UNIT - 01 Genetic Engineering

Introduction

* Recombinant DNA Technology (also known as genetic engineering, molecular biology technology or cloning) is the set of technique that enables DNA from different sources to be identified, isolated and recombined so that new characteristics can be introduced into an organism.

* The main technique in recombinant DNA Technology is DNA cloning.

* The recombinant DNA technique was engineered by Stanley Norman Cohen and Herbert Boyer in 1973.

what is DNA cloning ?

* DNA cloning is the production of large number of identical DNA molecules from a single ancestral DNA molecule.

* The essential characteristics of DNA cloning is that the desired DNA fragments must be selectively amplified. resulting in a large increase in copy number of selected DNA sequences.

* This involves multiple rounds of DNA replication catalysed by a DNA polymerase acting on one or more types of template DNA molecule.

- Essentially two Different DNA cloning approaches are used.

Cell based DNA cloning

- This was the first form of DNA cloning to be Developed, and is an in vivo cloning method.
- The first step in this approach involves attaching foreign DNA fragments in vitro to DNA sequence which are capable of independent replication.
- The recombinant DNA fragments are then transferred into suitable host cell where they can be propagated selectively.

Cell free DNA cloning

The polymerase chain reaction (PCR) is a newer form of DNA cloning which is enzyme mediated and is conducted entirely in vitro.

The essence of cell based DNA cloning involves following steps.

Cell-based DNA cloning requires attachment of DNA fragments to purified replicons in vitro condition and propagation of suitable host cells.

Construction for recombinant DNA molecule

Recombinants are hybrid DNA molecules consisting of autonomously replicating DNA segment plus inserted elements. such hybrid molecules are also called chimera.

Construction of recombinant DNA molecules by in vitro covalent attachment (ligation) of the desired DNA fragments (Target DNA) to a replicon (any sequence capable of independent DNA replication).

This step is facilitated by cutting the target DNA and replicon molecules with specific restriction endonucleases before joining the different fragments using the enzyme DNA ligase.

Transformation

The recombinant DNA molecules are transferred into host cells (often bacterial or yeast cells) in which the chosen replicon can undergo DNA replication independently of the host cell chromosome.

selective propagation of cell clones

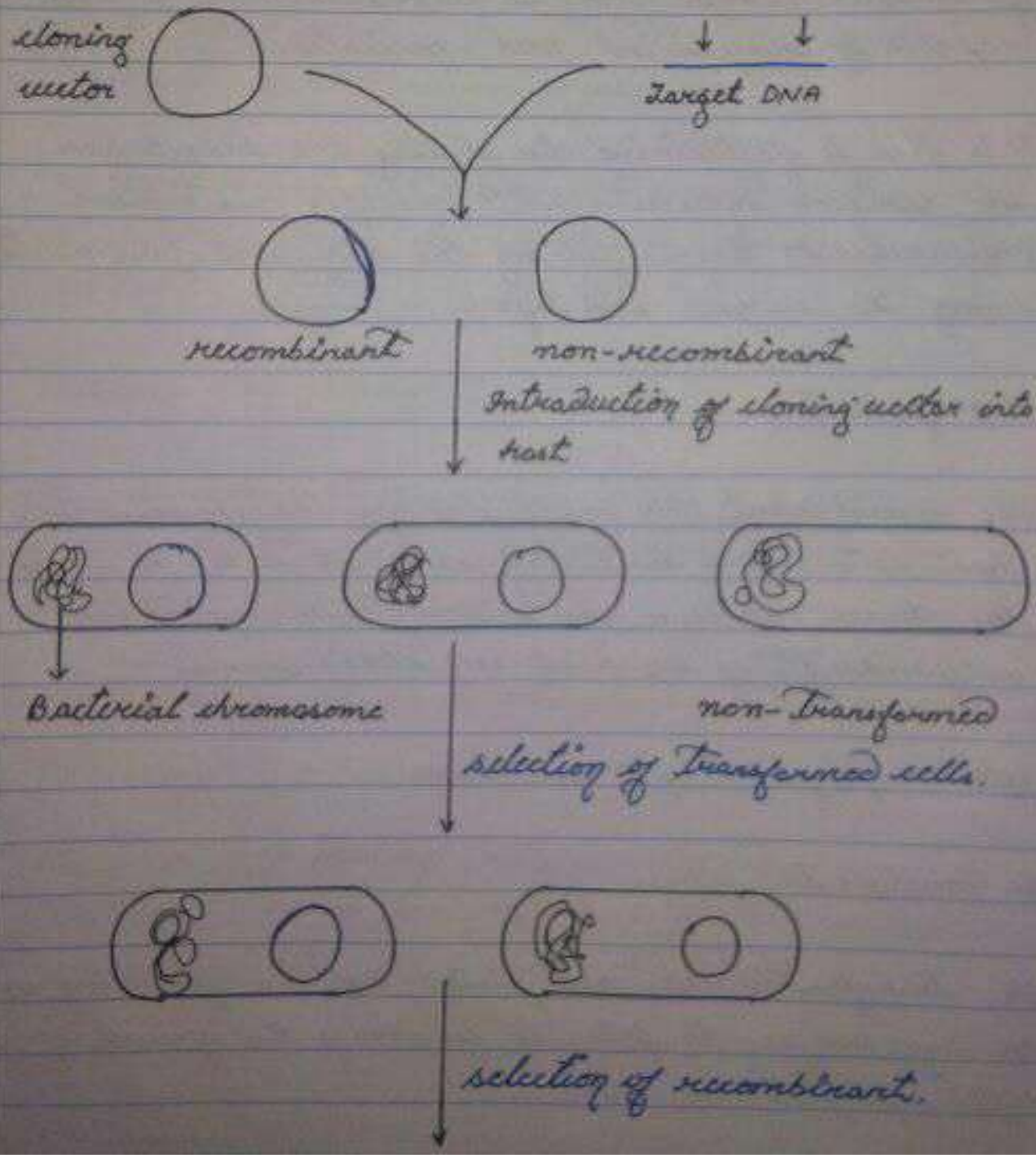
It involves two stages.

