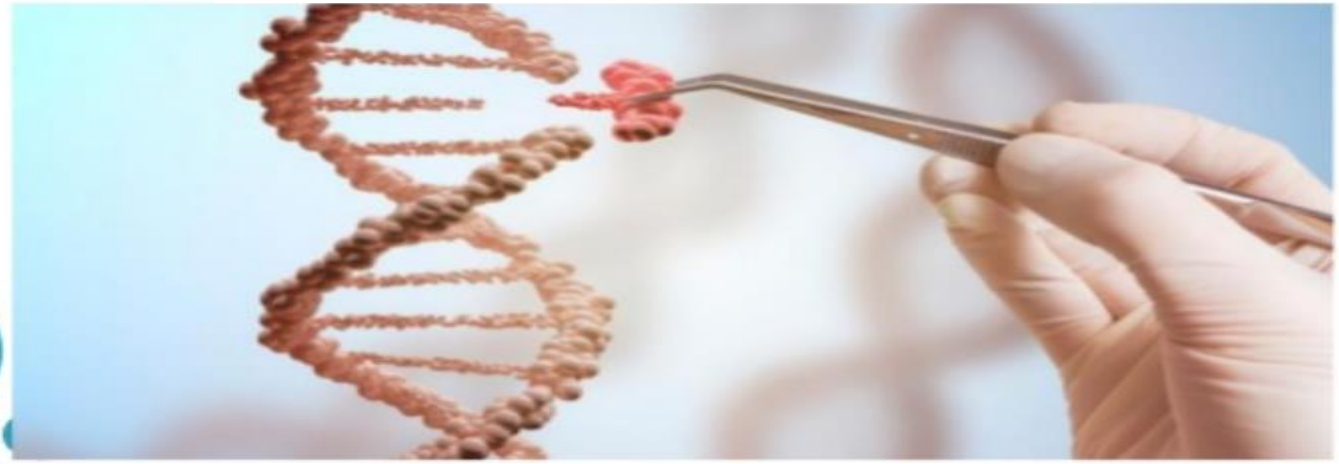
Gene Cloning



Essential Components for cloning a gene:

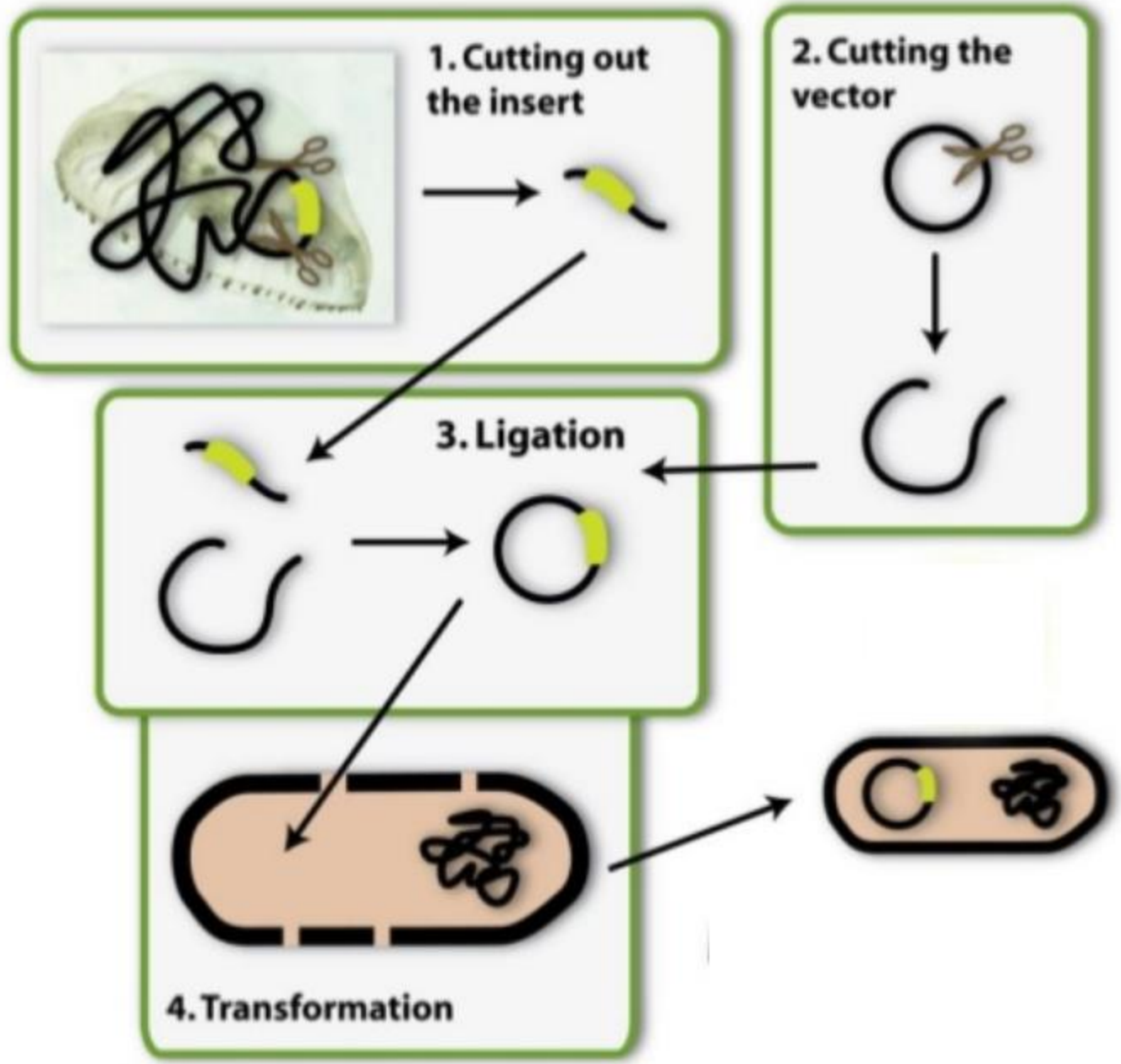
- Vehicles for introducing the recombinant molecule into host cell: Vector

- DNA fragments: Gene libraries

- Enzyme of cutting DNA fragments: Restriction endonuclease (RE)

- Enzyme of joining DNA fragments: DNA ligase

- Selection: Selection of the transformed cells for the presence of the rDNA


1. Cutting out the insert
2. Cutting the vector
3. Ligation
4. Transformation

Types of Cloning vectors:

