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TOPIC: RECOMBINANT DNA TECHNOLOGY TOOLS &TECHNIQUES – PART I

COURSE : M.Sc. ZOOLOGY

ELECTIVE PAPER : CELL & MOLECULAR BIOLOGY

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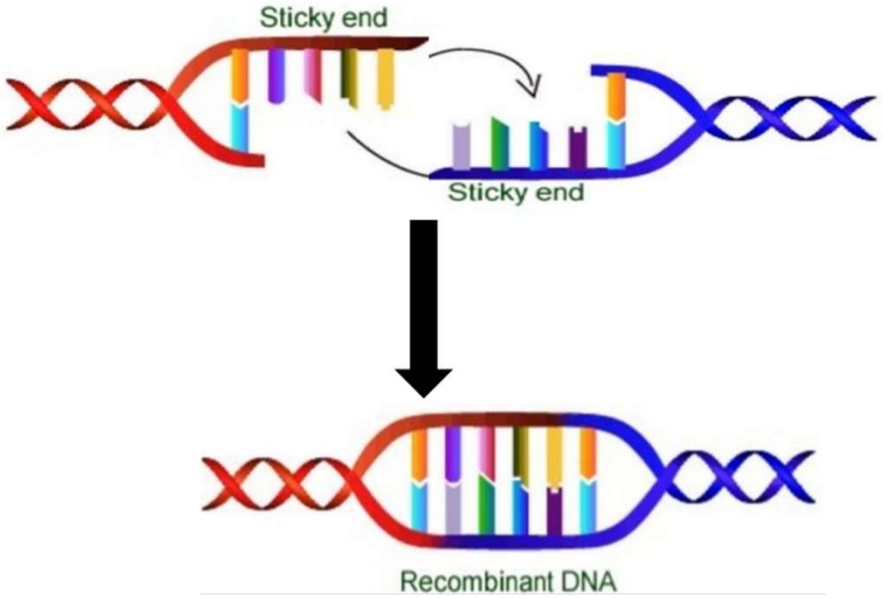
RECOMBINANT DNA TECNOLOGY

Recombinant DNA Technology (RDT) is the genetic engineering technique of joining together of distinct DNA molecules to produce new genetic combinations as "Recombinant DNA" (rDNA).



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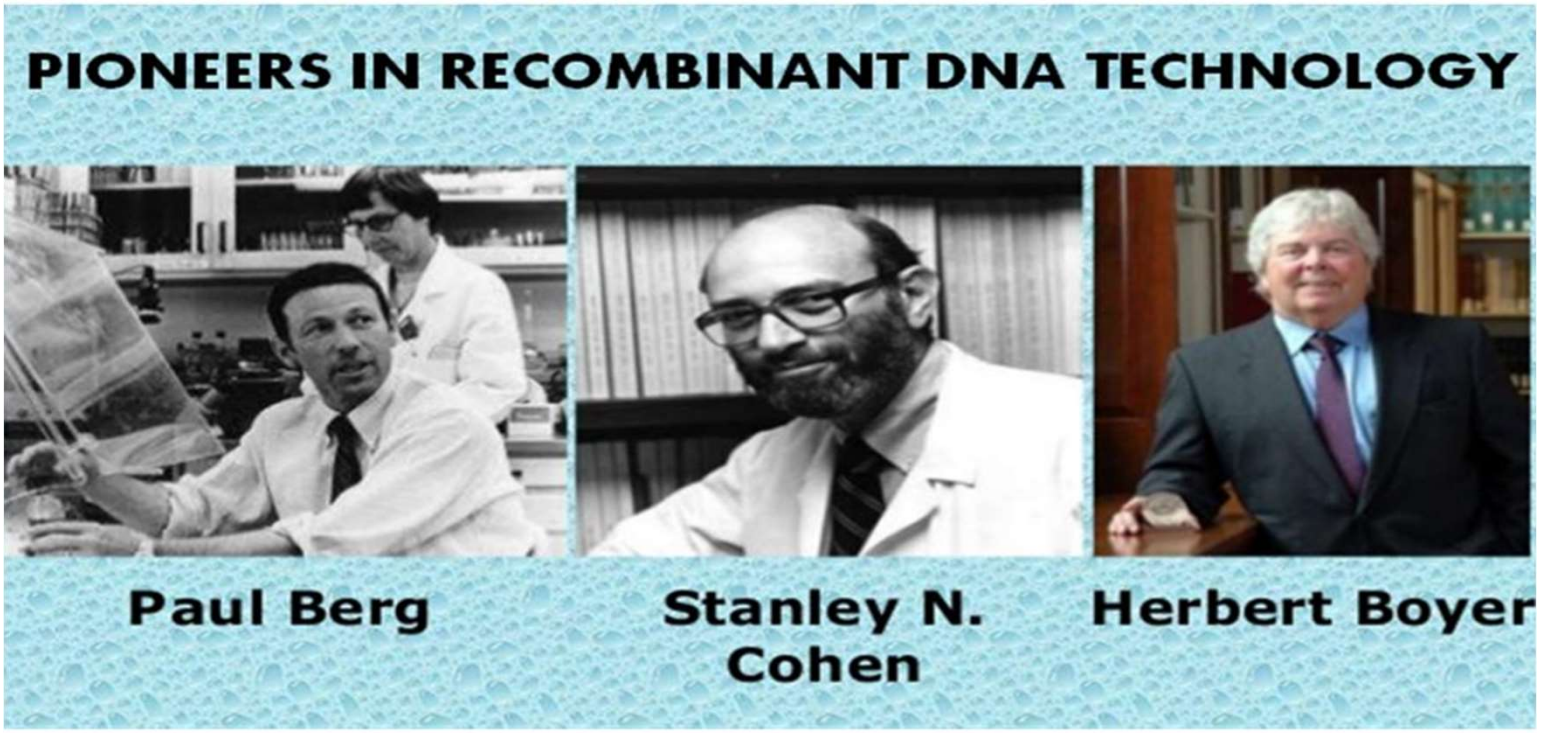

First discovered RDT in 1977 when they first successfully expressed somatostatin in bacteria.

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STEPS INVOLVED IN RDT

Step 1. Obtaining a copy of desired gene or DNA segment of interest by either of by following ways:

- ✓Chopping up of DNA with Restriction Enzyme.
- ✓Using Reverse Transcriptase (for preparation of cDNA).
- ✓Synthesizing the gene artificially.

Step 2. Inserting the gene in a suitable Vector.

Step 3. Introducing the vector with gene in a host cell.