The transformed cells are plated out by spreading on an agar surface in order to encourage the growth of well-separated cell colonies.

These are cell clones. subsequently, individual colonies can be picked from a plate and the cells can be further expanded in liquid culture.

Isolation of recombinant DNA clones

Isolation of recombinant DNA clones by haucsling expanded cell cultures and selectively isolating the recombinant DNA.



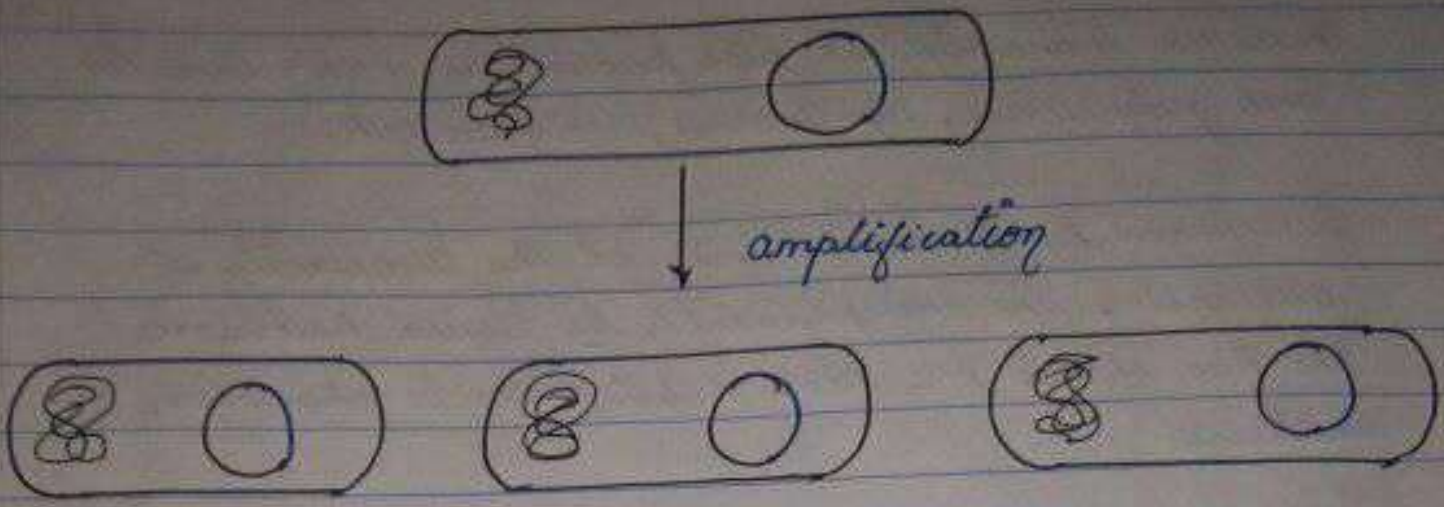


figure: DNA cloning bacteria using a plasmid vector.