Size

Copy number

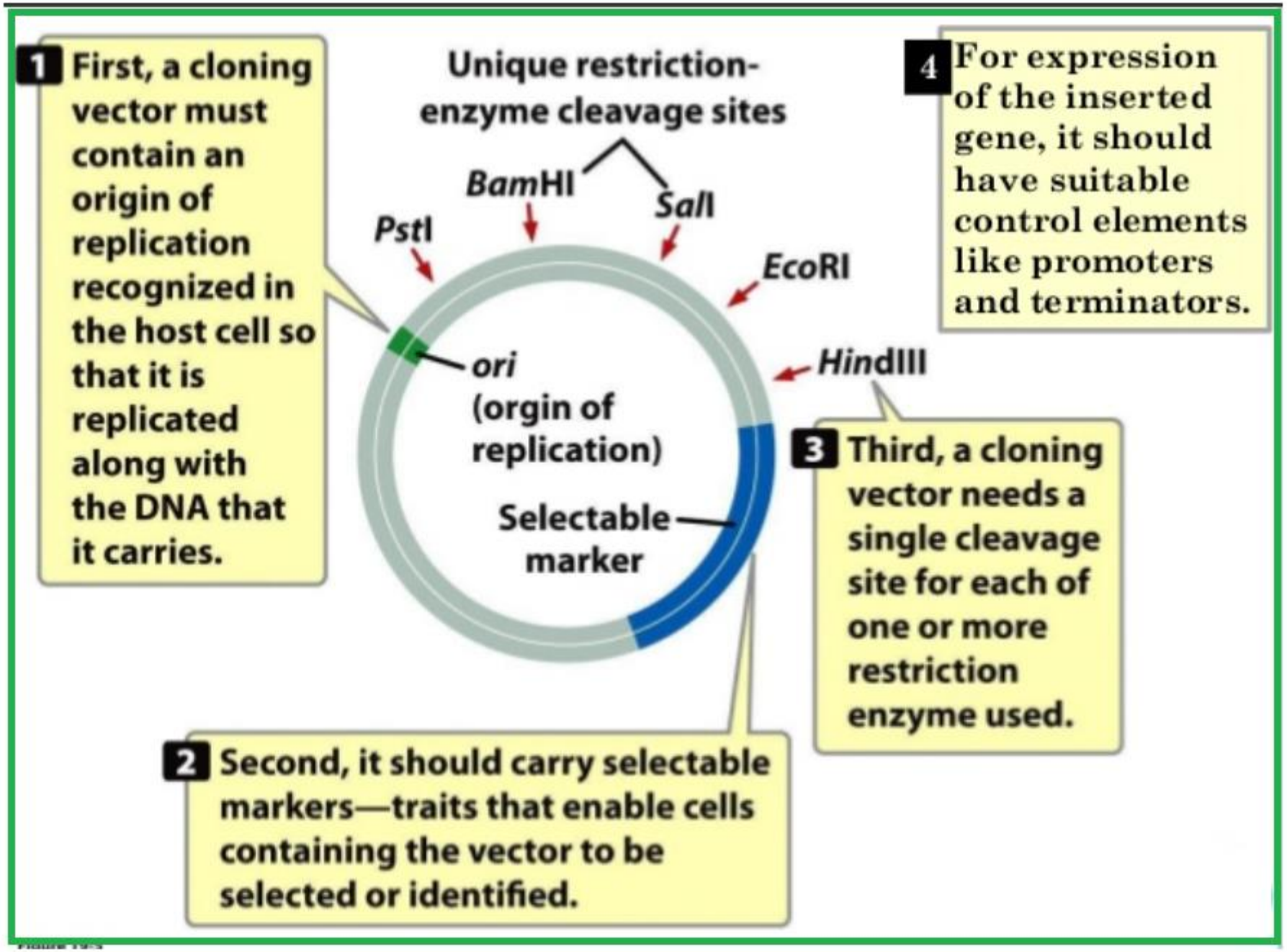
Cloning method

Characteristics of a Cloning Vector

- Origin of replication
- Antibiotic resistance gene
- Cloning site (MCS)
- Promoters and terminators for expression of the cloned gene

⇩

Expression vector


1 First, a cloning vector must contain an origin of replication recognized in the host cell so that it is replicated along with the DNA that it carries.
Unique restriction-enzyme cleavage sites
BamHI
SalI
PstI
EcoRI
HindIII
ori
(orgin of replication)
Selectable marker
4 For expression of the inserted gene, it should have suitable control elements like promoters and terminators.
3 Third, a cloning vector needs a single cleavage site for each of one or more restriction enzyme used.
2 Second, it should carry selectable markers—traits that enable cells containing the vector to be selected or identified.

Types of Cloning vectors based on Insert Size:

1. **Plasmids (about 10kb)**
2. Bacteriophage (bacterial viruses), 30-50kb inserts
3. Cosmids (35-50kb insert)
4. Bacterial Artificial Chromosomes (BACs)
    - Use fertility F plasmid
    - 75-300kb inserts possible
    - Developed during the human genome project
5. Yeast Artificial Chromosomes (YACs)
    - Mimics yeast chromosome
    - Contains all regions for replication (yeast ori and centromere)
    - 100-1000kb inserts poss.
    - developed during the human genome project

Plasmid

- Plasmids are double stranded, closed, circular DNA molecules which exist in the host cell as extrachromosomal units.
- They are self-replicating, can be single or multi-copy per cell.
- 1-200 kb in size
- Depend on the host proteins for replication and maintenance.
- All naturally occurring plasmids do not always contain all the essential properties of a suitable cloning vector.