Step 4. Selection of the Transformed Host Cell.

Step 5. Cloning of Gene.

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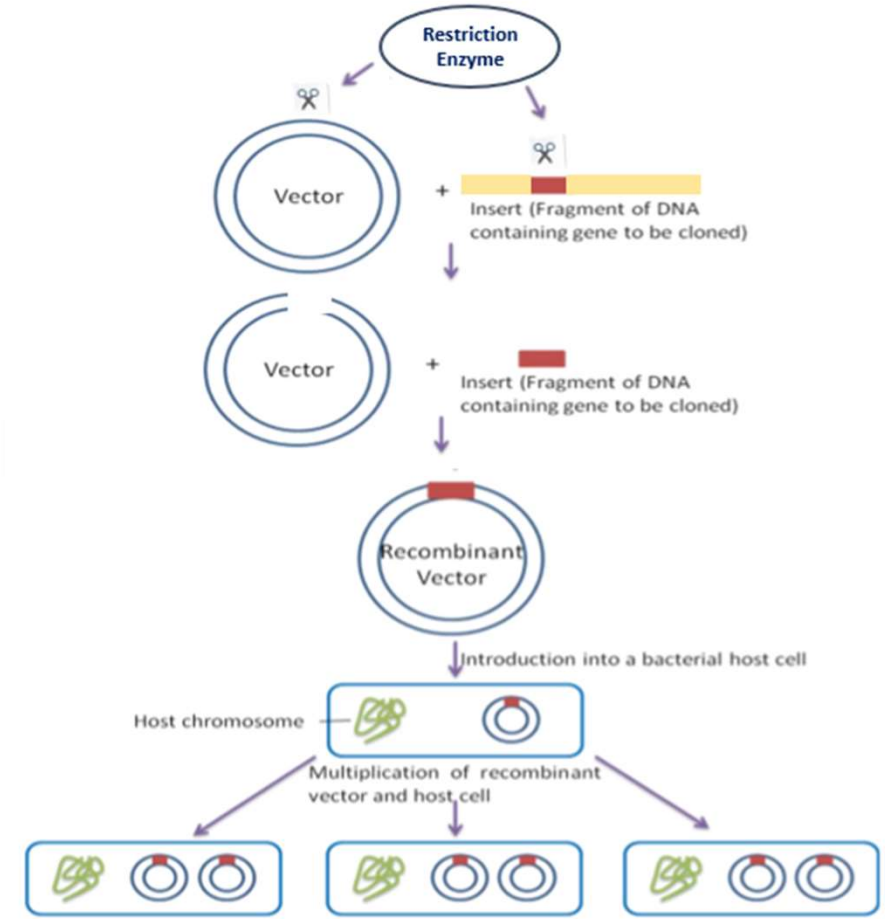
SCHEMATIC REPRESENTATION OF BASIC STEPS OF RDT



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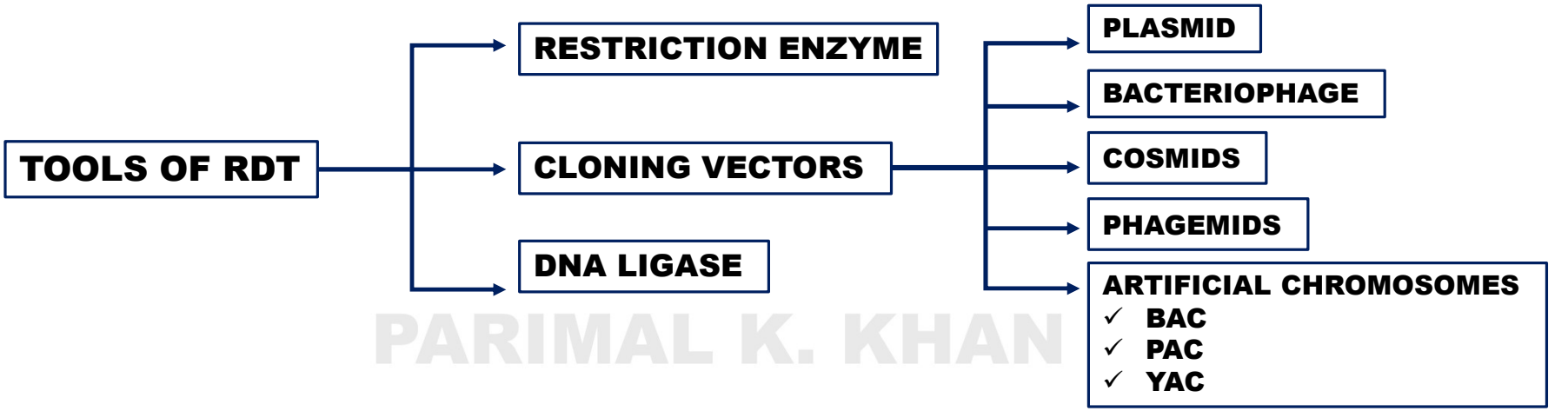
TOOLS AND TECHNIQUES OF RECOMBINANT DNA TECHNOLOGY (RDT)



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RESTRICTION ENZYME

HISTORICAL ASPECTS

- **Werner Arber (1960) : Hypothesized that certain enzymes protects the bacteria from viral infection by cutting up the viral DNA into small fragments and restricting the action of enzyme on its own DNA by modification (methylation), so known as the "Restriction Enzyme".**
- **Hamilton O. Smith (1970) : Isolated restriction enzyme *Hind* II from *Haemophilus influenzae.***
- **Daniel Nathan (1973) : Describe the working principle of restriction enzyme.**


Werner Arber


Hamilton O. Smith


Daniel Nathan

Received Nobel Prize in 1978 for the discovery of Restriction Enzyme

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RESTRICTION ENZYME IN RDT

- Restriction Enzyme (also known as Restriction Endonuclease/Molecular Scissors) act as a tool to cut the genomic DNA into smaller and manageable pieces.
- It recognizes a specific nucleotide pair sequence in DNA within or near the sequence.