Tools for recombinant DNA Technology.

Enzymes for manipulation

The enzymes used in the molecular biology fall into four broad categories.

Template - dependent DNA polymerase.

DNA polymerase enzymes that synthesize new polynucleotides complementary to an existing DNA or RNA Template.

Different type of DNA polymerase used in gene manipulation.

DNA polymerase I (Kornberg polymerase) has both the 3'-5' and 5'-3' exonuclease activity and 5'-3' polymerase activity

Reverse Transcriptase also known as RNA-Directed DNA polymerase, synthesizes DNA from RNA.

Discovered by Howard Temin at the University of Wisconsin, and independently by David Baltimore. The two shared the 1975 Nobel price in physiology of medicine.

Taq DNA polymerase DNA polymerase derived from a thermostable bacterium (Thermus aquaticus).

Operates at 72°C and is reasonably stable above 90°C and used in PCR.

Nucleases

Enzymes which degrade DNA molecules by breaking the phosphodiester bonds that link one nucleotide to the next.
There are two different kinds of nuclease.

- exonucleases remove nucleotides one at a time from the end of a DNA molecule.
- endonucleases are able to break internal phosphodiester bonds within a molecule.

restriction enzymes : molecular scissors with a Twist.

restriction enzyme also called restriction endonucleases, discovered by Werner Arber, Daniel Nathans and Hamilton smith, for which they received the 1978 nobel prize in medicine.

they are the enzymes that cut DNA molecule in specific places.

restriction enzymes vary considerably.

hundreds of different kinds of restriction enzymes are known (recognizing different DNA sequence)

required sequence length varies (most common are "4-base cutters and 6-base cutters.")

Placement of cut varies, some leave sticky ends others blunt ends.

most recognized sequence are palindromic.

* the sequence on one strand matches that of the complementary strand read in the opposite direction.

* Thus, 5'-AGCGCT-3' would have a complementary strand 3'-TCGCGA-5' on reading from 5'-to-3' 5'-AGCGCT-3'.

01 Type I enzymes

- cleaves at sites remote from a recognition sites.
- requires both ATP and S-adenosyl-L-methionine to function.
- multifunctional protein with both restriction and methylase activity.

02 Type II enzymes

- cleaves within or at short specific distances from a recognition sites.
- most require magnesium.
- single function (restriction) enzymes independant of methylase.

03 Type III enzymes

- cleaves at sites a short distance from recognition sites.
- requires ATP (but does not hydrolyse it)
- S-adenosyl-L-methionine stimulates the reaction but is not required.
- It exists as part of a complex with a modification methylase.

Type IV enzymes.

target modified DNA, eg. methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA.

Nomenclature of restriction enzymes.

Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial genus, species and strain.
example - The name of the ECORI, restriction enzyme was derived as

E → Escherichia Genus
Co → specific species coli
R → RY13 strain
I → first identified order of identification in the bacterium.

some examples of restriction enzymes

01 5' AAGCTT 3' Hind III 5' A 3' 5' AGCTT 3'
3' TTCGAA 5' digest 3' TTCGA 3' A 5'

5 protruding ends.

02. 5' GATATC 3' EcoRv 5' GAT 3' 5' ATC 3'
3' CTATAG 5' digest 3' CTA 5' 3' TAG 5'

blunt ends.