⇩ Derived from naturally occurring plasmids

- Can hold up to 10 kb fragments

⇩ Altered features

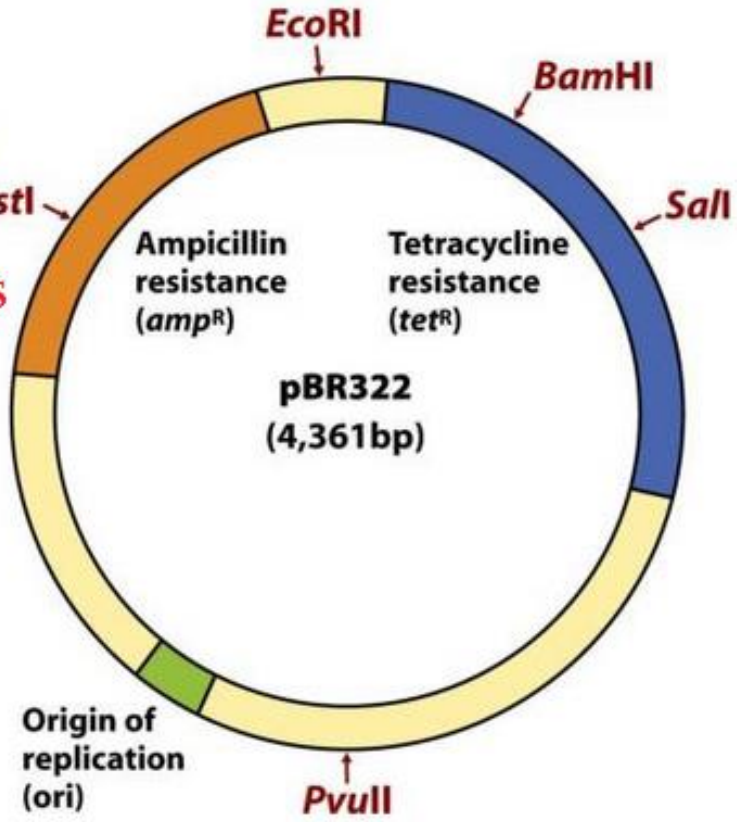
(removal noneseential, MCS , Selectable marker, Promoters, etc.)

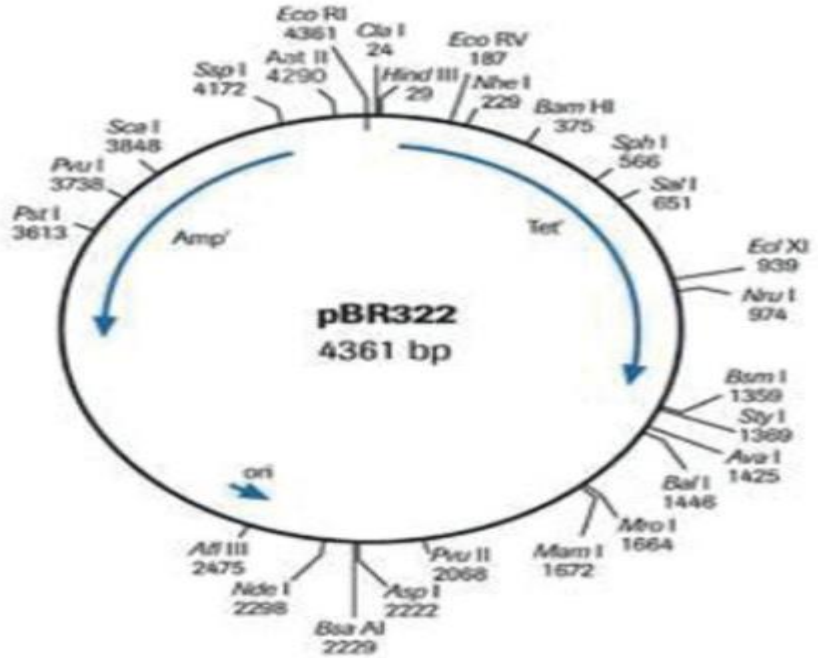
The constructed *E. coli* plasmid pBR322

artificial cloning vector

Features of pBR322

1.An origin of replication (ori)
2.Two genes that confer resistance to different antibiotics (tet^R, amp^R)
3. Several unique recognition sequences (EcoRI, BamH1) = 20 RS
4. Small size (4,361 bp)

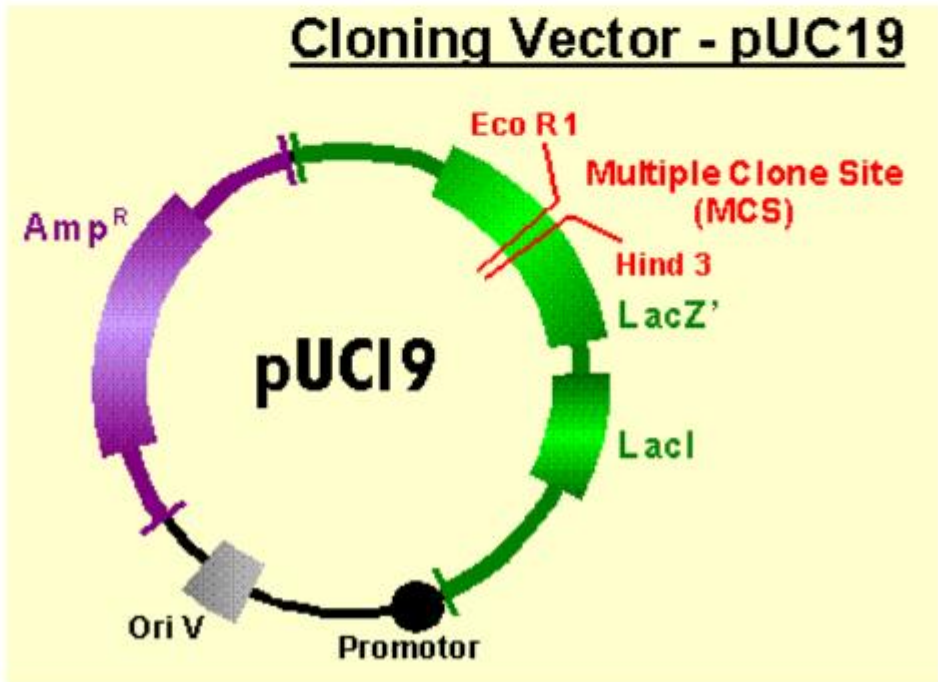




Enzyme	Cleavage sites	Position of Cleavage Sites				
Acc I	2	652	2247			
Asp 700	2	2035	3967			
Ban II	2	475	489			
Drd I	2	2170	2583			
Hind II	2	653	3909			
Ksp 6321	2	2359	4163			
Ppu MI	2	1439	1481			
Bgl I	3	935	1169	3488		
Dra I	3	3234	3253	3945		
Rsa I	3	165	2283	3848		
Sno I	3	2291	2789	4035		
Avi II	4	262	1358	1456	3590	
Bbe I	4	417	438	552	1209	
Bsp HI	4	489	3195	4203	4308	
Dra II	4	524	1439	1481	4344	
Eco 47 III	4	234	496	777	1729	
Nae I	4	403	771	931	1285	
Nar I	4	414	435	549	1206	
Nsp I	4	566	1820	2114	2479	
Mae I	5	230	1489	2970	3223	3558