TYPES OF RESTRICTION ENZYME

Type I Restriction Enzyme

- ✓ Not used in RDT
- ✓ Cleaves Bipartite and Asymmetric sequence of DNA other than recognition site randomly
- ✓ The same enzyme possess both restriction and modification activity

Type II Restriction Enzyme

- ✓ Only used in RDT
- ✓ Cleaves Palindromic sequence of DNA (4-6 bp) at specific restriction site
- ✓ The system has separate enzyme for restriction and modification activity

Type III Restriction Enzyme

- ✓ Not used in RDT
- ✓ Cleaves Asymmetric sequence of DNA (5-7bp) other than recognition site randomly
- ✓ The same enzyme possess both restriction and modification activity

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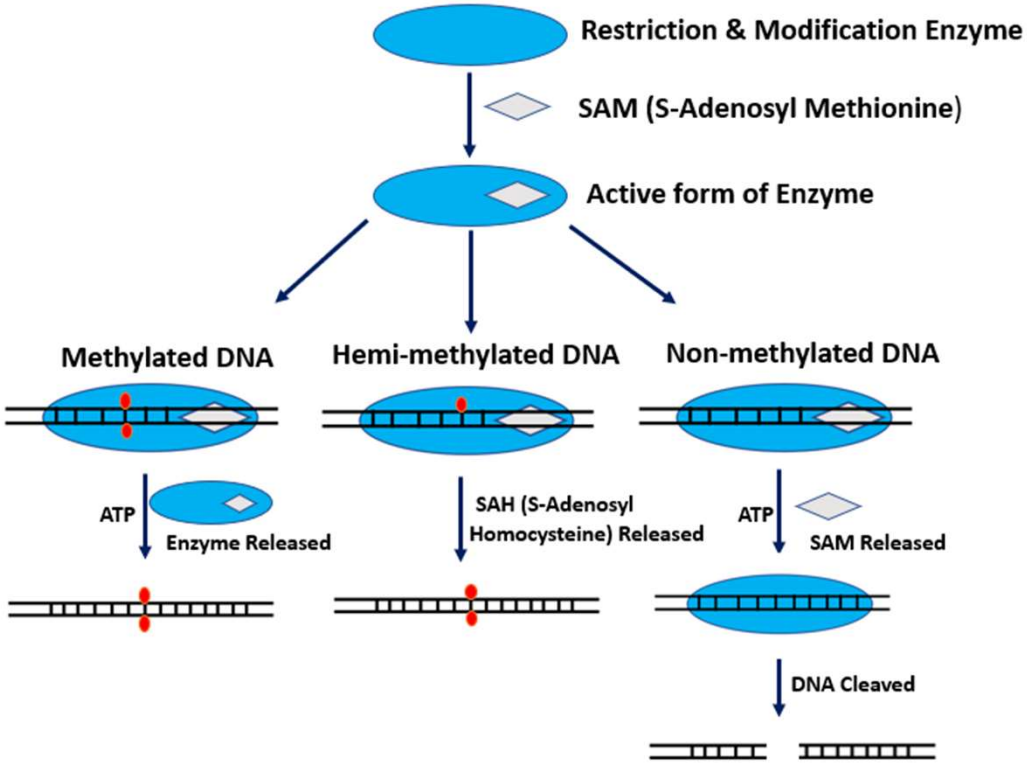
OUTLINE OF WORKING MECHANISM OF RESTRICTION ENZYME



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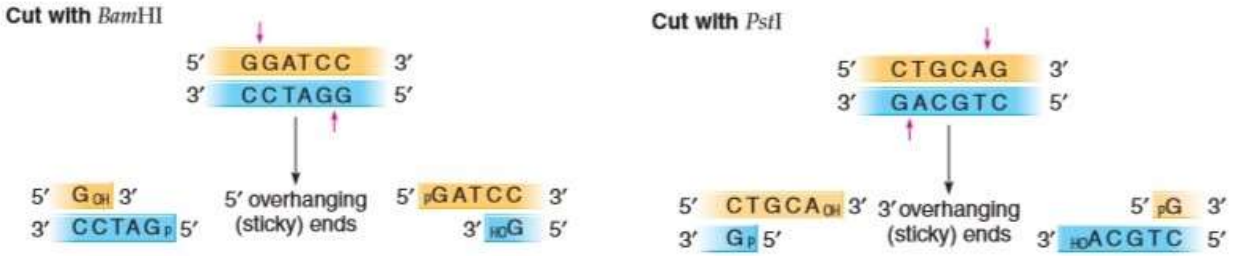
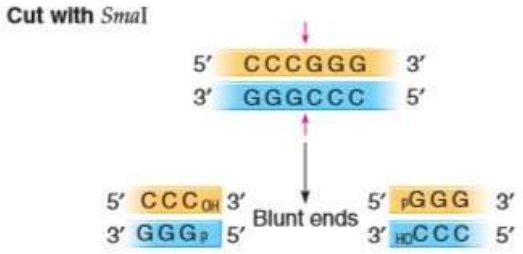
CUTS PRODUCED BY RESTRICTION ENZYME

Staggered Cuts : Produces 5'/3' overhanging sticky ends



Blunt Cuts : Produces blunt ends



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SOME REPRESENTATIVE RESTRICTION ENZYME

- About 3500 restriction enzyme till date isolated and about 200 restriction enzyme now produced commercially.

- It is named after the organism from which they are isolated (1st letter- the genus, 2nd & 3rd letter- the species).The letters italicized or underlined followed by a Roman Numerical that signify a specific restriction enzyme from that organism. Additional letters are also added to signify bacterial strain.

	Enzyme Name	Pronunciation	Organism in Which Enzyme Is Found	Recognition Sequence and Position of Cut[a]
Enzymes with 6-bp Recognition Sequences	*Bam*HI	"bam-H-one"	*Bacillus amyloliquefaciens H*	5′-G↓GATCC-3′ 3′-CCTAG↑G-5′
	*Bgl*II	"bagel-two"	*Bacillus globigi*	5′-A↓GATCT-3′ 3′-TCTAG↑A-5′
	*Eco*RI	"echo-R-one"	*Escherichia coli RY13*	5′-G↓AATTC-3′ 3′-CTTAA↑G-5′
	*Hae*II	"hay-two"	*Haemophilus aegypticus*	5′-RGCGC↓Y-3′ 3′-Y↑CGCGR-5′
	*Hind*III	"hin-D-three"	*Haemophilus influenzae R_d*	5′-A↓AGCTT-3′ 3′-TTCGA↑A-5′
	*Pst*I	"P-S-T-one"	*Providencia stuartii*	5′-CTGCA↓G-3′ 3′-G↑ACGTC-5′
	*Sal*I	"sal-one"	*Streptomyces albus*	5′-G↓TCGAC-3′ 3′-CAGCT↑G-5′
	*Sma*I	"sma-one"	*Serratia marcescens*	5′-CCC↓GGG-3′ 3′-GGG↑CCC-5′
Enzymes with 4-bp Recognition Sequences	*Hae*III	"hay-three"	*Haemophilus aegypticus*	5′-GG↓CC-3′ 3′-CC↑GG-5′
	*Hha*I	"ha-ha-one"	*Haemophilus haemolyticus*	5′-GCG↓C-3′ 3′-C↑GCG-5′
	*Hpa*II	"hepa-two"	*Haemophilus parainfluenzae*	5′-C↓CGG-3′ 3′-GGC↑C-5′
	*Sau*3A	"sow-three-A"	*Staphylococcus aureus 3A*	5′-↓GATC-3′ 3′-CTAG↑-5′
Enzyme with 8-bp Recognition Sequences	*Not*I	"not-one"	*Nocardia otitidis-caviarum*	5′-GC↓GGCCGC-3′ 3′-CGCCGG↑CG-5′
Enzyme with Recognition Sequence Containing a Nonspecific Spacer Sequence	*Bst*XI	"b-s-t-x-one"	*Bacillus stearothermophilus*	5′-CCANNNNN↓NTGG-3′ 3′-GGTN↑NNNNNACC-5′