Enzyme	Organism	recognition sequence	Blunt or sticky ends
EcoRI	Escherichia coli	GAATTC	sticky
BamHI	Bacillus amyloliquefaciens	GGATCC	sticky
BglII	Bacillus globigi	AGATCT	sticky
PvuI	Proteus vulgaris	CGATCG	sticky
HindIII	Haemophilus Influenza Rd	AAGCTT	Blunt
HinfI	" Rx	GANTC	sticky
Sau3A	staphylococcus aureus	GATC	sticky
AluI	arthrobacter luteus	AGCT	Blunt
TaqI	Thermus aquaticus	TCGA	sticky
HaeIII	Haemophilus aegyptius	GGCC	Blunt
NotI	Nocardia otitidis-caviarum	GCGGCCGC	sticky

vector

vector is a DNA molecule that has ability to replicate automatically in an appropriate host cells and into which the DNA fragment to be clone (called DNA insert) is integrated for cloning.

Properties of vector

- It should be able to replicate autonomously.
- A vector should be ideally less than 10 Kbp in size.
- A vector should be easily isolate and purify.
- It should be easily introduced into host cell
- A vector should have unique target site.

Types Cloning vector

vector used for propagation of DNA insert in a suitable host are called cloning vector.

Types of cloning vector

01. plasmids (about 20 Kb)
02. bacteriophage (bacterial viruses), 30-50 Kb inserts
03 cosmids (30-50 kb) inserts
04 BACs 75-300 kb inserts possible
use fertility F plasmids
developed during the human genome project.

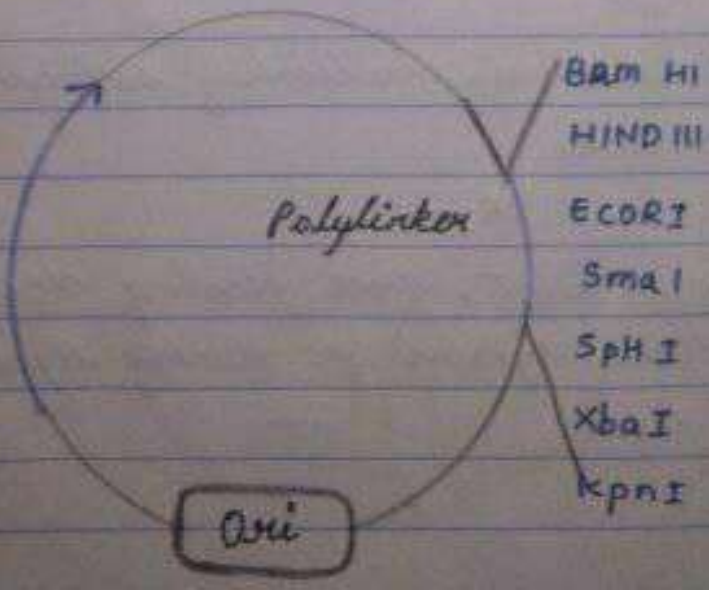
05 YACs mimics yeast chromosome
contains all region for replication (yeast ori and centromere)
100 - 1000 kb inserts
Developed during the human genome projects

Common features of a vector.

Origin of replication is a DNA segment recognized by the cellular DNA-replication enzymes. Without replication origin, DNA cannot be replicated in the cell.

selective markers is required for maintainance of plasmid in the cell. Because of the presence of the selective marker the plasmid becomes useful for the cell. Under the selective conditions, only cells that contain plasmids with selectable markers can survive.

multiple cloning site / Polylinker many vectors contain a MCS (DNA segment with several unique sites for restriction nucleases located next to each other).



The polylinker advantage

- The inclusion of polylinker into plasmid vectors
- Polylinker is a tandem of array of restriction endonuclease sites in a very short exparse of DNA.

example- pUC18's polylinker

- sites for 13 RE's
- Region spans the equivalent of 20 amino acids or 60 nucleotides

advantages
- Unique sites (usually)
- Insert excision facilitated
- R·E mapping and subcloning made easier

What determines choice of vectors ?

01 insert size
02 vector size
03 restriction sites
04 cloning efficiency

vector	Insert size (Kb)
Plasmid	<10 kb
Bacteriophage	9-15 kb
cosmids	33-50 kb
YACs	100-1000 kb

Plasmid are autonomously replicating extrachromosomal circular DNA molecules.

