

# RECOMBINANT DNA TECHNOLOGY



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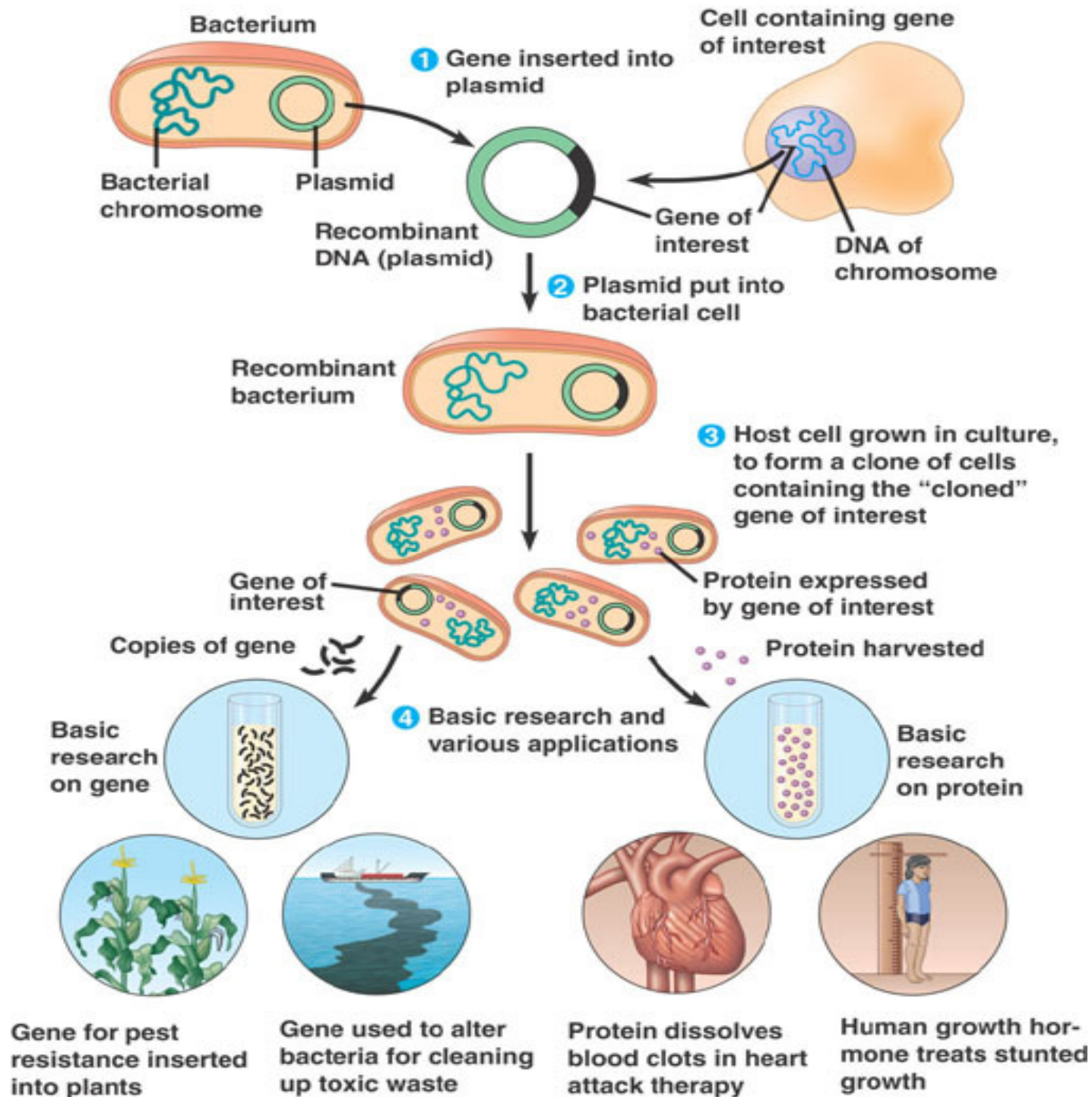
# Recombinant DNA Technology

- Recombinant DNA technology includes the procedures for creating recombinant DNA (rDNA).

## What is Recombinant DNA?

- Production of a unique DNA molecule by joining together two or more DNA fragments not normally associated with each other
- DNA fragments are usually derived from different biological sources

- **rDNA is a recombinant molecule where the vector is joined with a natural or a synthetic DNA segment of interest to make a molecule that can replicate in a living cell.**
- **To produce rDNA one must be able to cut the vector at precise sites so that the DNA of interest can be inserted.**



# Recombinant DNA Technology and DNA Cloning

- 1970s: Gene cloning became a reality
  - **Clone** – a molecule, cell, or organism that was produced from another single entity
- Made possible by the discovery of:
  - **Restriction Enzymes** – DNA cutting enzymes (molecular scissors)
  - **Plasmid DNA Vectors** – circular form of self-replicating DNA
    - Can be manipulated to carry and clone other pieces of DNA

# Tools of Recombinant DNA Technology

## Requirements for recombinant DNA technology

- ❖ **Enzymes**
- ❖ **Vector System**
- ❖ **Gene of Interest (DNA to be cloned)**
- ❖ **Cloning system (host)**

# Enzymes used in Recombinant DNA Technology

1. Nucleases
2. Ligases
3. Polymerases
4. DNA modifying enzymes

# Nucleases

Nucleases are enzymes that degrade DNA molecules by breaking the phosphodiester bonds that link one nucleotide to the next in a DNA strand.

Nucleases can be broadly categorized into

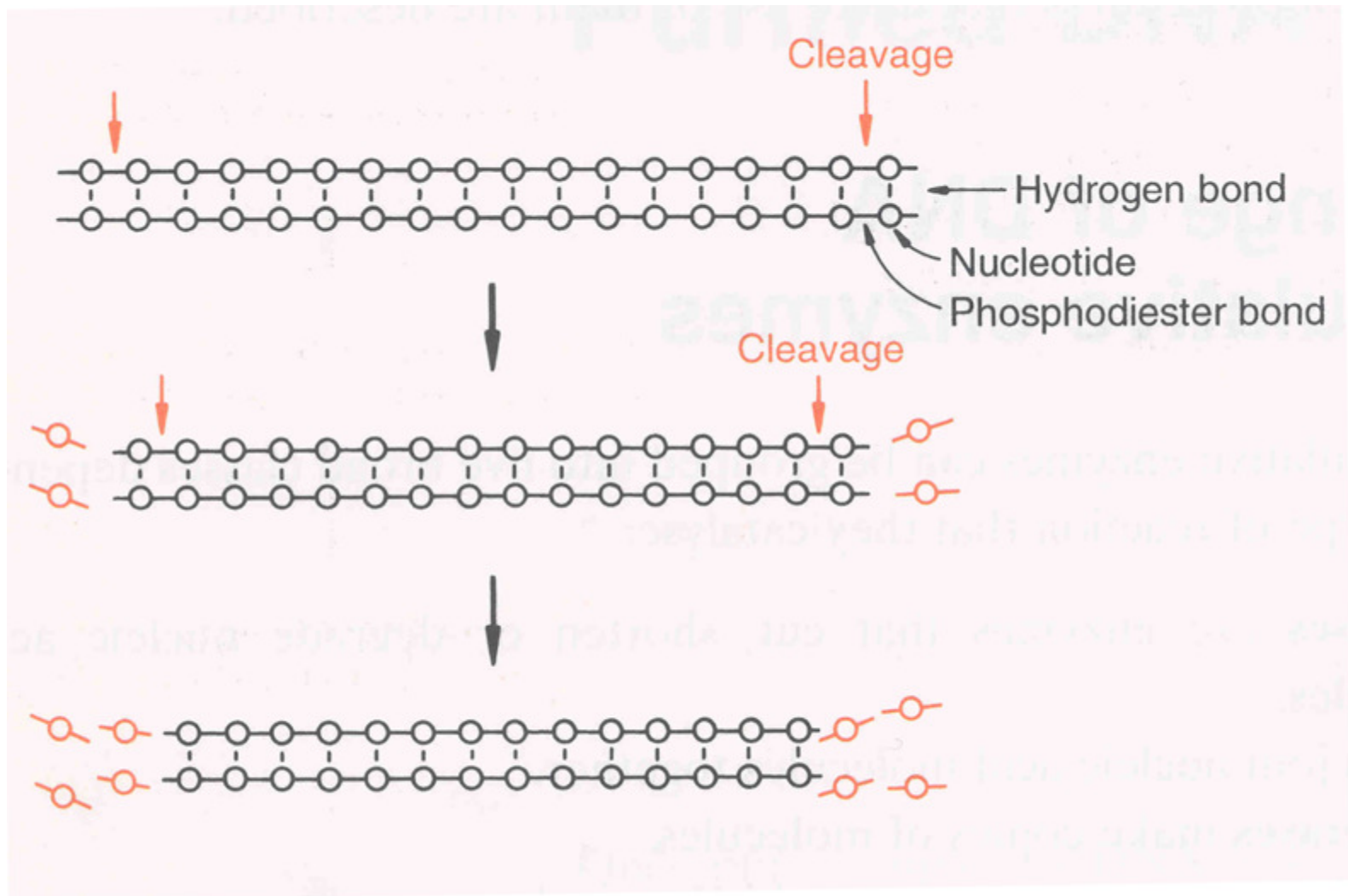
- (i) Exonucleases
- (ii) Endonucleases

Exonuclease removes the terminal nucleotide of the DNA molecule by breaking the phosphodiester bond

Endonuclease breaks the internal phosphodiester bond.

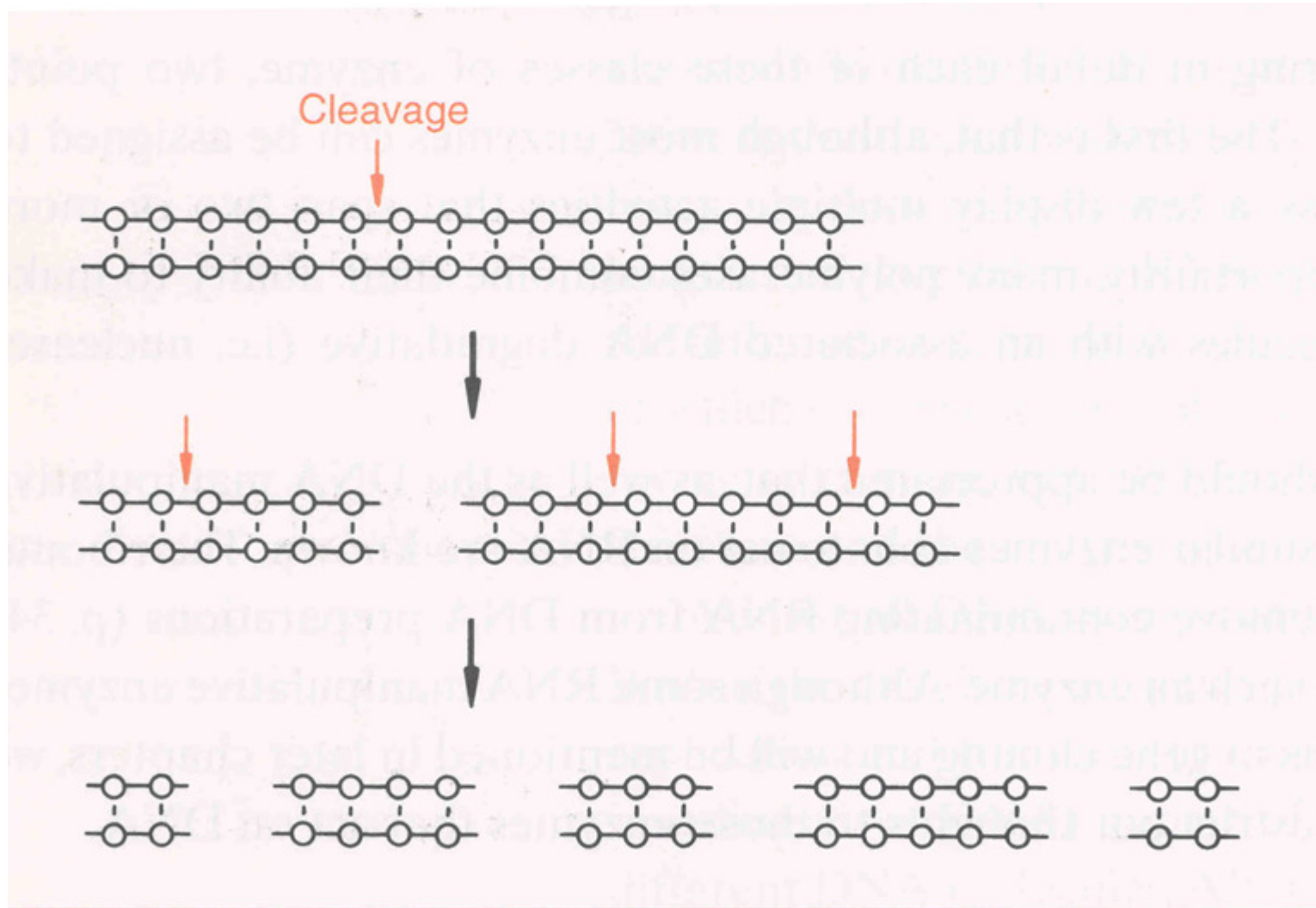
# EXONUCLEASES

(removes nucleotides one at a time from the end)