[a]In this column the two strands of DNA are shown with the sites of cleavage indicated by arrows. Since there is an axis of twofold rotational symmetry in each recognition sequence, the DNA molecules resulting from the cleavage are symmetrical. Key: R = purine; Y = pyrimidine; N = any base.

Characteristics of some Restriction Enzymes

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ISOSCHIZOMERS & NEOSCHIZOMERS

- **Restriction Enzymes that have the same recognition sequence as well as same cleavage sites are "Isoschizomers" .**

  Example: *Sph* I (CGTAC/G) & *Bbu* I (CGTAC/G)

- **Restriction Enzymes that have the same recognition sequence but cleavages DNA at a different site within that sequence are "Neoschizomers" .**

  Example: *Sma* I (CCC/GGG) & *Xma* I (C/CCGGG)

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CLONING VECTORS

- Vectors are those, that transfer donar DNA fragment with gene of interest to host cell (recipient) and are capable of replicating in the host cell.
- Cloning vectors include plasmids, bacteriophages, cosmids, phasmids, Bacterial Artificial Chromosomes (BACs) &Yeast Artificial Chromosomes (YACs).

CHARACTERISTIC FEATURES OF CLONING VECTORS

1. An Ori (Origin of Replication) sequence.
2. A selectable marker.
3. One or more restriction sites
4. Antibiotic resistance genes

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VARIOUS TYPES OF VECTORS AND THEIR INSERT SIZE

CLONING VECTORS	INSERT SIZE (kb)
Plasmid	0.5-8
Bacteriophage	5-25
Cosmids	35-45
BAC	50-300
YAC	200-1000

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PLASMIDS

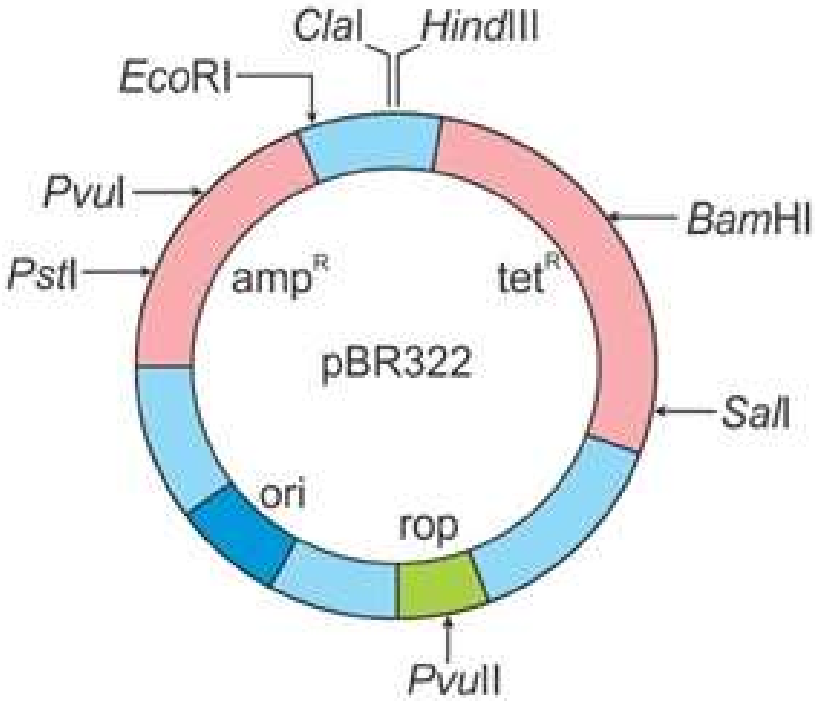
- Plasmids are extrachromosomal elements that replicate autonomously within cells.
- Found naturally in bacteria.
- Act as DNA carrier up to 8kb.

EXAMPLES:

pBR322

- First plasmid vector developed and generated by Bolivar & Rodriguez and on whom the plasmid was named.
- Characteristics features:

1. Small in size, i.e., 4363bp, thus, it can be purified with ease.
2. Carries two sets of antibiotic resistance genes –ampicillin (amp^R) and tetracycline (tet^R) that allows simple identification of transformants.
3. Has several restriction sites (*Eco*RI, *Bam*HI etc.).



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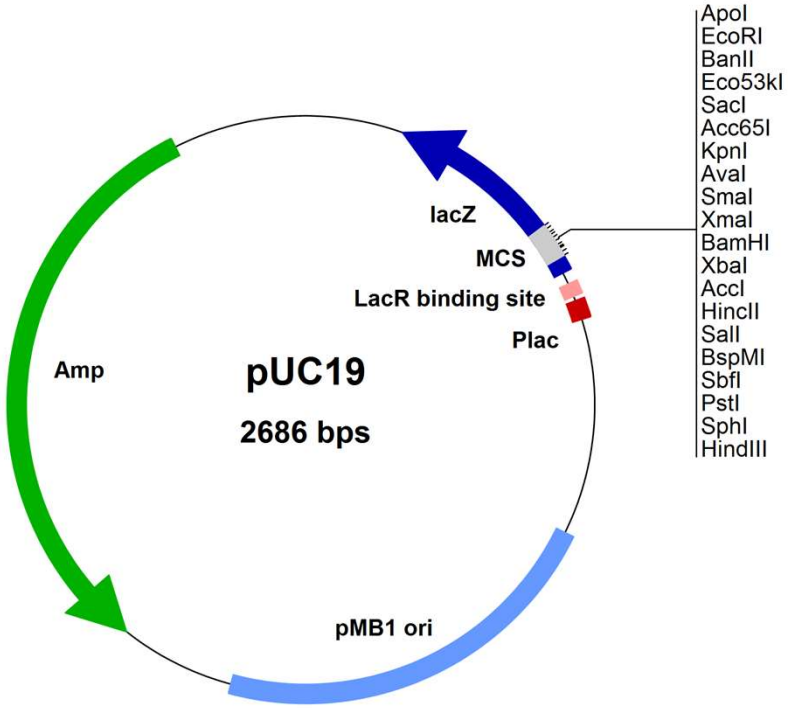
pUC