OR

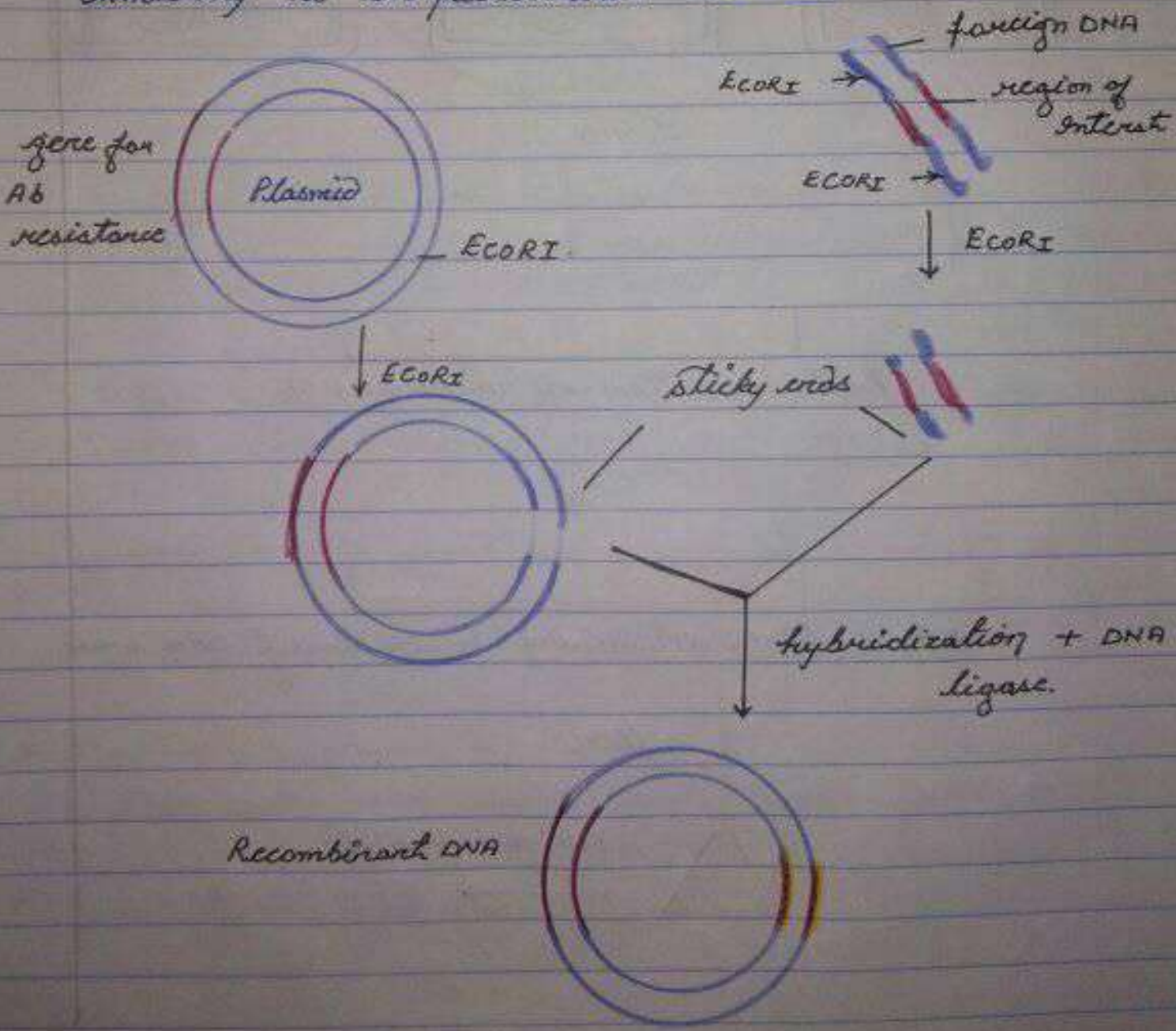
Plasmids are independent, free-floating circular pieces of DNA in a bacteria capable of making copies of itself in a host cell.

