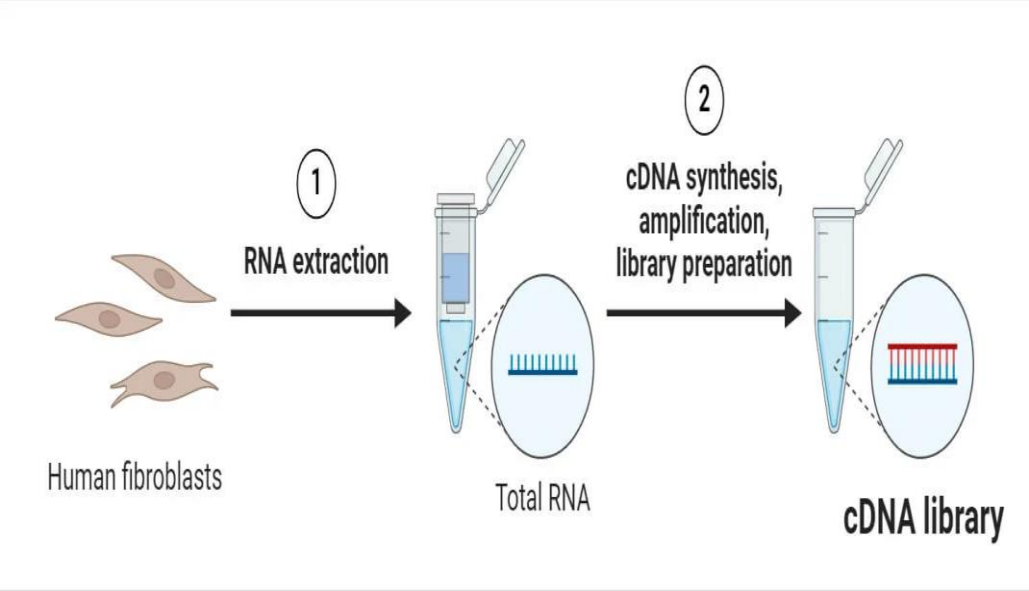
- A cDNA (complementary DNA) library is a collection of cDNA molecules derived from mRNA

- Unlike genomic libraries, cDNA libraries represent only the expressed genes of an organism,
- excluding non-expressed genomic regions such as introns and other noncoding sequences.

- cDNA libraries are useful for studying gene expression, protein functions, and producing recombinant proteins
- . Since cDNA libraries exclude noncoding regions, they provide a more focused view of the expressed genetic information.

- The disadvantages of the cDNA library include the limitation of studying gene regulation due to the absence of regulatory sequences, limited gene diversity, and bias toward highly expressed genes.
- cDNA libraries are specifically created from eukaryotes to study the expressed genes.
- Prokaryotes do not contain introns. Therefore, creating cDNA libraries for prokaryotes
- is generally not necessary, as their genomic DNA directly corresponds to their mRNA.





Recombinant Selection and Library Screening

- Recombinant DNA library represents an organism's mRNA or genomic DNA, that are cloned into the vectors as well aRecombinant DNA library represents an organism's mRNA or genomic DNA, that are cloned into the vectors as well as stored as the collection of transformants. s stored as the collection of trans formants.
- In the recombinant selection process, pressure would be applied during the growth stage of host cells that contain recombinant DNA
- It can be done in the presence of antibiotics. Cells with desired traits are then selected by scientists.

- However, recombinants in the genome library identify the various propagate
- sequences of fragments of the entire organism related to the genome

- Although, the genomic library of DNA is huge as compared to the cDNA library.
- Moreover, the recombinant library has been used to identify and demonstrate the various study topics.

Recombinant selection and library screening

- After the process of introducing r-DNA into a proper host cell, the cells, which have received the rDNA molecule, need to be identified.
- This process can be determined as the screening.
- Vector DNA, that is present within the recombinant cells, generally expresses characters.
- On the other hand, non-recombinants ones do not provide any type of traits or characters.
- A huge genomic library generally contains millions of cloned sequences.