# ENDONUCLEASES

(breaks phosphodiester bonds within a DNA molecule)



## **Deoxyribonucleases**

**Deoxyribonucleases degrading ssDNA and ds DNA**

**Ex. DNAase I**

## **Ribonucleases**

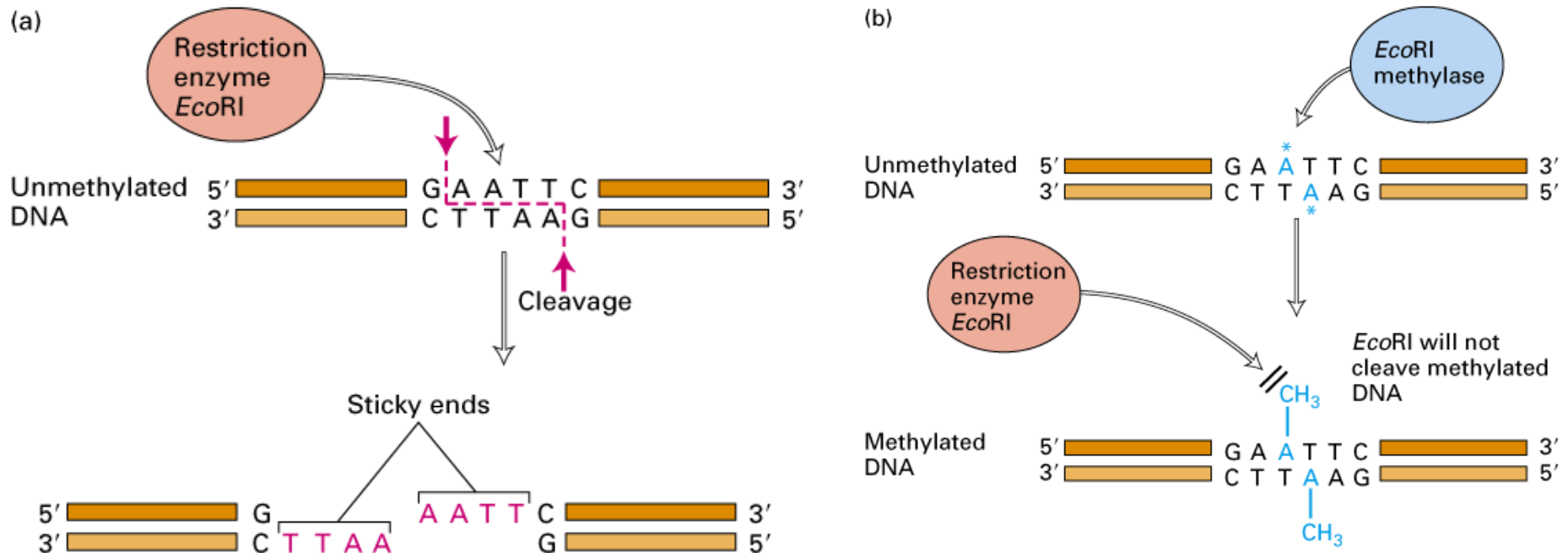
**Enzyme which degrade RNA**

**Ex. RNAase A, RNAase H**

## **Restriction Endonucleases**

- ❖ **The restriction enzymes is also called as “Molecular Scissors”**
- ❖ **These acts as foundation of recombination DNA technology – discovered by Werner Arber (1965)**
- ❖ **Primarily found in bacteria (they use these for defense)**
- ❖ **Endonuclease which recognize and cut DNA within specific sequences of bases called a restriction site**

# Restriction enzymes & DNA methylation



## Why don't restriction enzymes digest bacteria DNA?

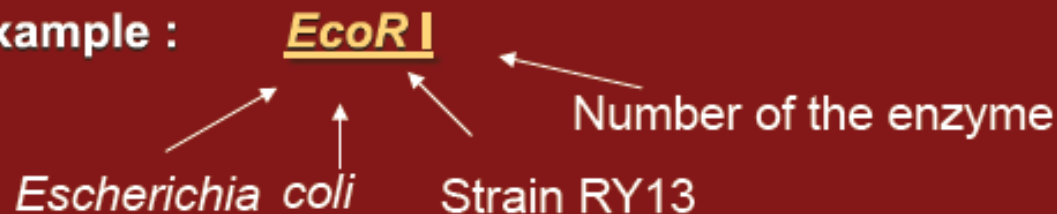
- Methylases protect host from cleavage by corresponding restriction endonuclease.
- Site-specific DNA methylases at N6 position of adenine and C5 position of cytosine

# Nomenclature of Restriction endonucleases

- ❖ Naming of enzyme is as follows:

- ❖ first letter: genus name, italicized and capitalized
- ❖ second two letters: species name, italicized and not capitalized
- ❖ Other variant designations follow: designates particular strain
- ❖ Roman numeral identify multiple enzymes from the same bacterium

- ❖ Example :



# Different Classes of Restriction Endonucleases

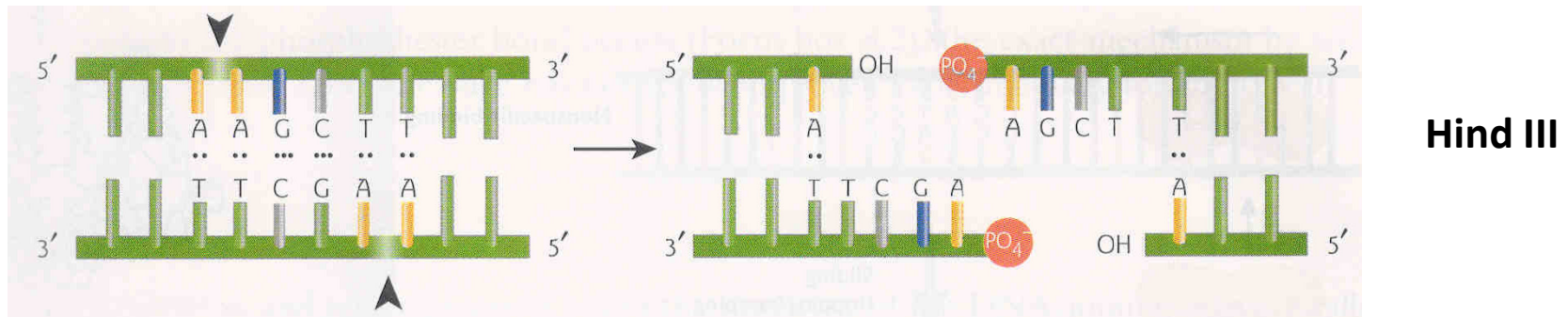
Type	Subunit structure	Cofactors & activators	Recognition site	Cleavage site
I	One enzyme with different subunits for recognition, cleavage & methylation (RMS)	Mg <sup>+2</sup> AdoMet, ATP	Interrupted Bipartite	Distant (>1000 bp) and variable from recognition site <i>EcoKI</i> : <b>AAC(N6)GTGC(N&gt;400) ↓</b> <b>TTG(N6)CACG(N&gt;400) ↑</b>
II	Two different enzymes, one for recognition (Homodimer) & other for methylation (monomer)	Mg <sup>+2</sup>	Palindromic	Defined, within recognition site, may result in a 3' overhang, 5'overhang, or blunt end <i>EcoRI</i> : <b>G ↓ A A T T C</b> <b>C T T A A ↑ G</b>
III	One enzyme with 2 different subunits, one for recognition and modification and other for cleavage (RM dimer competes)	Mg <sup>+2</sup> ,ATP	Non Palindromic	Cuts approx 25 bases downstream to recognition site <i>EcoP15I</i> : <b>CAGCAG(N) 25–26 ↓</b> <b>GTCGTC(N) 25–26 ↑</b>

## Characteristic Properties of Type II Class of REs

- ❖ Most commonly found and highly useful in recombinant DNA research
- ❖ Each restriction site of Type II endonucleases is a palindrome  
(reads same forward and backward on opposite strands of DNA)  
For exp.      5'-GAATTC-3'  
                    3'-CTTAAG-5'      (Palindromic sequence)
- ❖ Highly specific cleavage of DNA strands
- ❖ Recognition sites are usually
  - 4 bases long- Bfal, Alul, HpaII
  - 6 bases long- EcoRI, BamHI, PvuII
  - 8 bases long- NotI, PmeI, SbfI
- ❖ Frequency of restriction sequences in a DNA molecules  
 $4^n$  where  $n$  = no of bases in particular restriction site,  
for an example  $n=4$ ,  $n=256$ , if  $n=6$ ,  $n=7776$  nucleotides

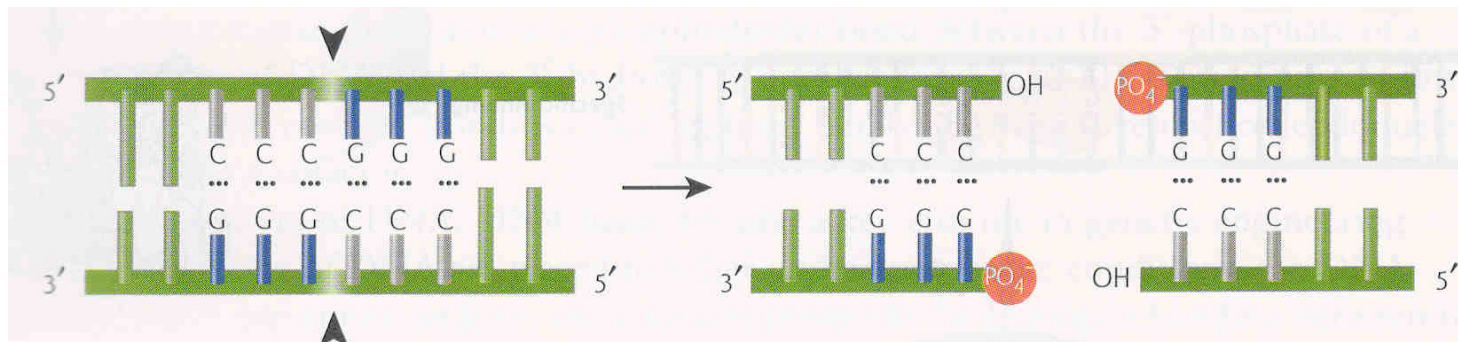
## Characteristic Properties of Type II Class of REs