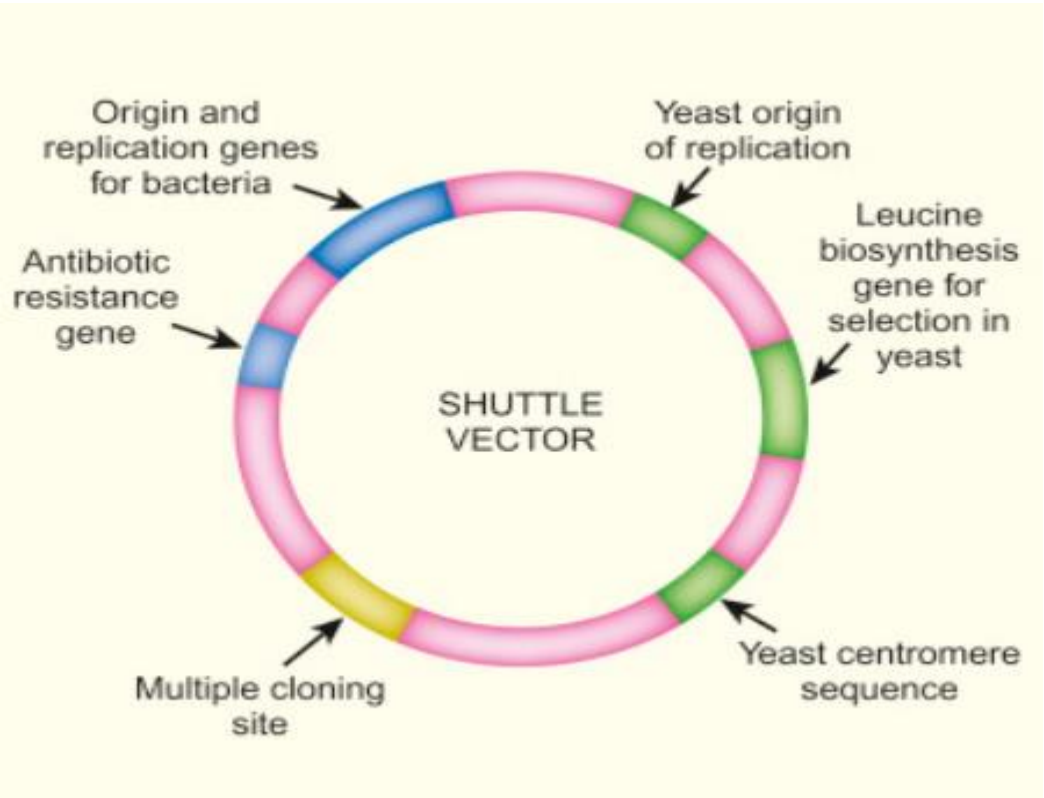
pUC19 Cloning vector

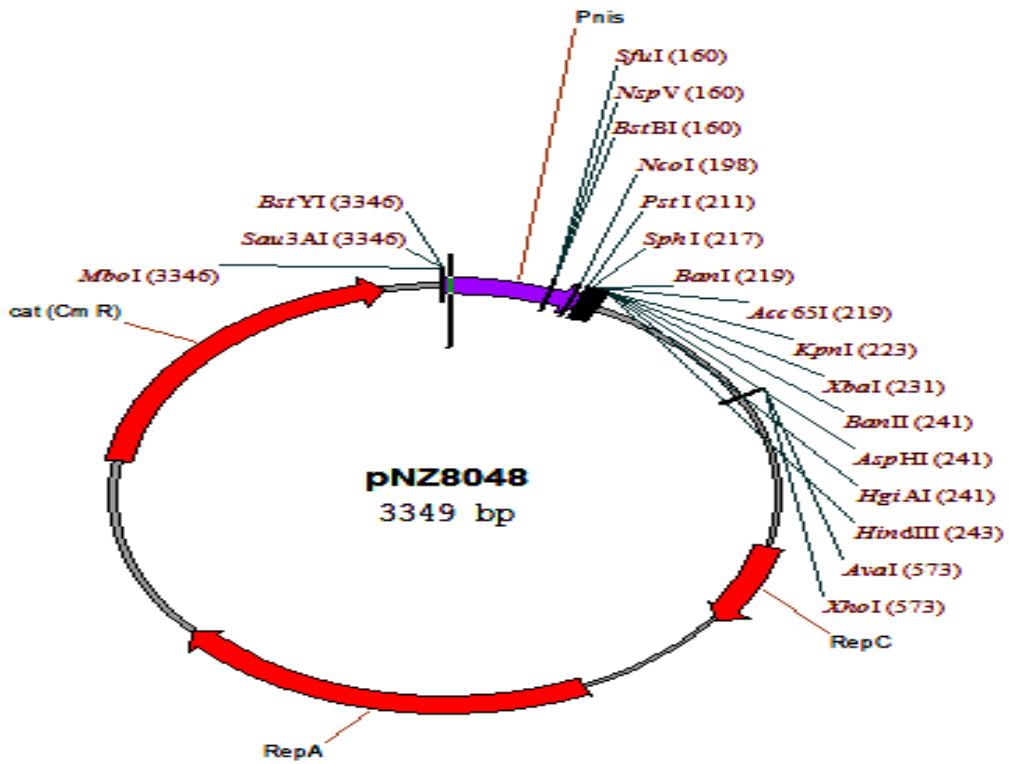
- Its name originated from University of California from where it was developed.
- 2.7 kb in size and possess:
  - ✓ Gene for ampicilin resistance
  - ✓ lacZ gene (coding for ß-galactosidase gene) for blue/white selection
  - ✓ Origin of replication from pBR322
- Within the lacZ region there is a polylinker having unique RE sites



Shuttle vectors

A shuttle vector is a vector (usually a plasmid) that can propagate in two different host species. Thus the DNA fragment inserted into it can be manipulated or tested in two different cell types.