Screening of cDNA library

- Several methods for library screening in terms of cDNA are based on appropriate pieces of materials either various nuclei and probes of acid to cDNA antibodies to the “polypeptide”

- product based on genes emerged by the cDNA. The DNA antibodies have been used for library screening by expressing the DNA library into protein.

- However, it is prepared by representing the various collections of genes that are properly coated with proteins by organisms.

- The main purpose of the cDNA library is to produce several genes that are expressed in recombinant libraries.
- Several steps of screening a cDNA or "Genomic library" can be classified into several ways as follows: "probe", "Homologous probe", "Heterologous probe", and many more to identify appropriately.

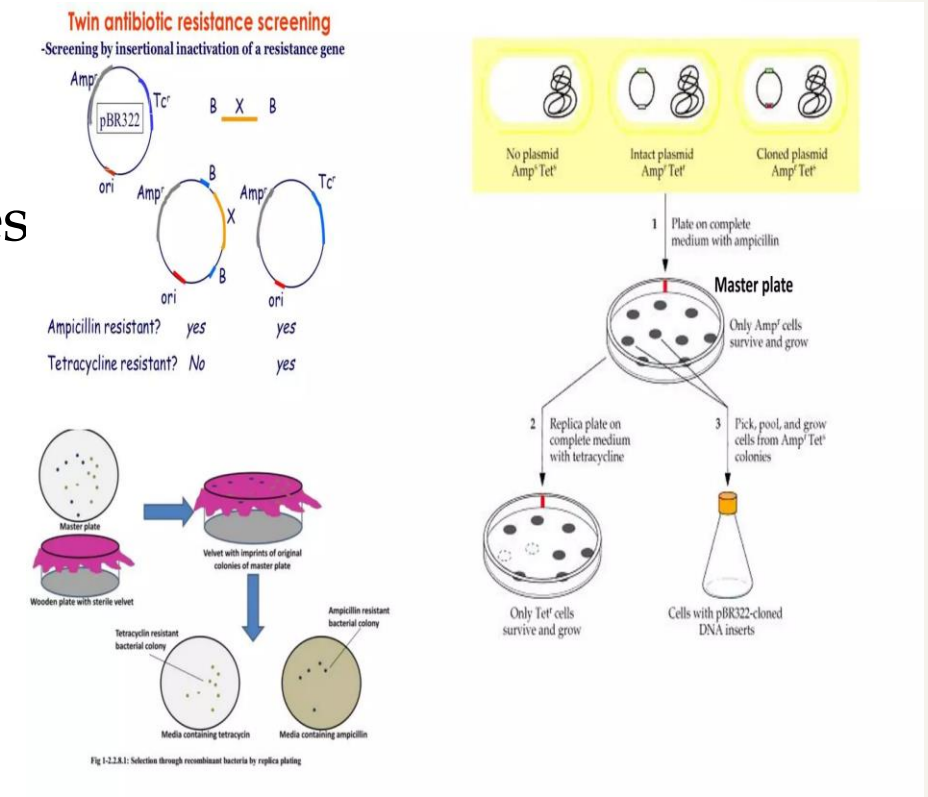
SCREENING OF A GENOMIC LIBRARY

- Screening based on library recombinant evaluates colonies combined with a particular gene.
- Library screening can be based on investing in various DNA sequencing of the "cloned gene".
- However, the genomic library is developed through separating the various cells and using DNA-based technology.
- Furthermore, the library related to genomics has been prepared in "cosmid vector"

- provides the partial size of suggested DNA fragments that are adjusted to approximately more than 42 kb.
- The various uses of genomic libraries are effective techniques in the biological field.

- However, this source and tools are critical to analysing the overall functions for the detection of various genes that are related to several sources

- Genomic libraries remain a significant tool for assembling the huge amount of appropriate information on genome sequencing.



THANK YOU

- REFERENCE :
- "Molecular Cloning: A Laboratory Manual" by Michael R. Green and Joseph Sambrook

"Screening Recombinant Clones“

by J. Sambrook and D. W. Russell (Cold Spring Harbor Protocols, 2006)