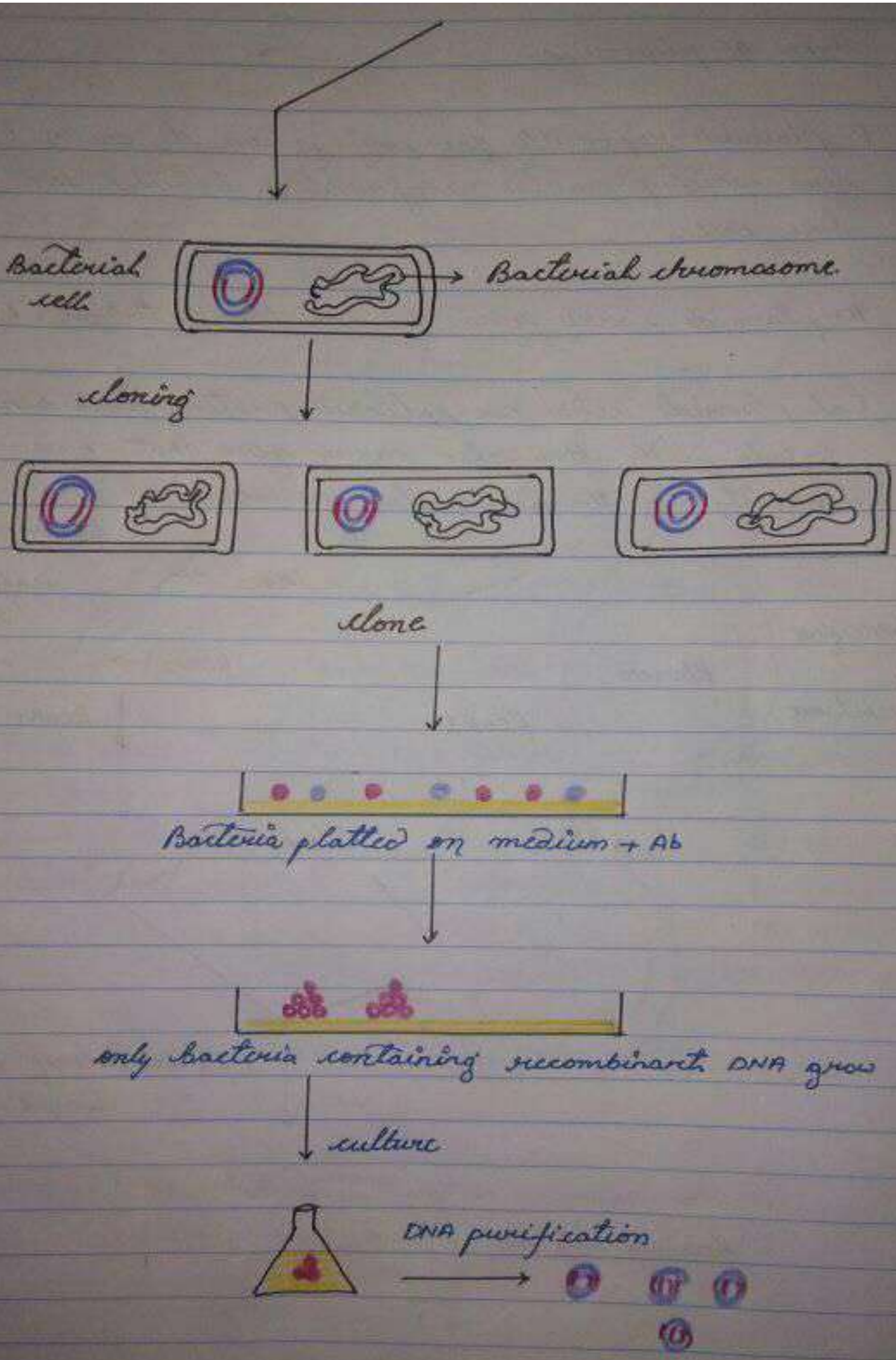
Types of plasmids

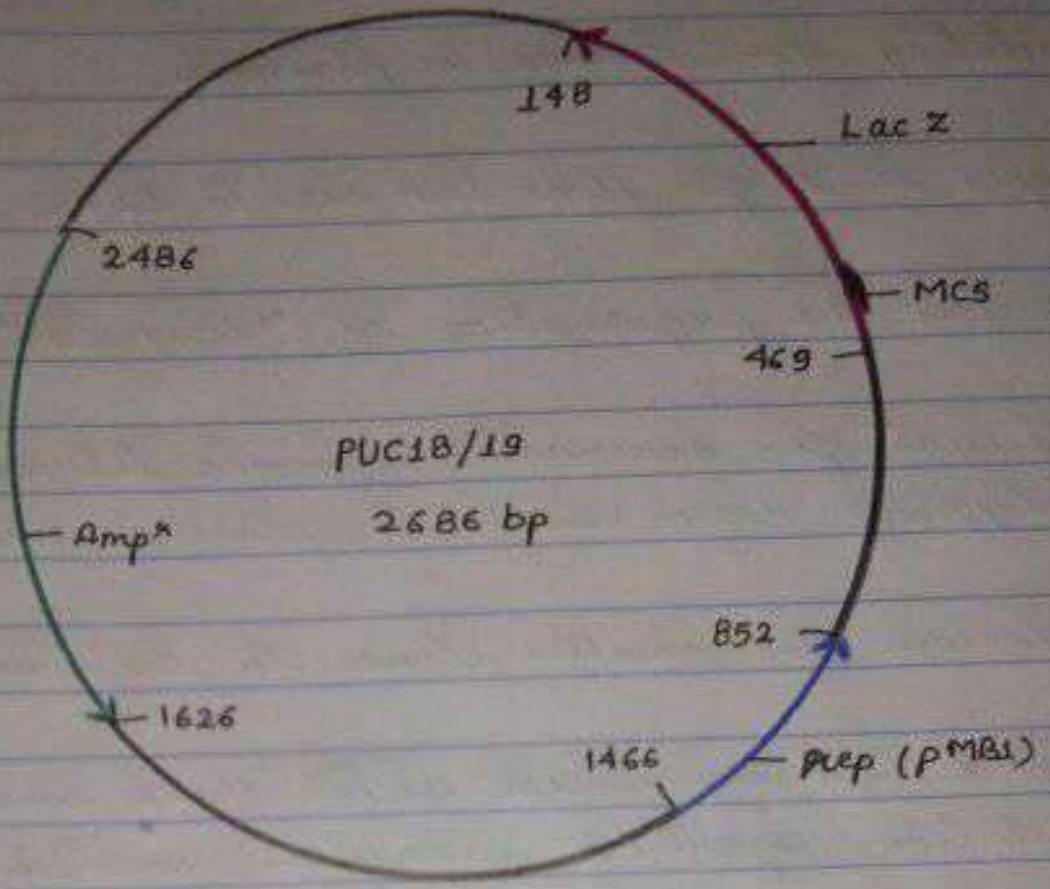
01 F- plasmids responsible for conjugation it carry tra gene which promotes conjugation between the two cell

02. R- plasmids carry gene for resistance to antibiotic

03. Col plasmids codes for colicans protein kills sensitive E-coli cells they also carry gene that provides immunity to the particular colicans




Bacterial cell
Bacterial chromosome
cloning
clone
Bacteria platted on medium + Ab
only bacteria containing recombinant DNA grow
culture
DNA purification



small size high copy number. (no. of plasmids maintained per cell) marker gene for easy selection in bacteria or other hosts.

usual markers for selecting bacteria carrying plasmids with inserts (blue white screening)

Promoters for in vitro transcription

Features of modern plasmids

- some are expression vectors and have sequences that allow RNA polymerase to transcribe genes.
- DNA sequencing primers.

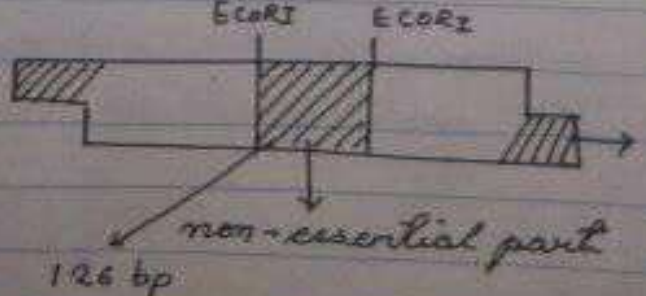
limitation for cloning in plasmids.

- Upper limit for clone DNA size is 12 Kb.
- requires the preparation of "competent" host cell.
- Ineffecient for generating genomic libraries as overlapping region needed to place in proper sequence.
- preference for smaller clones to be Transformed.
- If it is an expression vector there are often limitation regarding eukaryotic protein expression.