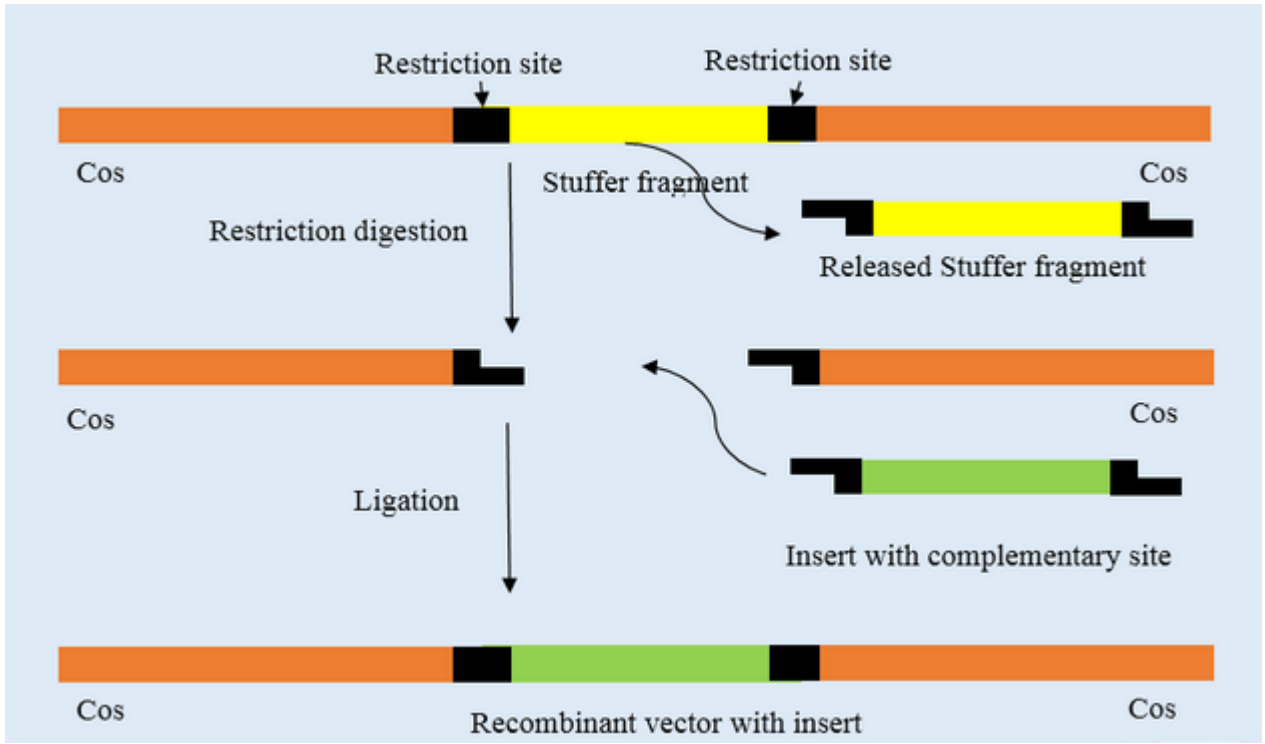
Major Limitation of Cloning in Plasmids

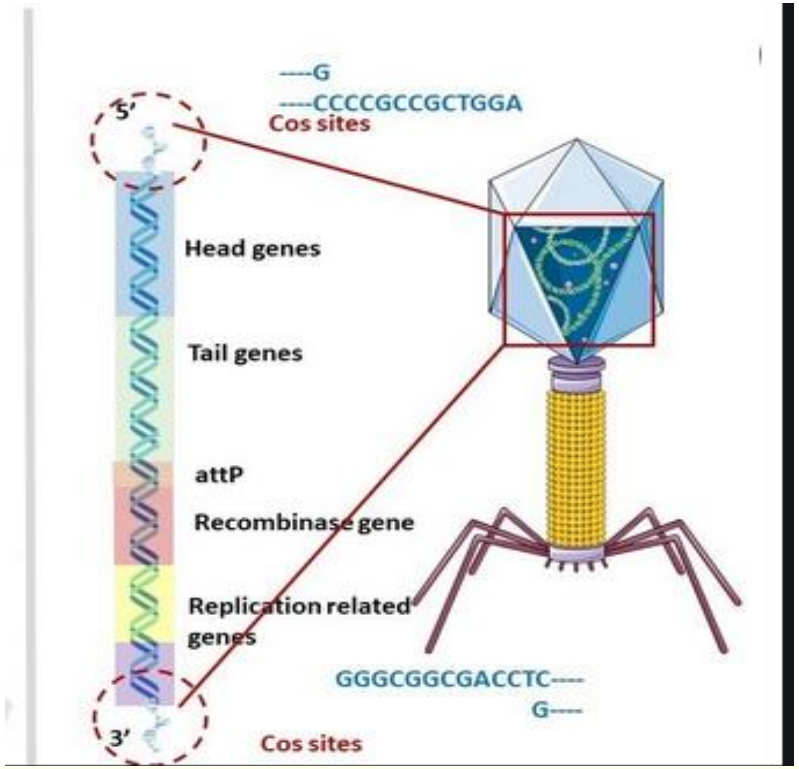
- Upper limit for insert DNA size is 12 kb
- Requires the preparation of "competent" host cells

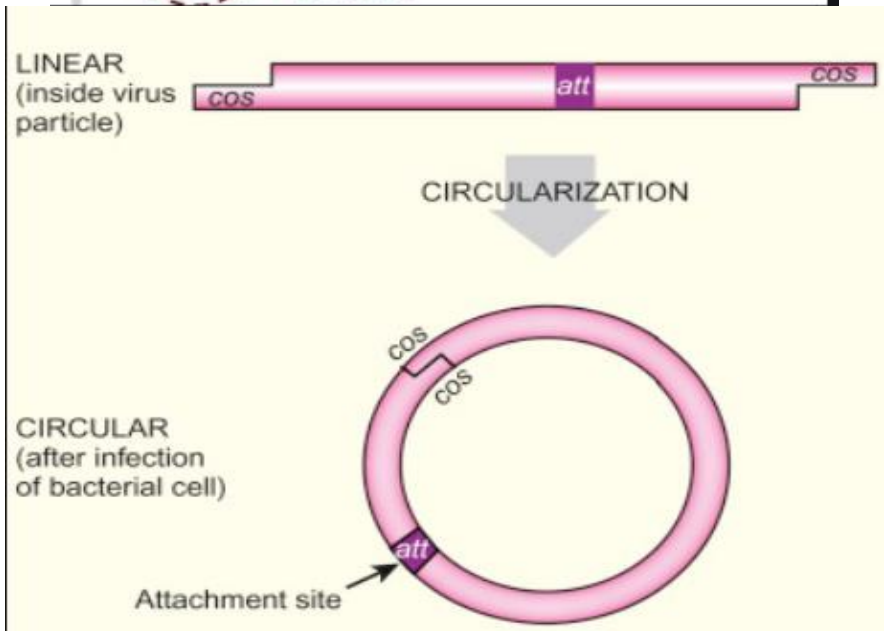
- If it is an expression vector there are often limitations regarding eukaryotic protein expression and post translational modification

Phage Cloning Vectors

- Fragments up to 23 kb can be may be accommodated by a phage vector
- Lambda is most common phage
- Lambda phage is a virus that infects bacteria (*E. coli*)
- In 1971 Alan Campbell showed that the central third of its genome was not required for lytic growth.