- Some cut DNA to create DNA fragments with overhanging single stranded ends called "sticky" or "cohesive" ends"
- Some cut DNA to generate fragments with double-stranded ends called "blunt" ends, they do not produce any overhang



Hind III

"sticky" or "cohesive" ends"



Sma I

**sticky ends** are preferred for cloning because DNA fragments with sticky ends can be easily joined together

## Characteristic Properties of Type II Class of REs

- ❖ Different enzymes cleave the same site at same position – *Isoschizomers*

*HpaII and MspI cleave as: C<sup>^</sup>CGG*

*Sph I and BbuI cleave as: GCATG<sup>^</sup>C*

- ❖ Different enzymes cleave the same site at different position - *Neoisoschizomers*

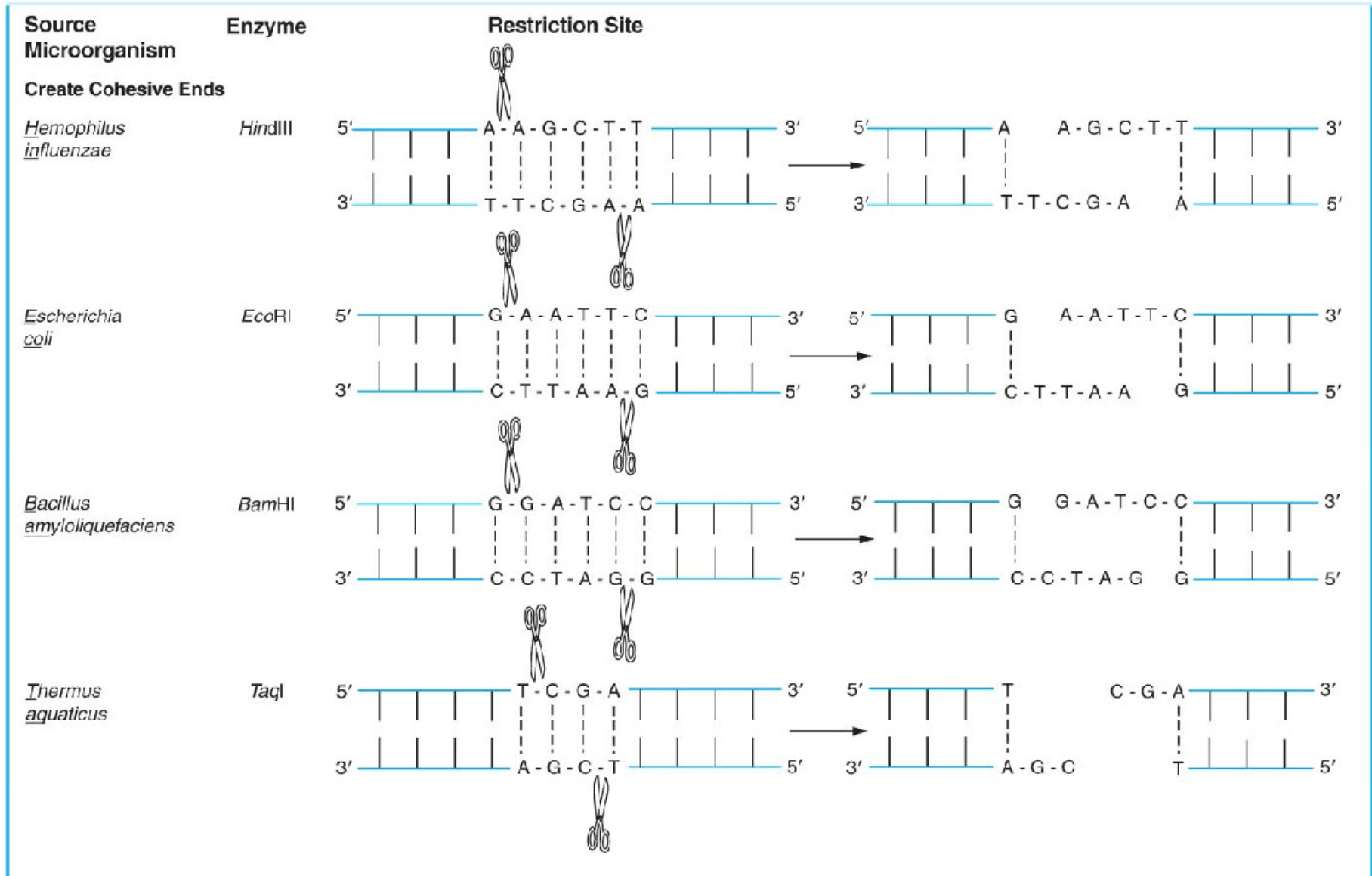
*SmaI: CCC<sup>^</sup>GGG*

*XmaI: C<sup>^</sup>CCGGG*

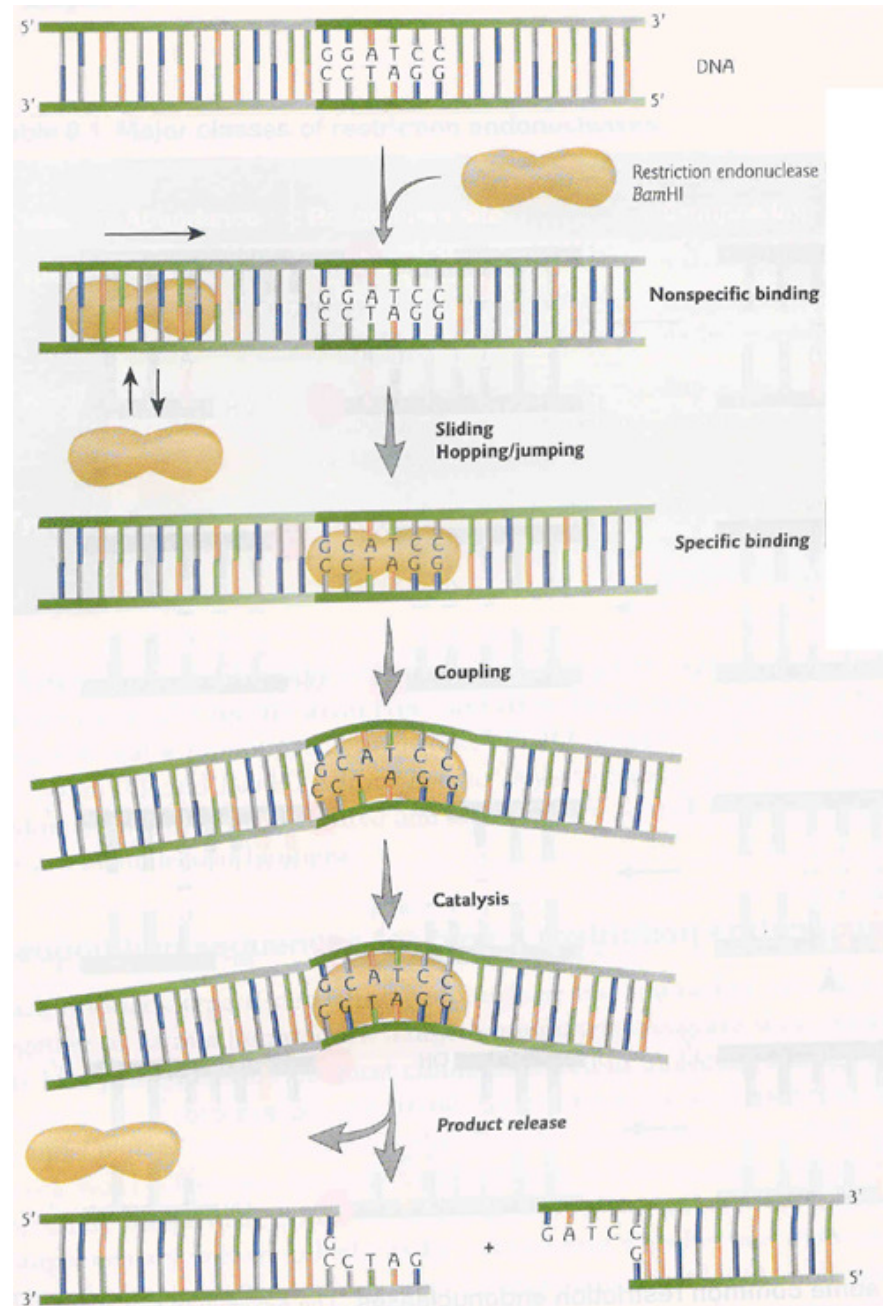
*AatII: GACGT<sup>^</sup>C*

*ZraI: GAC<sup>^</sup>GTC*

# Some most common restriction enzyme



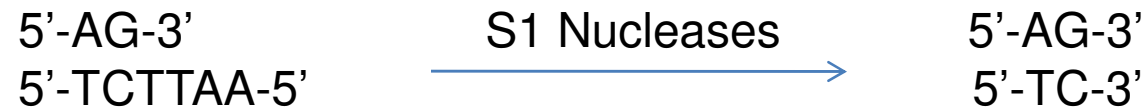
## Mechanism of Type II Class of REs



# S1 Nucleases

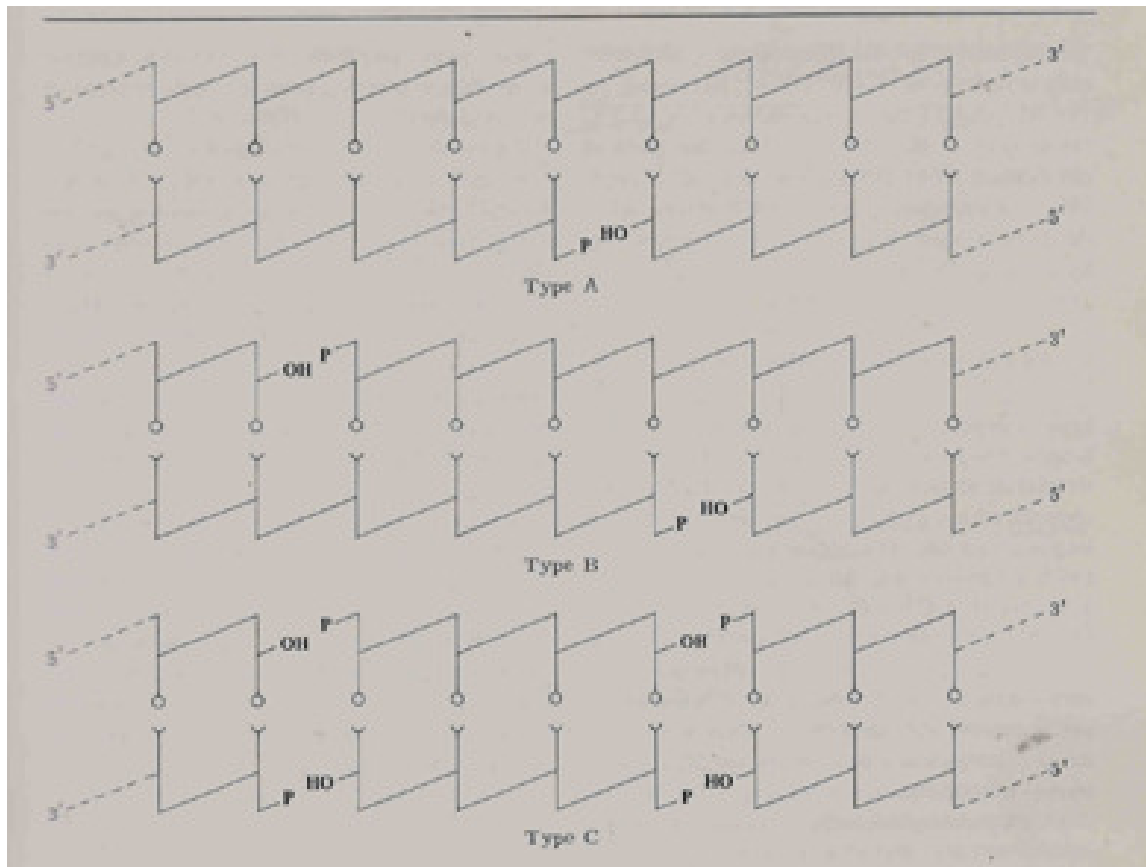
It degraded the single stranded DNA or single strand overhang of double stranded DNA