- An off-shoot of pBR322 with various advantages which includes:

1. Consists of polylinker or multiple cloning site (MCS) which carry sites for several restriction endonucleases extending the range of enzymes that can be used to generate a restriction fragment suitable for cloning.
2. Contains DNA sequence for lac Z' coding for β-galactosidase that permits rapid visual detection of an insert via blue-white selection method that will be discussed later in the chapter.
3. Has high copy number in contrast to pBR322 as the former can be maintained at 500700 copies per cell before amplification.



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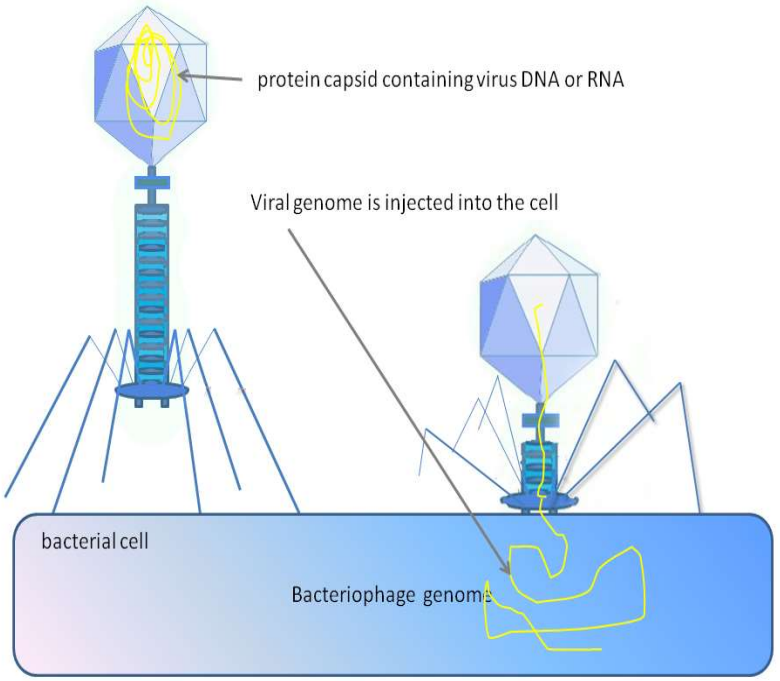
BACTERIOPHAGE

- Bacteriophage or λ phage is a virus that infect bacterial cell and replicate using the machinery of those infected cell.
- They are advantageous over plasmids as:

1. Infect cells with high efficiency than transformation by plasmids; hence, the quantity of clones yielded with phage vectors is usually greater.
2. Carry a larger fragment of size up to 25kb in contrast to limit of 8 kb in plasmids.

- M13 phage has the ability to isolate single stranded form of the cloned gene which is of utmost importance in both sequencing and mutagenesis.



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COSMIDS

- Cosmids are plasmids that incorporate cohesive end site (cos) of bacteriophage λ which contains elements required for packaging DNA into λ particles.
- It is used to clone large DNA fragments between 35 to 45 Kb.

PHAGEMIDS

- Phagemids are plasmids containing properties of plasmid and phage.
- They contain f1 origin of replication (ori) from filamentous 'f1' phage.
- They can be maintained as plasmids as well as can be packaged as single stranded DNA in viral particles.

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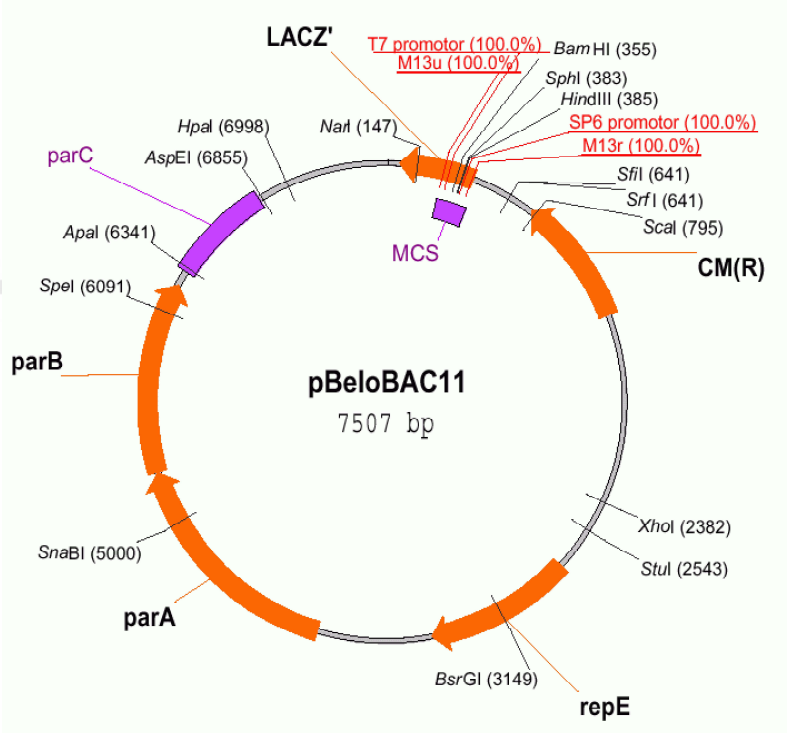
ARTIFICIAL CHROMOSOMES

- Artificial chromosomes are cloning vectors that can accommodate very large piecesof DNA, producing recombinant DNA molecules resembling small chromosomes.
- Example: BACs & YACs

BACTERIAL ARTIFICIAL CHROMOSOMES

- Bacterial artificial chromosomes (BACs, “backs”) are cloning vectors containing the origin of replication from a natural plasmid found in E. coli called the F factor, a multiple cloning site, and one or more selectable markers.
- pBeloBAC11, is a BAC vector similar to a plasmid vector with one or more selectable markers (chloramphenicol resistance), a multiple cloning site in part of the gene, but uses an origin derived from the F factor, which limits the copy number of the BAC to one per E. coli cell.