Bacteriophage lambda

Lambda genome is approximately 49 kb in length.

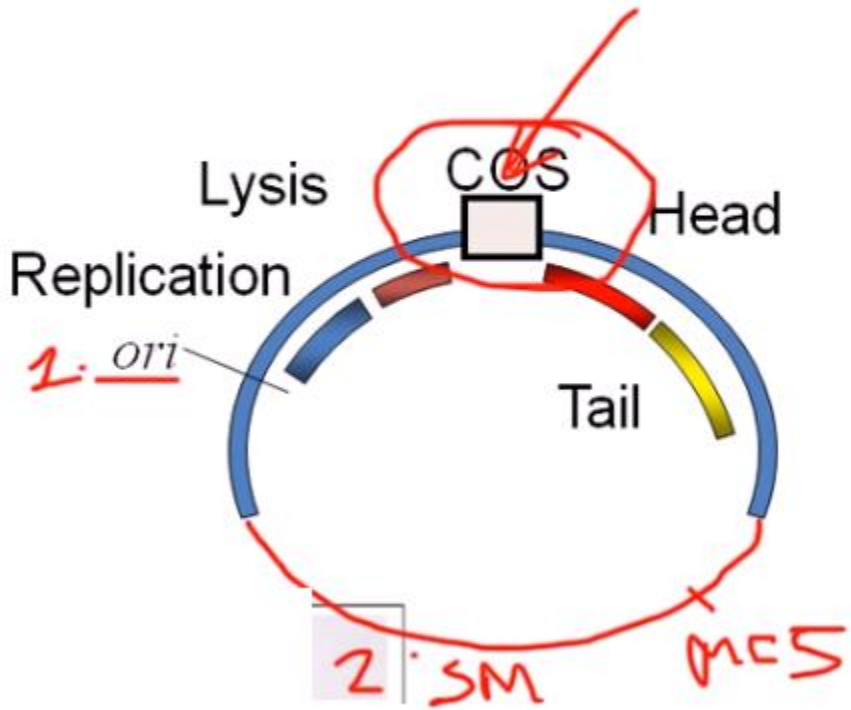
Only 30 kb is required for lytic growth.

Thus, one could clone 19 kb of "foreign" DNA.

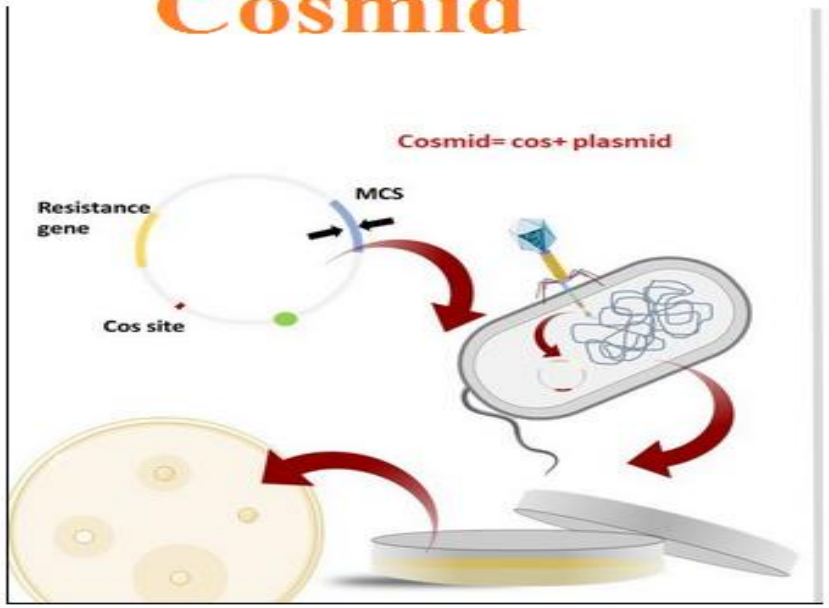
Packaging efficiency 78%-100% of the lambda genome.

Modification in Bacteriophage lambda

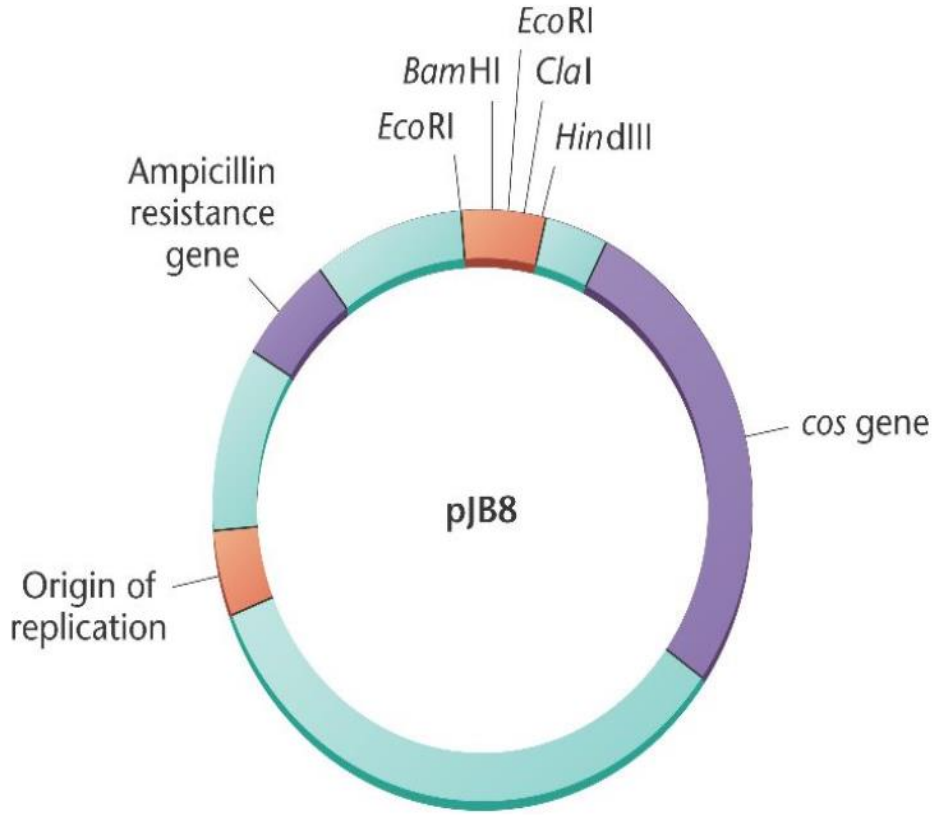
- **Eliminate the non-essential parts of lambda**
- **Can now insert large pieces of DNA (~ 20 kb)**