It converted cohesive end to blunt end



# LIGASES

- ❖ **Ligases are enzymes that join the nucleic acid molecules together.**
- ❖ **These nucleic acids can either be DNA or RNA, and the enzymes are thus called DNA ligase and RNA ligase, respectively.**
- ❖ **DNA ligase catalyses the formation of a phosphodiester bond between the 5' phosphate of one strand and the 3' hydroxyl group of another.**
- ❖ **In nature the function of DNA ligase is to repair single strand breaks (discontinuities) that arise as a result of DNA replication and/or recombination.**
- ❖ **In recombinant DNA technology, ligases catalyse the joining of DNA of interest called as 'insert', with the vector molecule and the reaction is known as ligation.**



**Repair single strand breaks  
(discontinuities)**

**Phosphodiester bond  
between duplex DNA  
containing cohesive ends**

**Phosphodiester bond  
between duplex DNA  
containing blunt ends**

## ***E. coli DNA Ligases***

- **Biological Source: *E. coli***
- **Catalyzes a phosphodiester bond between duplex DNA containing cohesive ends.**
- **Does not efficiently ligate blunt ended fragments.**
- **Requires NAD<sup>+</sup> as a cofactor.**

## ***T4 DNA Ligases***

- **Most common DNA ligases**
- **Biological Source: *T4 bacteriophage***
- **Catalyzes a phosphodiester bond between**
  - Duplex DNA containing cohesive ends.**
  - Duplex DNA containing blunt ends**
  - Joins nicks in RNA chains of ds DNA-RNA hybrids**
- **Requires ATP as a cofactor.**

# Polymerases

Catalyze formation of a new strand of DNA or RNA

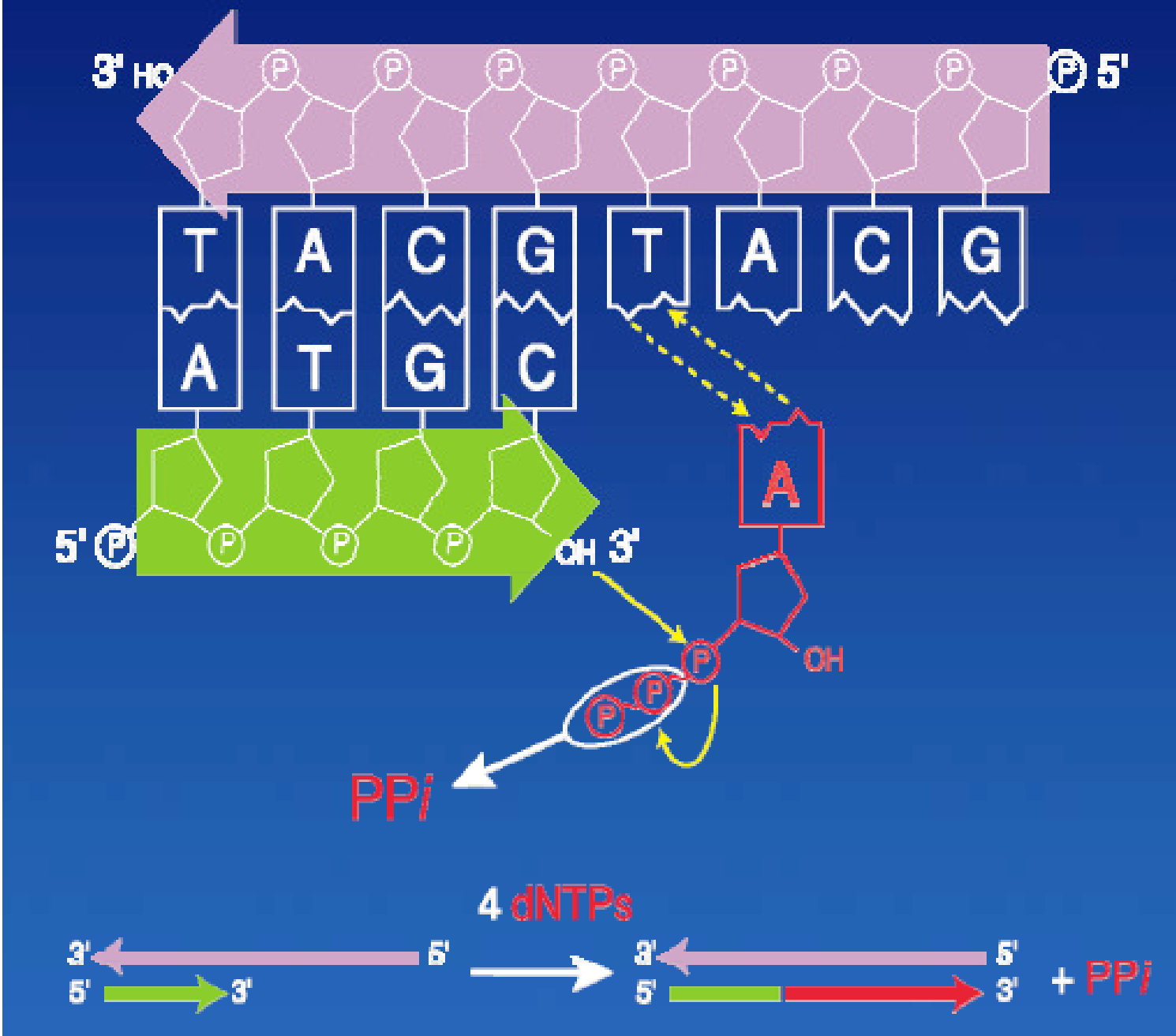
## DNA polymerases

DNA polymerases are enzymes that catalyse the synthesis of a new DNA strand from a pre-existing strand.

The enzyme adds deoxyribonucleotides to the free 3'-OH of the chain undergoing elongation. The direction of synthesis is 5'-3'.

It has three major requirements for its activity;

- (1) a template strand for which the enzyme synthesizes a complementary strand;
- (2) a primer with a free 3'-OH group that hybridizes with the template to form a double stranded region that initiates the polymerization and
- (3) a pool of all the four dNTPs that are used to synthesize the new DNA strand. In addition, some cofactors like  $Mg^{2+}$  ions may be required in a buffer solution with correct pH for optimum activity.



## Types of DNA polymerases used in recombinant DNA technology

- ❑ *E. coli* DNA Polymerase I
- ❑ Klenow polymerase
- ❑ Thermostable DNA Polymerase
- ❑ Reverse Transcriptase

# Role of different types of polymerases

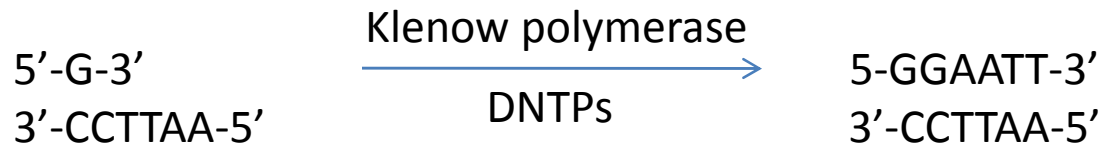
## ***E. coli* DNA polymerase I**

*E. coli* DNA polymerase I is an enzyme that has both DNA polymerase (5'-3' polymerase) as well as DNA nuclease (3'-5 and 5'-3' exonuclease activity) activity.

Used in DNA cloning

## **Klenow polymerase**

Filling of overhangs created by restriction enzyme to create blunt end

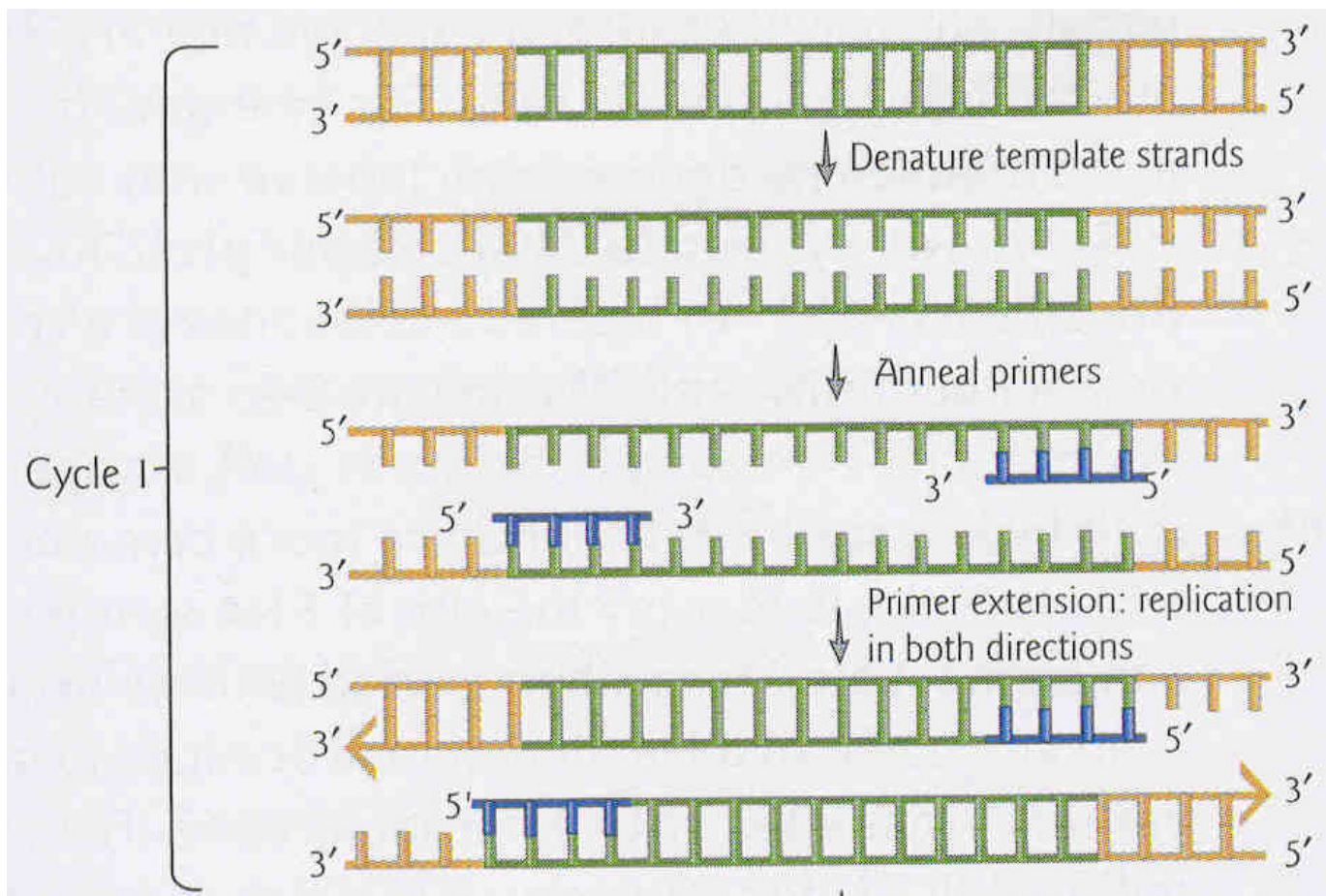


Used in DNA sequencing

## Thermostable DNA Polymerase (*Taq* polymerase)

A thermostable DNA polymerase enzyme that catalyzes the DNA amplification in polymerase chain reaction (PCR).