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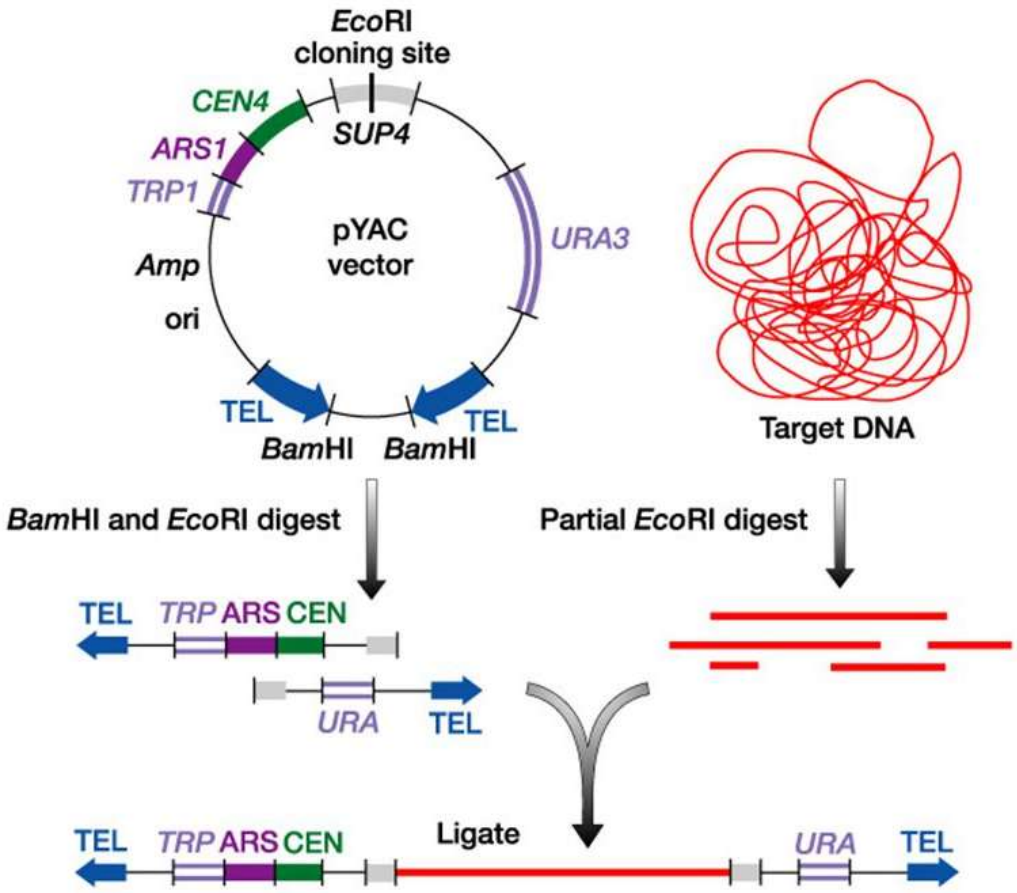
YEAST ARTIFICIAL CHROMOSOMES

- Yeast artificial chromosomes (YACs; "yaks") are cloning vectors that enable artificial chromosomes to be made and replicated in yeast cells.
- YAC vectors have been used to clone very large DNA fragments (between 0.2 and 2.0 Mb), for example, creating physical maps of large genomes such as the human genome.
- As linear form of vector has following features :

1. Autonomously Replicating Sequence (ARS) for plasmid replication.
2. Centromere (CEN) for uniform distribution of chromosome to daughter cells during cell division
3. Telomeres (TEL) for protection of ends of chromosomes.
4. TRP1 and URA3 for selection of recombinants



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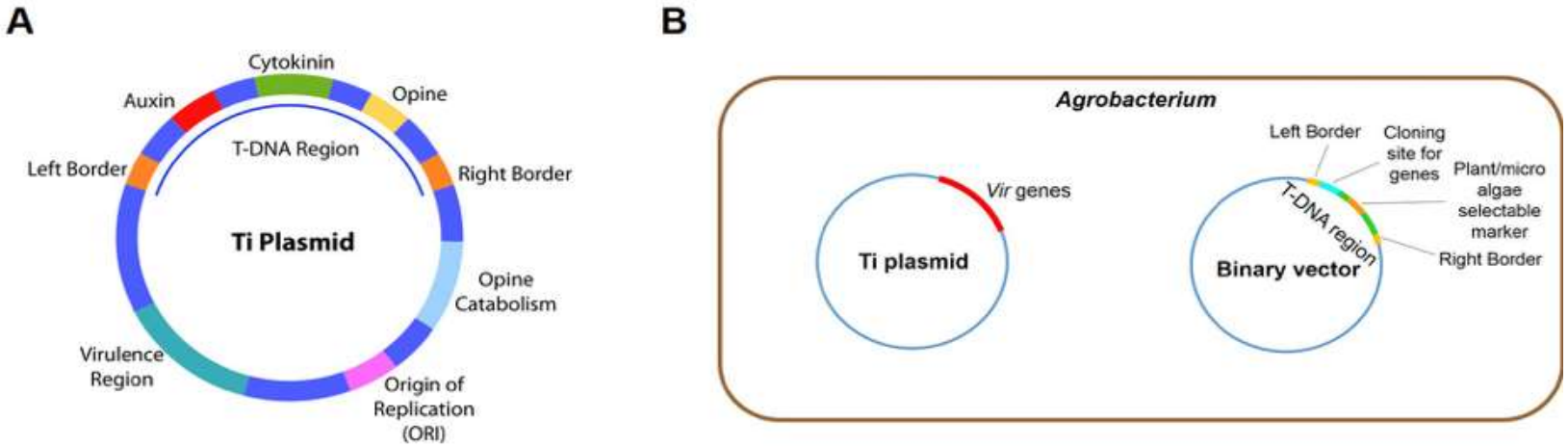
Ti-PLASMIDS

- Tumor inducing (Ti) plasmid is found in soil bacteria *Agrobacterium tumefaciens a*s the natural genetic engineer of plants.
- It causes crown gall disease in dicot plants due to its natural ability to transfer a portion of its plasmid called T-DNA which then, integrates into the plant chromosome. This inherent property of bacteria can be exploited in cloning wherein TDNA can be replaced with gene to be cloned and then can be delivered efficiently to the plants.