Cosmid



- Cosmids are plasmids that can be packaged into λ phage and they combine essential elements of a plasmid and λ systems (cos sites).
- Fragments from 30 to 46 kb can be accommodated by a 5 kb cosmid vector.



DNAخارجی وارد ناحیه ی MCS میشه و بعد کاسمید با

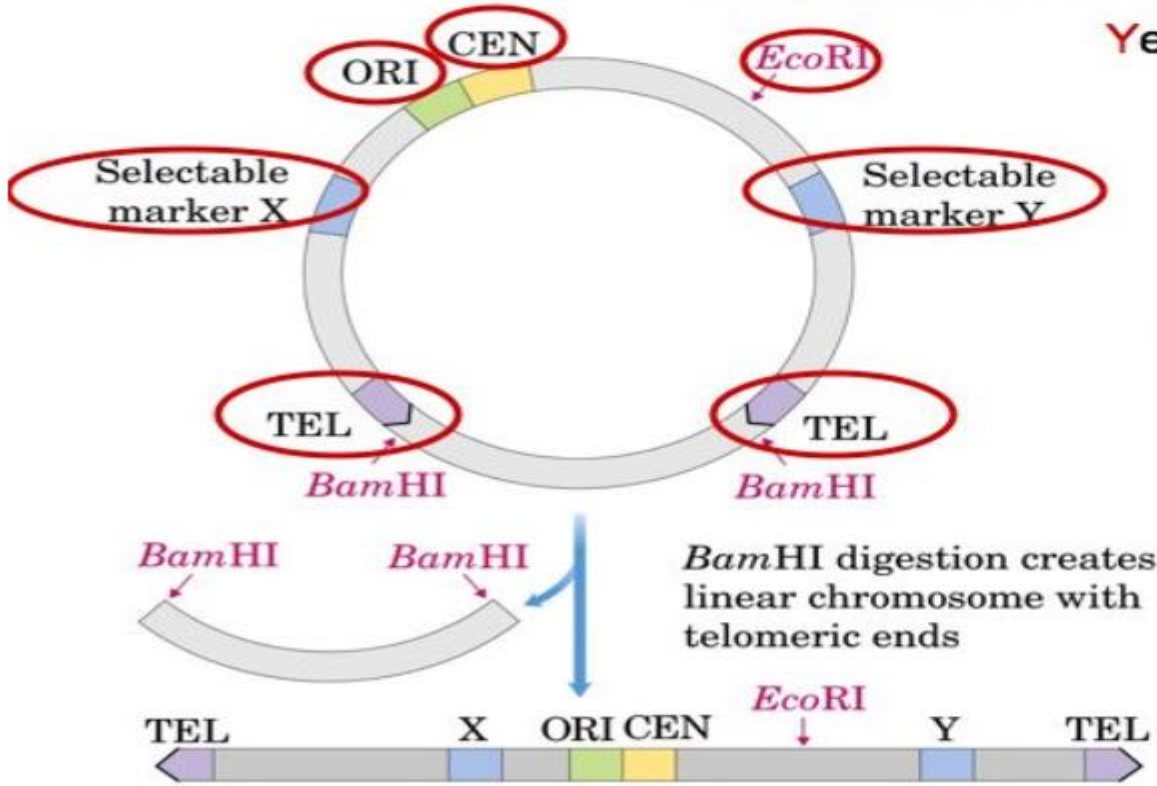
AP endonucleaseبرش داده میشه و ژنوم های خطی ایجاد میشه که

با روش بسته بندی در in vitro فاژ کامل حاوی کاسمید خطی

(به جای ژنوم فاژی خطی) تولید میشه که قابلیت ورود به میزبان رو داره اما قادر به بیمار کردن میزبان نیست.