- The polymerase chain reaction (PCR) is a technique allows us to amplify any DNA sequence of interest to high (thousands to millions) copy numbers.



## Reverse Transcriptase

- Reverse transcriptase (RT) is a RNA dependent DNA polymerase found in RNA viruses also called as retroviruses.
- This enzyme is involved in the replication of retroviruses, where the RNA genome is first converted into DNA and then integrated into the host.
- RT uses mRNA template instead of DNA for synthesizing new DNA strand.
- The complementary DNA strand formed on the mRNA template is called the complementary DNA (cDNA).
- RT also shows RNaseH activity that degrades the RNA molecule from a DNA-RNA hybrid.
- Formation of a double stranded cDNA from the mRNA molecule using RT finds applications in genetic engineering.
- The cDNA thus formed from any mRNA can be cloned in an expression vector and its respective protein can be made to express in large quantities.

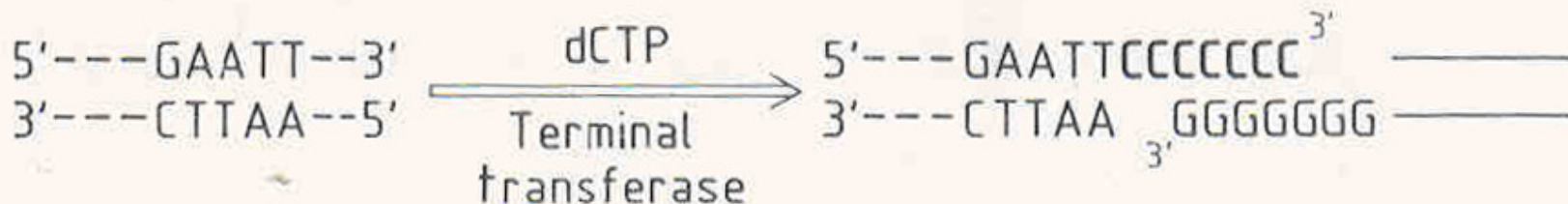
# DNA modifying enzymes

## Alkaline phosphatase (AP)

- This group of enzymes removes the phosphate group ( $\text{PO}_3^{2-}$ ) from 5' terminus of the DNA molecule.
- It is active at alkaline pH, hence the name 'alkaline phosphatase'.
- Commercially, it is obtained from *E. coli* (bacteria) and calf intestine
- Alkaline phosphatase inhibit the circularization of restricted vector DNA.

## Terminal Transferase

- Add oligodeoxynucleotide tail to 3'-OH end of double strand DNA
- It extend homopolymer tails (Homopolymer tailing)
- Used in DNA cloning



# Cloning vectors

- A cloning vector is a DNA molecule in which foreign DNA can be inserted or integrated and which is further capable of replicating within host cell to produce multiple clones of recombinant DNA
- Most vectors are genetically engineered.
- The cloning vector is chosen according to the size and type of DNA to be cloned

# Why 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 to amplify a single molecule of DNA into many copies.
- Cloning vectors are DNA molecules that are used to "transport" cloned sequences between biological hosts and the test tube.
- Without Cloning Vector, Molecular Gene Cloning is totally impossible.

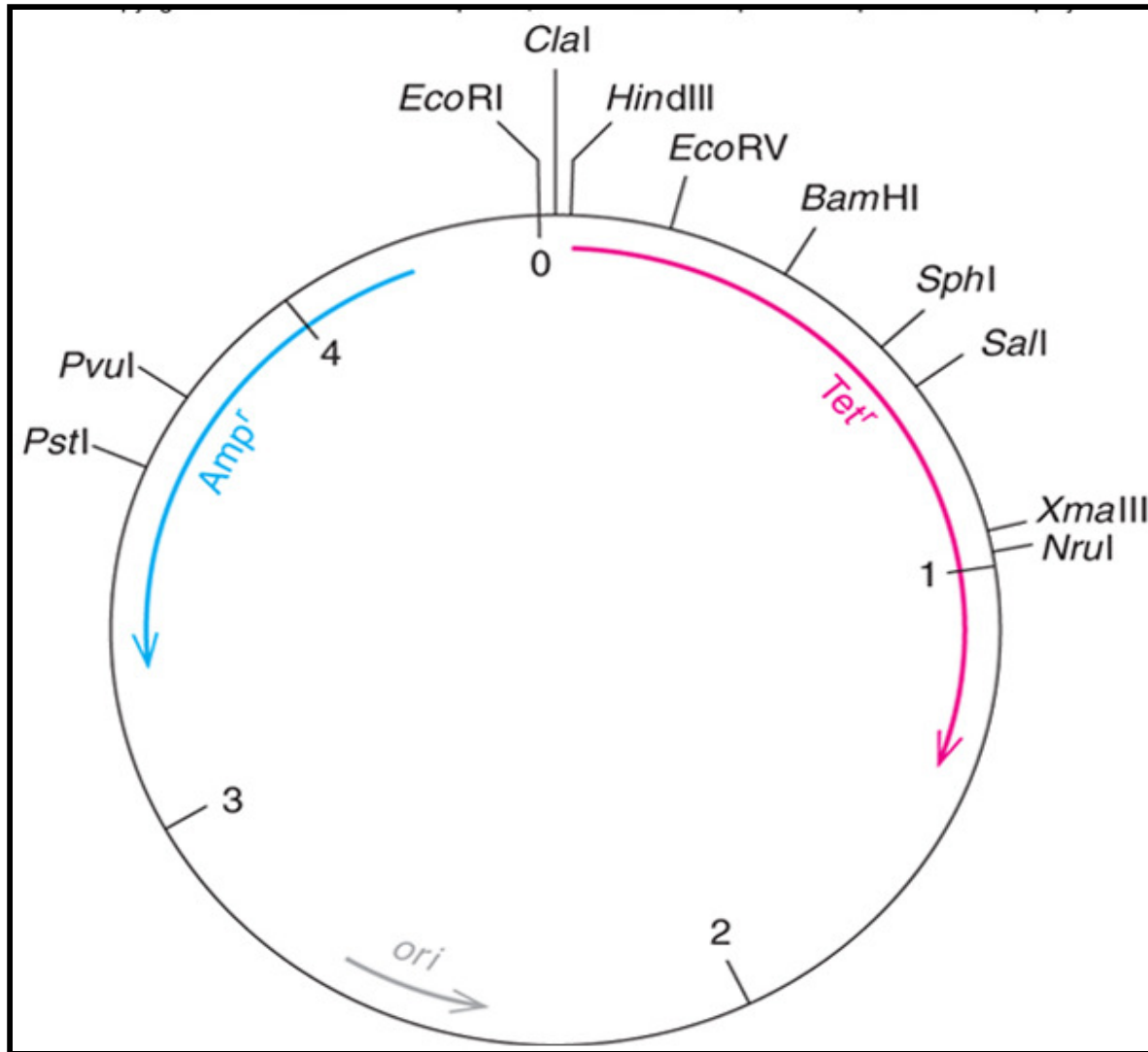
# History

- **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 for cloning purposes, pBR322 – a plasmid.
- This vector was small, ~4 kb in size, and had two antibiotic resistance genes for selection.

# Features of A Cloning Vector

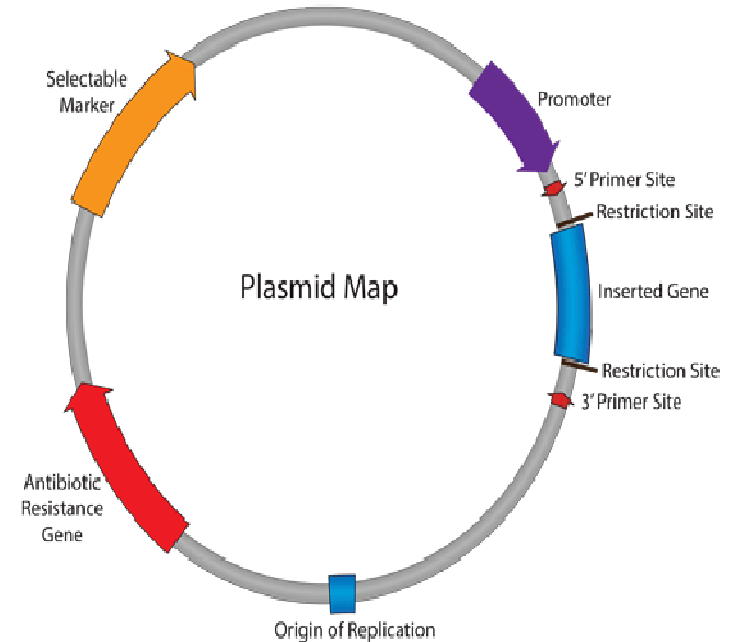
All commonly used cloning vectors have some essential features:

- It should be able to replicate autonomously
- Origin of replication (ori):
  - This makes **autonomous replication** in vector.
  - ori is a **specific 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.
- Restriction 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.



## **Additional Properties of Vectors**

- It should be short, small.
- Compatible with host cell.
- Incompatible with other vector.
- Should become high in copy number.
- It should be able to move under two system (Prokaryote and Eukaryote system).

# Types

1. Plasmid
2. Bacteriophage
3. Cosmid
4. Phagemid
5. Yeast Artificial Chromosomes (YAC)
6. Bacterial Artificial Chromosomes (BAC)

# Plasmid

- Plasmid is an **autonomously replicating circular double stranded extra-chromosomal DNA** which is physically separated from a chromosomal DNA and can replicate independently.
- They are most commonly found in **bacteria**, sometimes they are present in archaea and eukaryotic organisms.
- The size of the plasmid varies from **1 to over 200 kb**.
- Most general plasmids may be used to clone DNA insert of **up to 10 kb in size**.
- Many plasmids have **high copy number** and high copy number is useful as it produces greater yield of recombinant plasmid for subsequent manipulation
- However **low copy number** plasmids may be preferably used in certain circumstances, for example, when the protein from the cloned gene is toxic to the cells.
- **Example: pBR322, pUC18, F plasmid, Col Plasmid etc.**

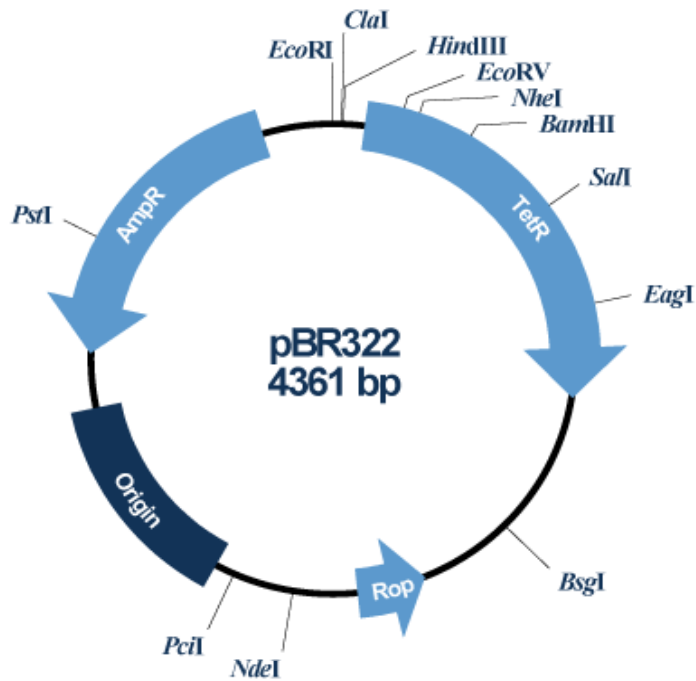


Table 2.1 Sizes of representative plasmids

Plasmid	Size		Organism
	Nucleotide length (kb)	Molecular wt (MDa)	
pBR345	0.7	0.46	<i>E. coli</i>
pBR322	4.362	2.9	<i>E. coli</i>
ColEI	6.36	4.2	<i>E. coli</i>
RP4	54	36	<i>Pseudomonas</i> + others
F	95	63	<i>E. coli</i>
TOL	117	78	<i>Pseudomonas putida</i>
pTiAch5	213	142	<i>Agrobacterium tumefaciens</i>



TABLE 4.2 Copy numbers of some plasmids

Plasmid	Approximate copy number
F	1
P1 prophage	1
RK2	4-7 (in <i>E. coli</i> )
pBR322	16
pUC18	~30-50
pIJ101	40-300

## **Why Plasmids are Good Cloning Vectors**

- Small size (easy to manipulate and isolate).
- Circular (more stable).
- Replication independent of host cell.
- Several copies may be present (facilitates replication).
- Frequently have antibiotic resistance (detection easy).