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DNA LIGASES

- The enzyme DNA Ligase joins the cloning vector and insert to form recombinant vector know as recombinant DNA (rDNA).
- Commonly used DNA Ligases in cloning includes- *E.coli* DNA Ligase and T4 DNA Ligase
- Sticky ends can base pair easily with the complementary overhangs, however, the ligation efficiency of blunt ends is very low.

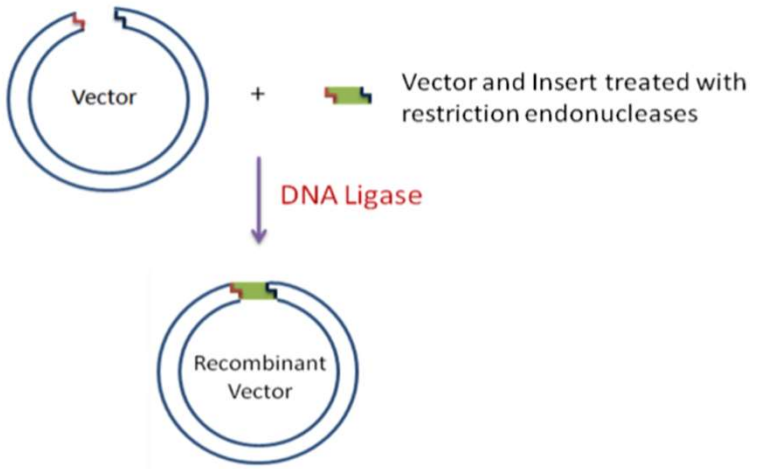

Fig. OVERVIEW OF DNA LIGASE & RECOMBINANT VECTOR

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APPROACHES FOR JOINING BLUT ENDS

I. **T4 DNA ligase** catalyzes the formation of phosphodiester bonds between blunt-ended fragments.
II. **Use of linkers or adaptors** Ligation of adaptors or linkers to the blunt ended insert creates cohesive end terminal that assist in joining.
III. **Use of 'terminal deoxynucleotidyl transferase'** that synthesize 3' homopolymer tails at the ends of fragments to be joined. Homopolymer tailing involves addition of homopolymer tails at the 3' end of insert along with vector with the help of an enzyme known as "Terminal deoxynucleotidyl transferase". These homopolymers facilitate the joining of insert and vector using DNA ligase.

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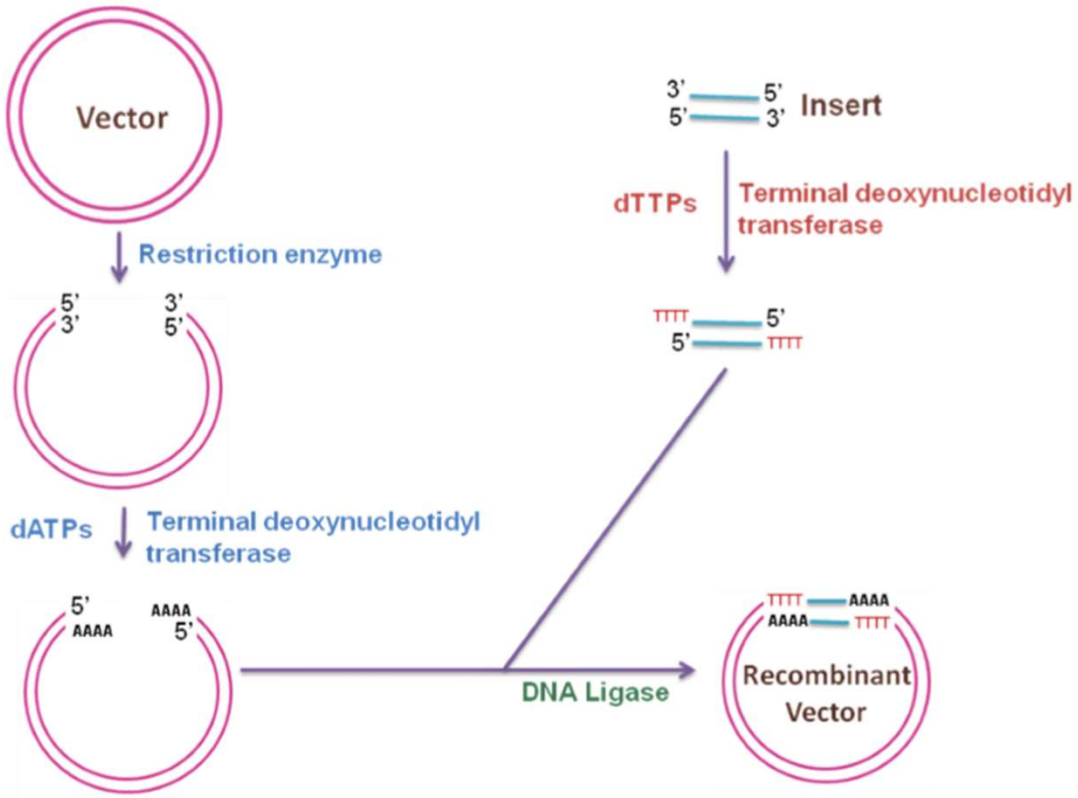

HOMOPOLYMER TAILING FOR JOINING BLUNT END DNA MOLECULES

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CLONING STRATEGIES

TECHNIQUES TO TRANSFER CLONED VECTORS INTO HOST CELLS

- **The process of transferring foreign DNA into the host cell is known as transformation.**
- **The transform ability can be induced artificially by various methods depending upon the host cell discussed below:**

(A) Bacterial cells

Calcium chloride (CaCl2) method : This method was developed by Mandell & Higa. In this, bacterial cells are treated with cold CaCl2 which make the cell competent for transformation. Ca2+ being positively charged bind to the negatively charged membrane and when DNA is added, it bind to the membrane through these calcium ions. After addition, cells are given heat shock treatment at 42°C for 90s which transiently create pores in the cell membrane allowing entry of foreign DNA.

Transfection : It is the transfer of foreign DNA into cultured host cell mediated through charged chemicals (cationic liposomes, calcium phosphate, DEAE dextran) which are taken & mixed with DNA molecules. The recipient host cell is overlayered by this mixture & foreign DNA is taken up by the host cell.
Bacterial

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(B) Animal cells

- **Microinjection :** It is a technique of delivering foreign DNA into a living cell (a cell, egg, oocyte, embryos of animals) through a glass micropipette. (Also used in plant cell)
- **Direct transformation :** In this, foreign DNA fragment is precipitated with calcium phosphate & mix with the cells to be transformed. DNA molecule passes through cell membrane & integrates with the mammalian chromosome.