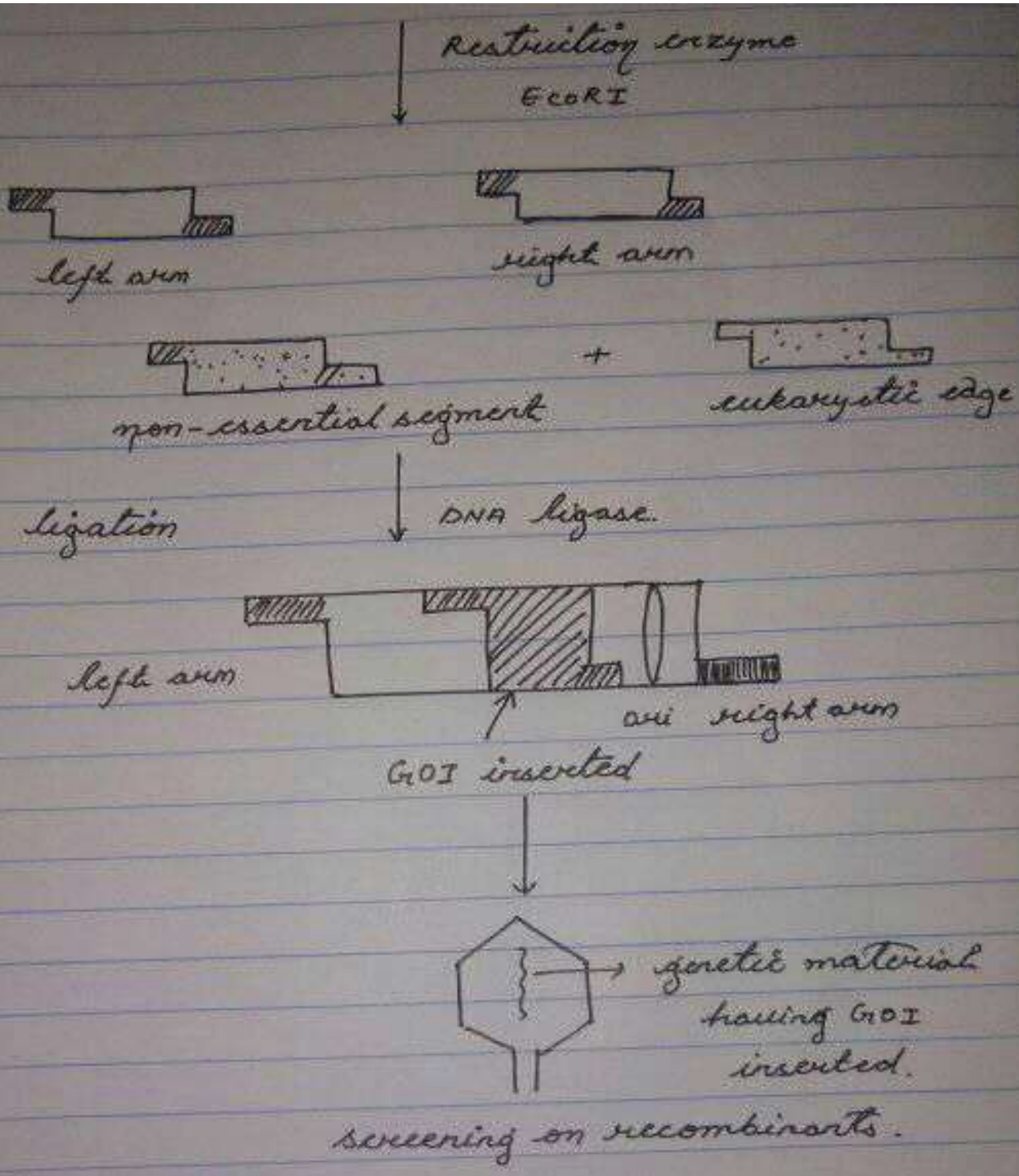
Bacteriophage.

Bacteriophages are viruses that attack bacteria. Most bacteria lies the bacteria cell they infect, But many other can choose to follow either a lytic or lysogenic cycle.

In the later situation the phage chromosomes integrates into the bacterial chromosomes and multiply with it as prophase.



(cohesive x sticky ends)



cos sequence. are 200 bp long and essential for packaging. they contain Cos N sites where DNA is nicked at each strand at 12 bp apart with the help of Terminase. this cause linearization of circular cosmid with two cohesive and sticky ends of 12 bp long.