## **Disadvantages Using Plasmids**

- Cannot accept large fragments.
- Sizes range from 0 – 10kb.
- Standard methods of transformation are inefficient.

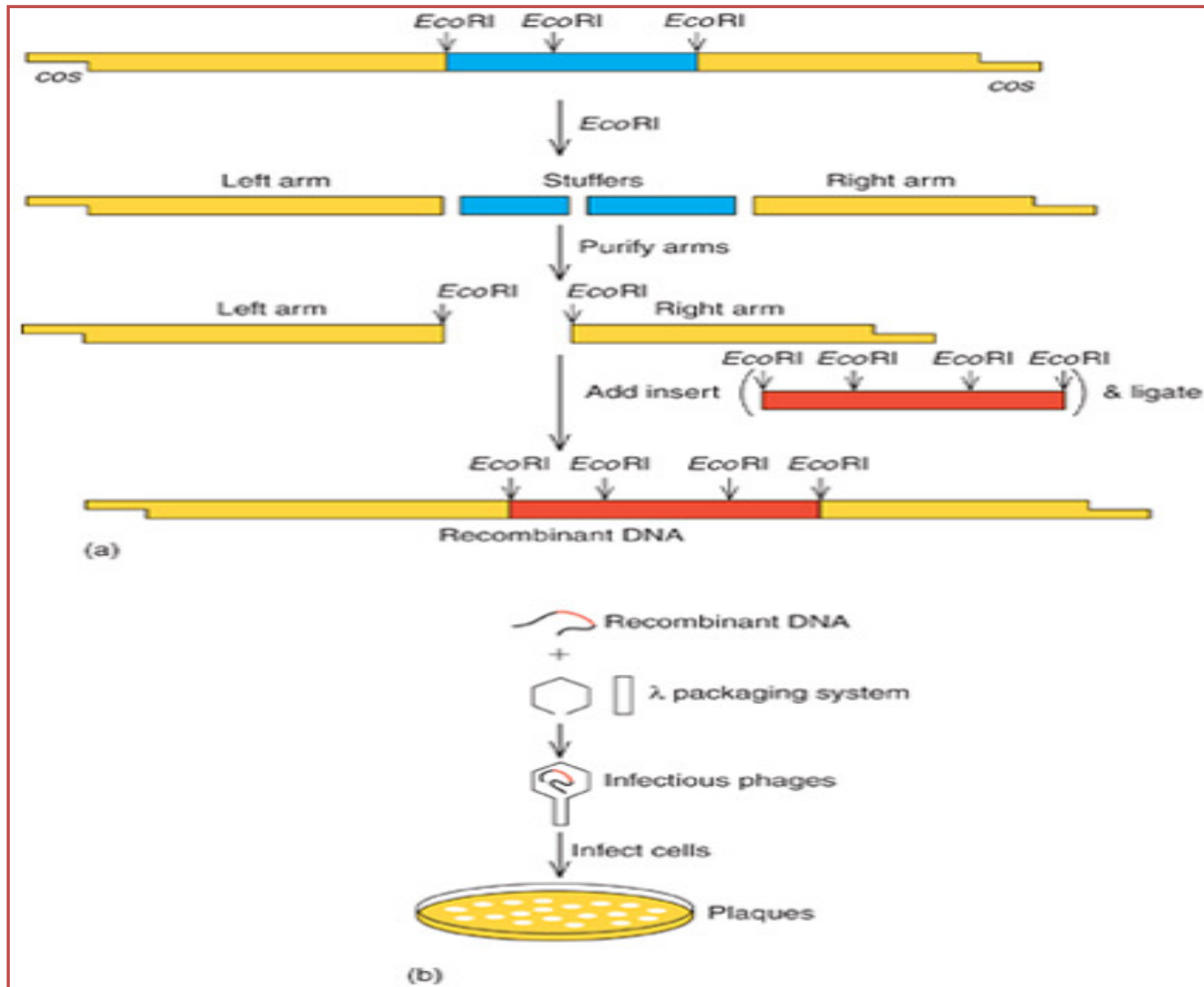
## Bacteriophage as a vector

- The bacteriophages used for cloning are the **phage  $\lambda$  and M13 phage**.
- There is an **upper limit** on the amount of DNA that can be packed into a phage (a maximum of 53 kb).
- There is also a **lower size limit (20 kb)** for DNA that can be packed into a phage, and vector DNA that is too small cannot be properly packaged into the phage.

# Phage Lambda vector

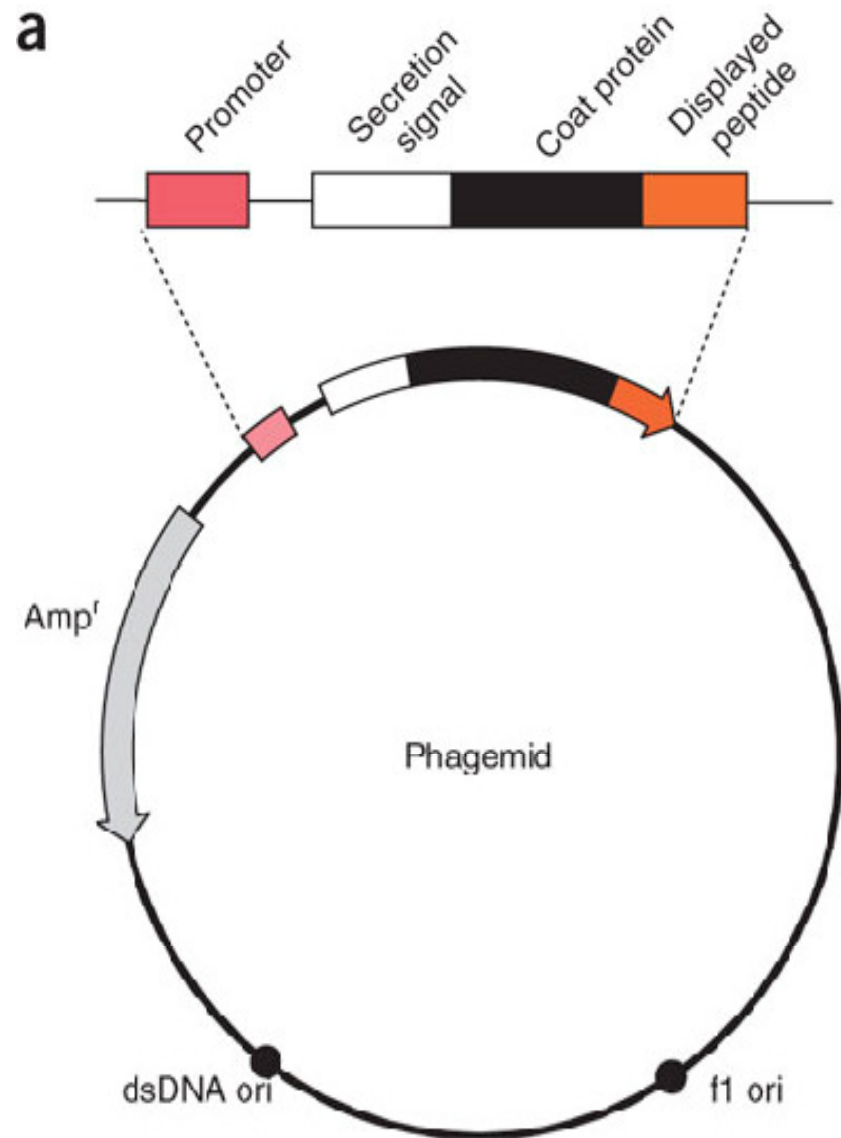
- Phage lambda is a **bacteriophage or phage**, i.e. bacterial virus, that uses *E. coli* as host.
- Its structure is that of a typical phage: **head, tail, tail fibres.**
- Lambda viral genome: **48.5 kb DNA** with a **12 base ssDNA "sticky end"** at both ends; these ends are complementary in sequence and can hybridize to each other (this is the **cos site**: cohesive ends).
- **Infection:** lambda tail fibres adsorb to a cell surface receptor, the tail contracts, and the DNA is injected.
- The DNA circularizes and lambda begins its life cycle in the *E. coli* host.

# DNA cloning using phages as vectors



# Phagemid Vector

- A **phagemid** or **phasmid** is a plasmid that contains an f1 origin of replication from an f1 phage.
- It can be used as a type of cloning vector in combination with filamentous phage M13.
- A **phagemid** can be replicated as a plasmid, and also be packaged as single stranded DNA in viral particles.

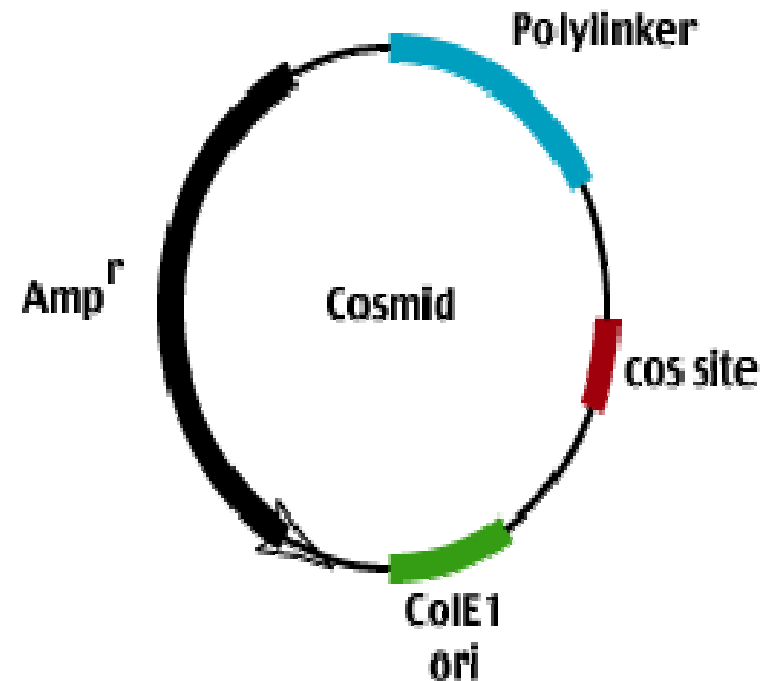


# Cosmid vector

- Cosmids are plasmids that incorporate a segment of **bacteriophage  $\lambda$  DNA** that has the **cohesive end site (cos)** which contains elements required for packaging DNA into  $\lambda$  particles.
- It is normally used to clone large DNA fragments between **25 and 45 Kb**.
- They can replicate as plasmids if they have a suitable origin of replication.
- They can also be packaged in phage capsids, which allows the foreign genes to be transferred into cells by **transduction**.

## Advantages

- High transformation efficiency.
- The cosmid vector can carry up to 45 kb whereas plasmid and Lambda phage vectors are limited to 10-20 kb.