(C) Plant cells

- **Vector-mediated or indirect gene transfer method:** This is mediated by Ti plasmid of a soil bacterium, Agrobacterium tumefaciens which is known as natural genetic engineer of plants.
- **Electroporation :**This involves application of a pulse of high voltage (~350 V) to protoplasts/ cells/ tissues which induce the formation of transient (temporary) pores in the plasma membrane which facilitates the uptake of foreign DNA.
- **Particle bombardment method (biolistics) :**The foreign DNA is coated onto the surface of minute gold or tungsten particles & bombarded (shot) onto the target tissue/ cells using a particle gun (gene gun/ shot gun/ microprojectile gun).

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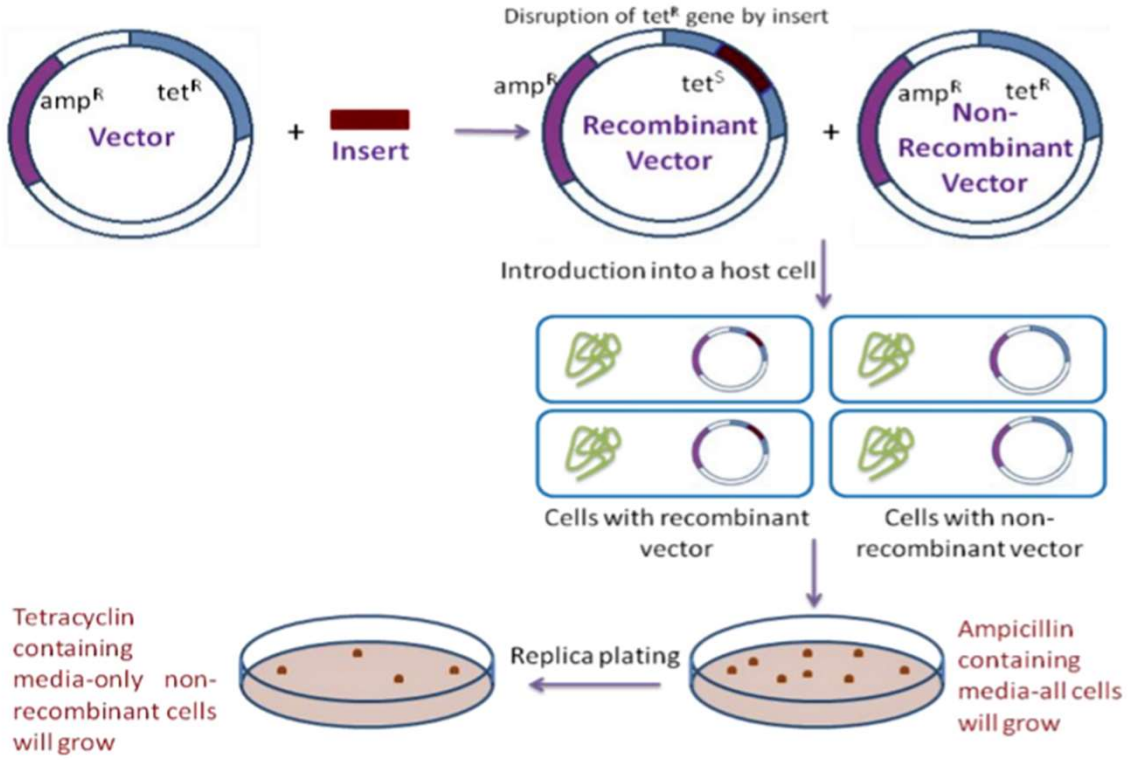
TECHNIQUES FOR SELECTION TRANSFORMED CELLS

I. Insertional Inactivation Method



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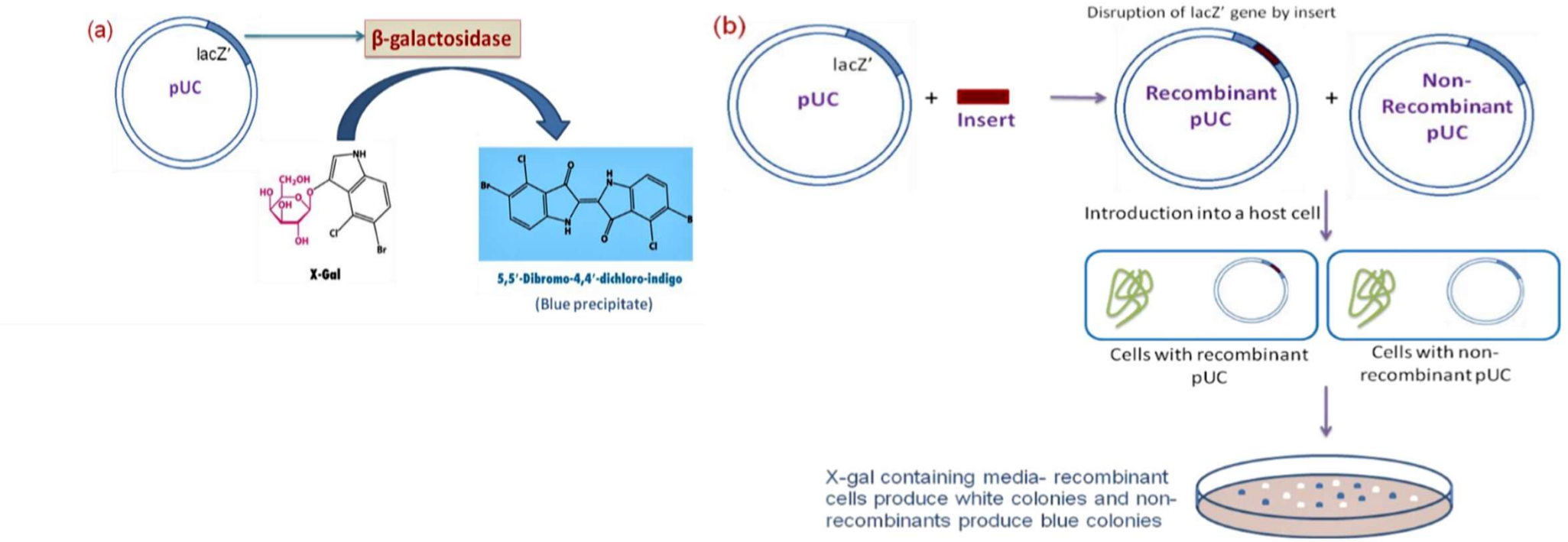
II. Blue-White Selection Method



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