# Bacterial Artificial Chromosome (BAC)

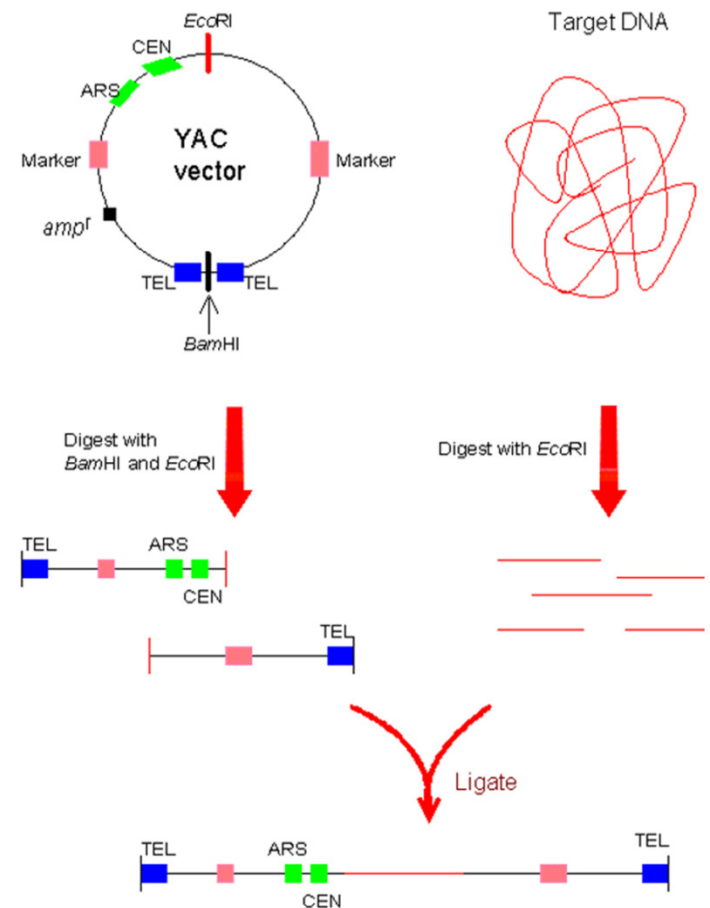
- BAC vectors are similar to standard *E. coli* plasmid vectors.
- Contain the origin and genes encoding the ori binding proteins required for plasmid replication.
- Derived from a naturally occurring large plasmid, **the F' plasmid.**
- **Low copy number** (1-2 copies per cell)
- The bacterial artificial chromosome's usual insert size is **150-350 kb.**
- BACs are preferred for different kind of genetic studies of inherited or infectious diseases because **they accommodate much larger sequences without the risk of rearrangement**, and are therefore more stable than other types of cloning vectors.

# Yeast Artificial Chromosome (YAC)

- The yeast artificial chromosome (YAC) vector is capable of carrying a large DNA fragment (up to 200 Kb), but its **transformation efficiency is very low**.
- Cloning vehicles that propagate in eukaryotic cell hosts as eukaryotic chromosomes.
- Final chimeric DNA is a linear DNA molecule with telomeric ends:

## Artificial Chromosome

- YAC cloning vehicles often have a bacterial origin of DNA replication (**ori**) and a selection marker for propagation of the YAC through bacteria.
- The YAC can use both yeast and bacteria as a host.



## What Determines Choice of Vector?

- Insert size
- Vector size
- Restriction size
- Cloning efficiency

Vector	Insert size (kb)
Plasmid	10 kb
Bacteriophage	10 – 20 kb
Cosmids	23 – 45 kb
BACs	$\leq$ 300 kb
YACs	100 – 3000 kb

# Gene cloning

Isolation of genomic DNA (Gene of interest) and plasmid DNA (Vector)



Cut vector and gene of interest with same restriction enzyme



Integration of vector and gene of interest and ligation with DNA ligase



Preparation of Recombinant DNA



Introduced Recombinant DNA in Bacterial host cell

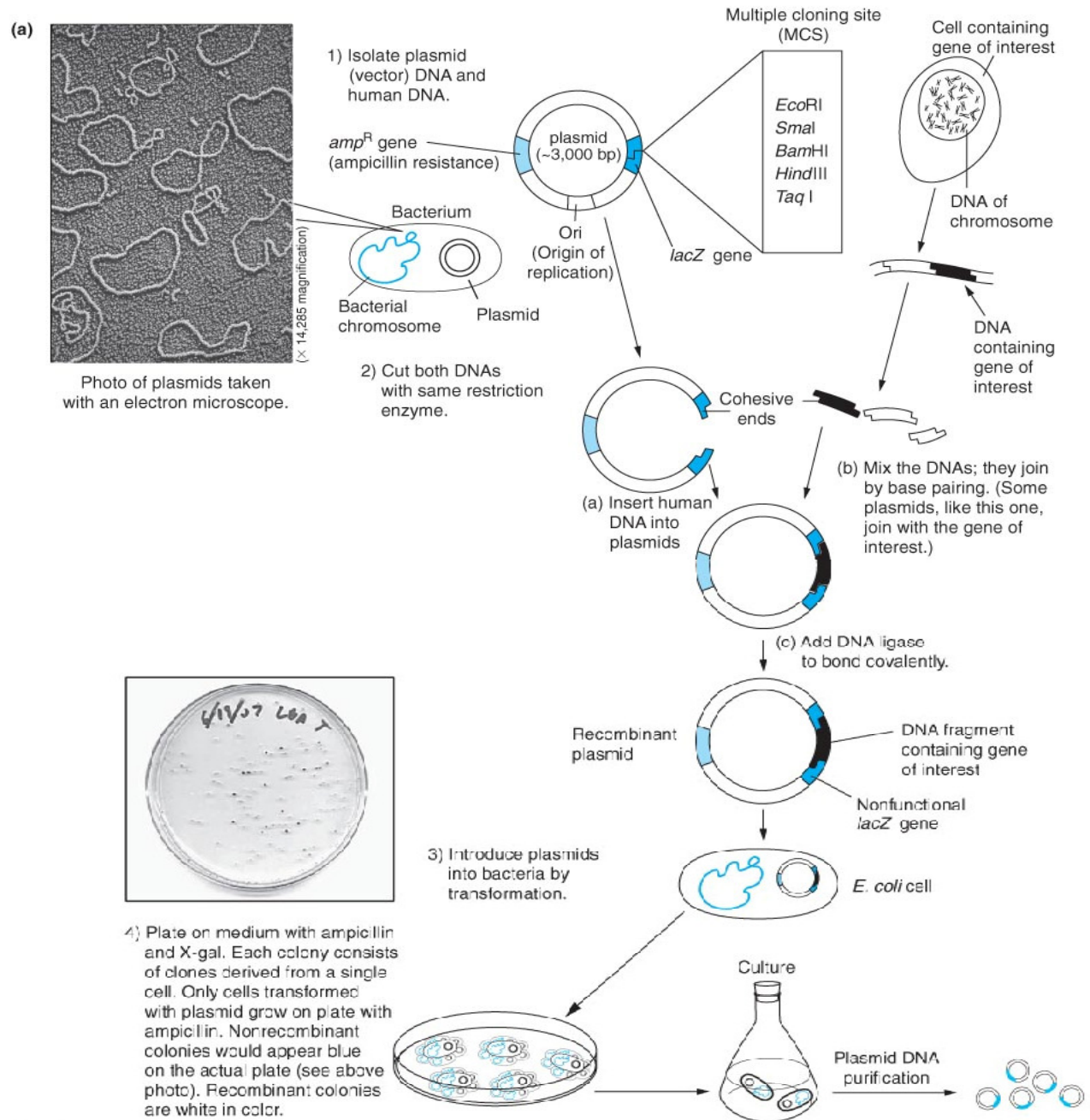


Identification and selection of transformed cell



Cell multiplication

# Basic steps of gene cloning



# How Do You store a Gene of Interest?

- Creating DNA Libraries
  - Collections of cloned DNA fragments from a particular organism contained within bacteria or viruses as the host
  - Screened to pick out different genes of interest
- Two Types of Libraries
  - **Genomic DNA libraries**
  - **Complementary DNA libraries (cDNA libraries)**

# Genomic Libraries

- Chromosomal DNA from the tissue of interest is isolated and digested with a restriction enzyme which produces many fragments that include the entire genome
- Vector is digested with same enzyme
- DNA ligase is used to ligate genomic DNA fragments and vector DNA
- Recombinant vectors are used to transform bacteria and theoretically each bacteria will contain a recombinant plasmid

**Foreign genome**

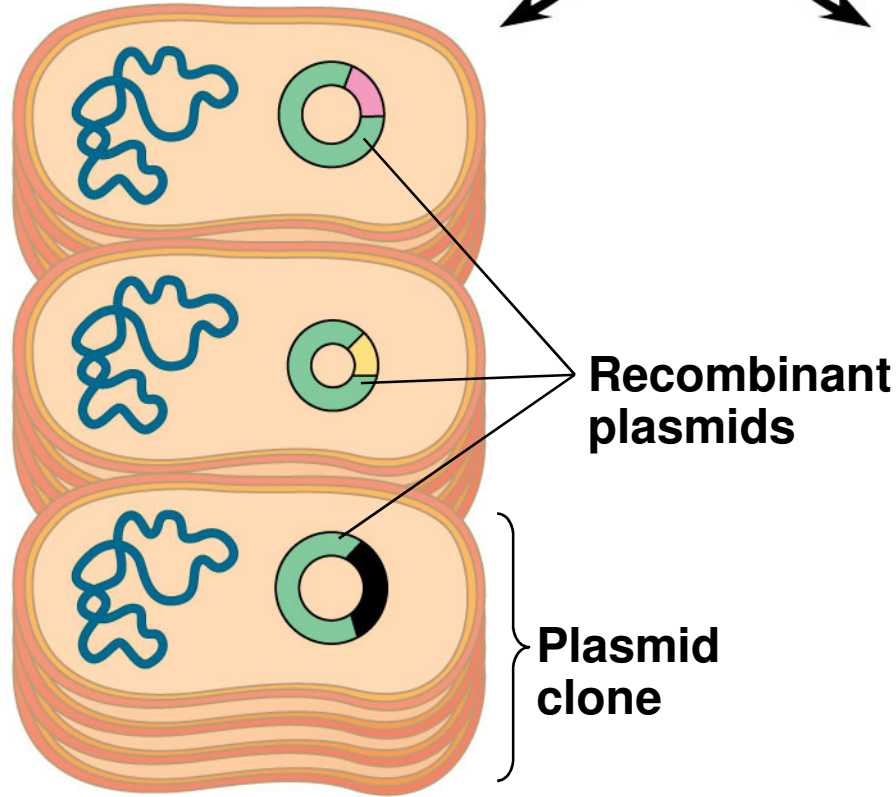


Cut with restriction enzymes into either

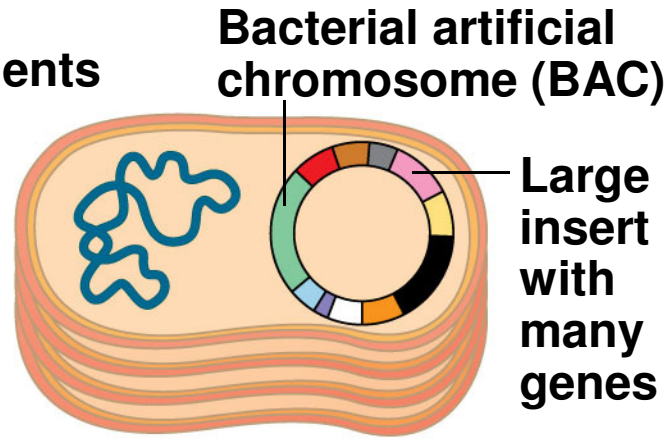
small fragments

or

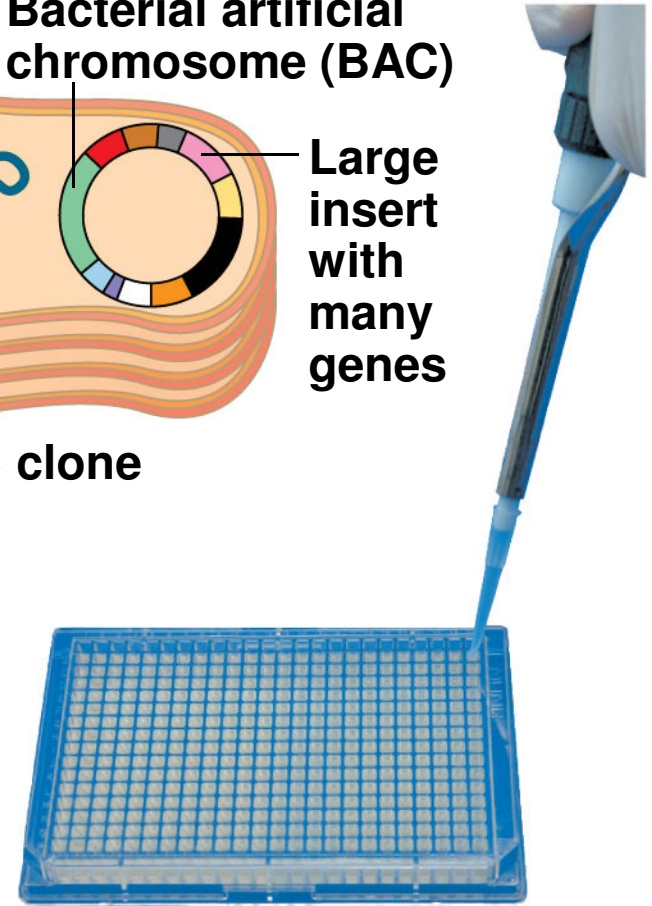
large fragments



**(a) Plasmid library**




**(b) BAC clone**



**(c) Storing genome libraries**

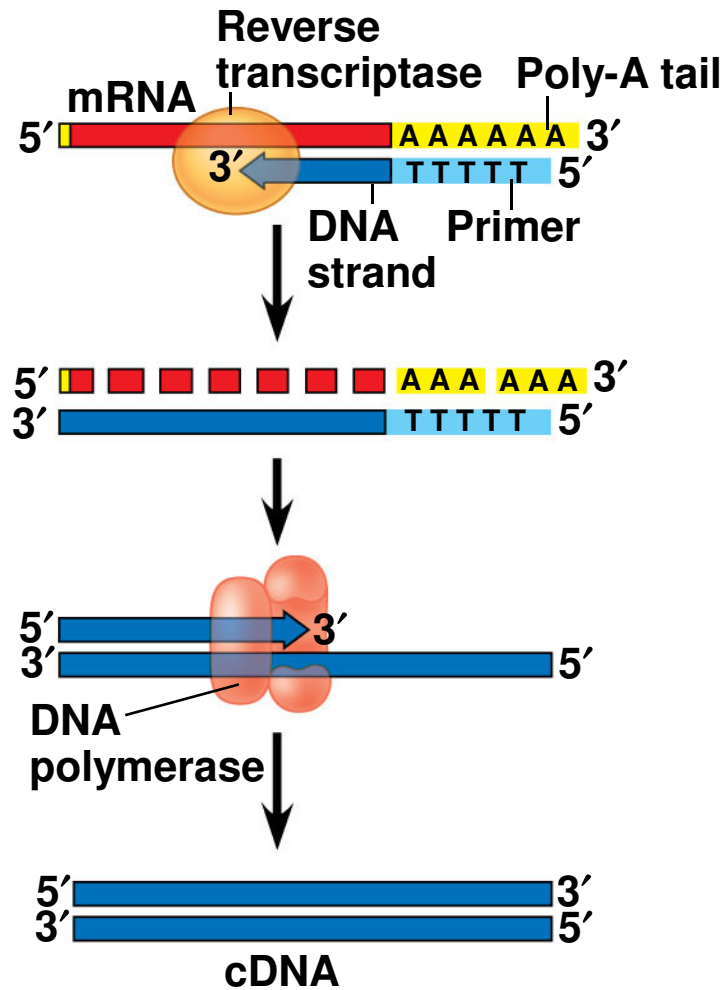
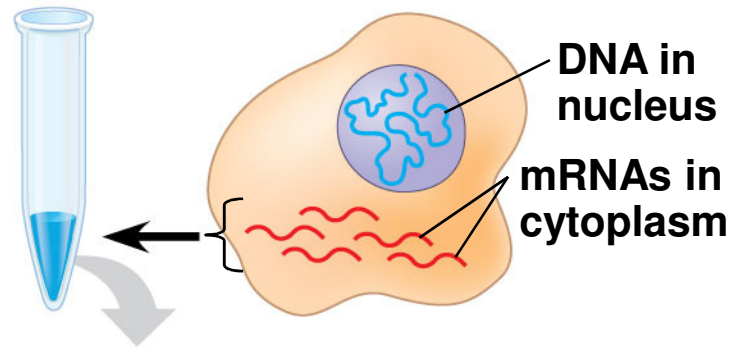
## – **Disadvantages of genomic libraries**

- Introns are cloned in addition to exons;
  - Majority of genomic DNA is introns in eukaryotes so majority of the library will contain non-coding pieces of DNA
- Many organisms have very large genome, so searching for gene of interest is difficult
- Time consuming!

- 
- A **complementary DNA (cDNA)** library is made by cloning DNA made *in vitro* by reverse transcription of all the mRNA produced by a particular cell
  - A **cDNA library** represents only part of the genome—only the subset of genes transcribed into mRNA in the original cells

- **cDNA Libraries**

- mRNA from tissue of interest is isolated
- Need to make double stranded DNA from mRNA: How?
  - a. enzyme **reverse transcriptase** catalyzes synthesis of complementary single stranded DNA from mRNA
    - i. Called complementary DNA (cDNA) because it is an exact copy of the mRNA
  - b. mRNA is degraded either with an enzyme or alkaline solution
  - c. **DNA Pol** is used to synthesize second strand of DNA to create double stranded cDNA
- Short **linker** double stranded DNA sequences which contain restriction enzyme recognition sites are added to the ends of the cDNA
- Cut with **restriction enzyme**, cut vector with same enzyme, ligate fragments to create recombinant vectors
- Then transform bacteria with recombinant vectors



- **cDNA Libraries**

- Advantage over genomic libraries

- Collection of actively expressed genes in the cells or tissues from which the mRNA was isolated
    - Introns are NOT cloned
    - Can be created and screened to isolate genes that are primarily expressed only under certain conditions in a tissue

- Disadvantage

- Can be difficult to make the cDNA library if a source tissue with an abundant amount of mRNA for the gene is not available

# Screening a Library for Clones Carrying a Gene of Interest

## (1) Selection marker gene (Antibiotic resistance gene)

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.

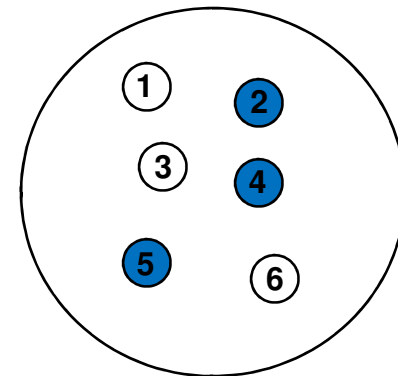
A cloning vector contains a selectable marker, which confer on the host cell an ability to survive and proliferate in a selective growth medium containing the particular antibiotics.

## (2) Blue white selection method

*LacZ* gene encodes B-galactosidase, give blue colour

*LacZ* gene present in restriction enzymes

*LacZ* gene inactivated if inserted with gene of interest



### 3. Colony hybridization or nucleic acid hybridization

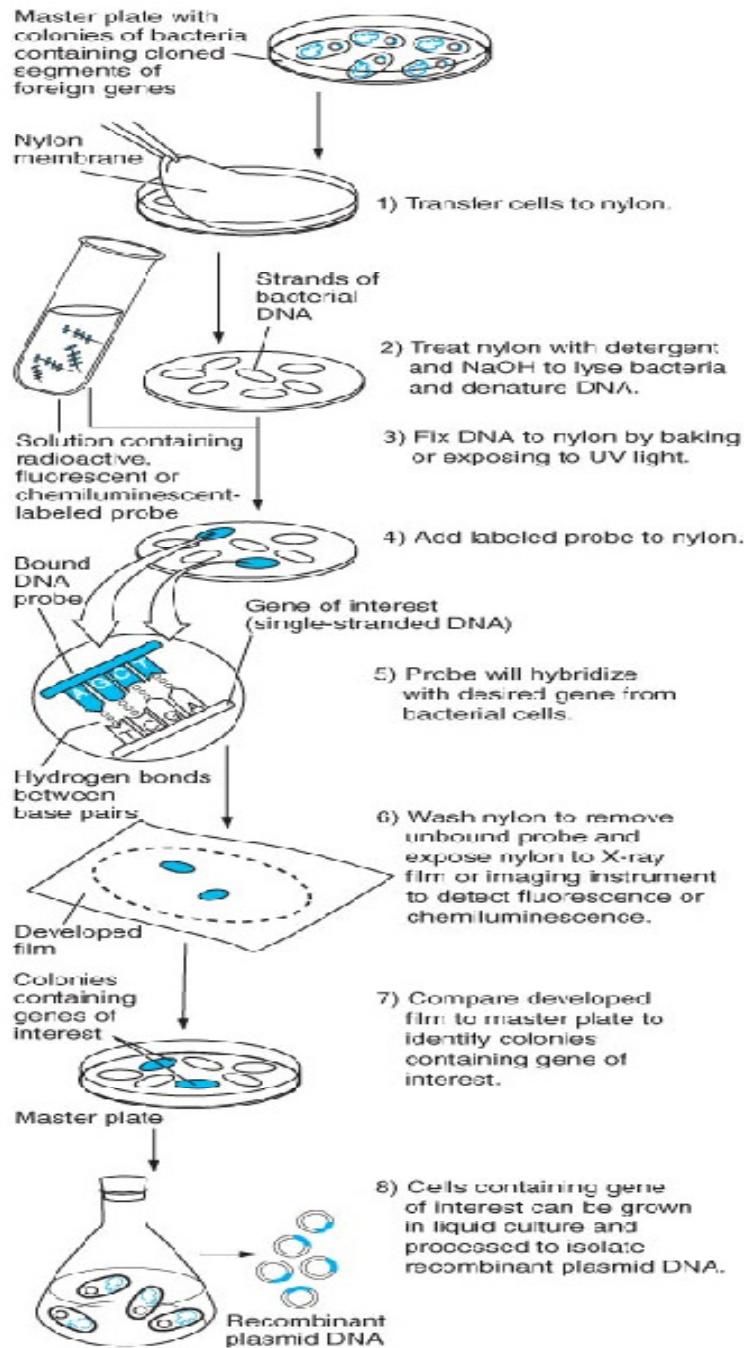
- A clone carrying the gene of interest can be identified with a **nucleic acid probe** having a sequence complementary to the gene
- This process is called **nucleic acid hybridization**
  - A probe can be synthesized that is complementary to the gene of interest
  - For example, if the desired gene is

5' ... CTCATCACCGGC ... 3'

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– Then we would synthesize this probe a

3' GAGTAGTGGCCG 5'



# Expressing Cloned Eukaryotic Genes

- After a gene has been cloned, its protein product can be produced in larger amounts for research
- Cloned genes can be expressed as protein in either bacterial or eukaryotic cells