Yeast Artificial Chromosomes as Cloning Vectors

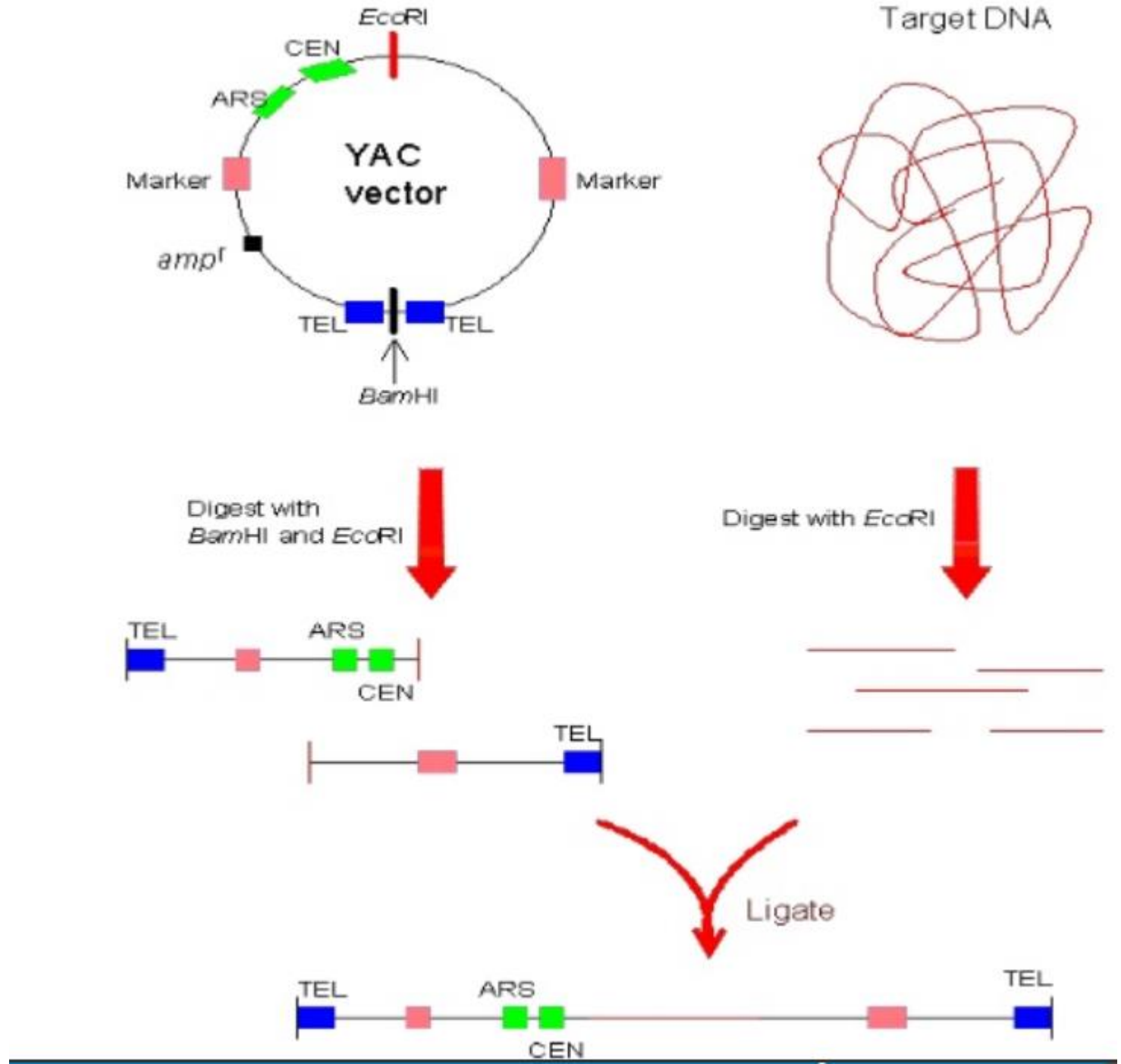
Artificial Chromosomes allow for cloning of large pieces of DNA



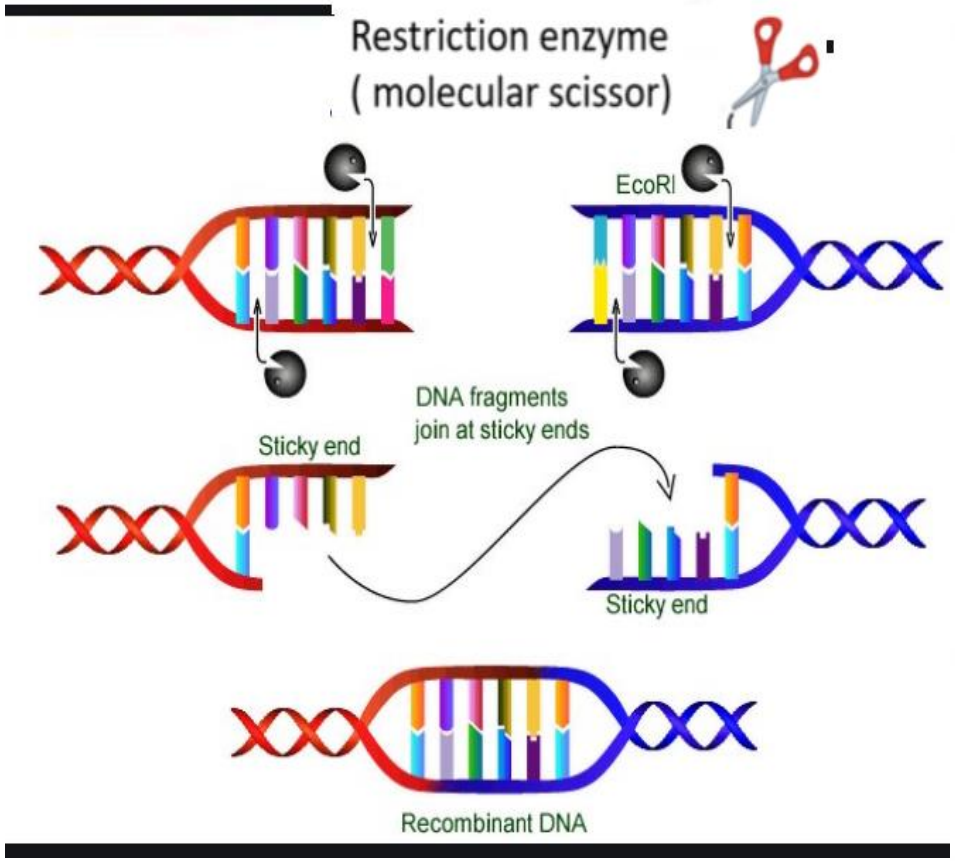
Yeast Artificial Chromosome

1. *ori* allows for replication in bacteria (generate more DNA for cloning)
2. *cen* helps segregate YAC evenly between daughter cells
3. TEL needed for stability and proper replication of YAC
4. X & Y allow for selection of transformed cells
5. EcoR1 provides cloning site

- The amount of DNA that can be cloned into a YAC is, on average, from 200 to 500 kb.
- However, as much as 1 Mb (mega, 106) can be cloned into a YAC.


EcoRI
CEN
ARS
YAC
vector
Marker
Marker
ampr
TEL
TEL
BamHI
Target DNA
Digest with
BamHI and EcoRI
Digest with EcoRI
TEL
ARS
CEN
TEL
Ligate
TEL
ARS
TEL
CEN

RESTRICTION ENDONUCLEASE (RE)



- Also called restriction enzymes
- 1962: "Molecular scissors" discovered in bacteria
- *E. coli* bacteria have an enzymatic immune system that recognizes and destroys foreign DNA
- 3,000 enzymes have been identified, around 200 have unique properties, many are purified and available commercially
- RE: Molecular scissors that cut double stranded DNA molecules at specific points.
- An important tool for manipulating DNA

Werner Arbor, Hamilton Smith and Daniel Nathans shared the 1978 Nobel prize for Medicine and Physiology for their discovery of Restriction Enzymes.

Nomenclature:

Named for bacterial genus, species, strain, and type:

Example: *HinD*II (1st RE isolated)

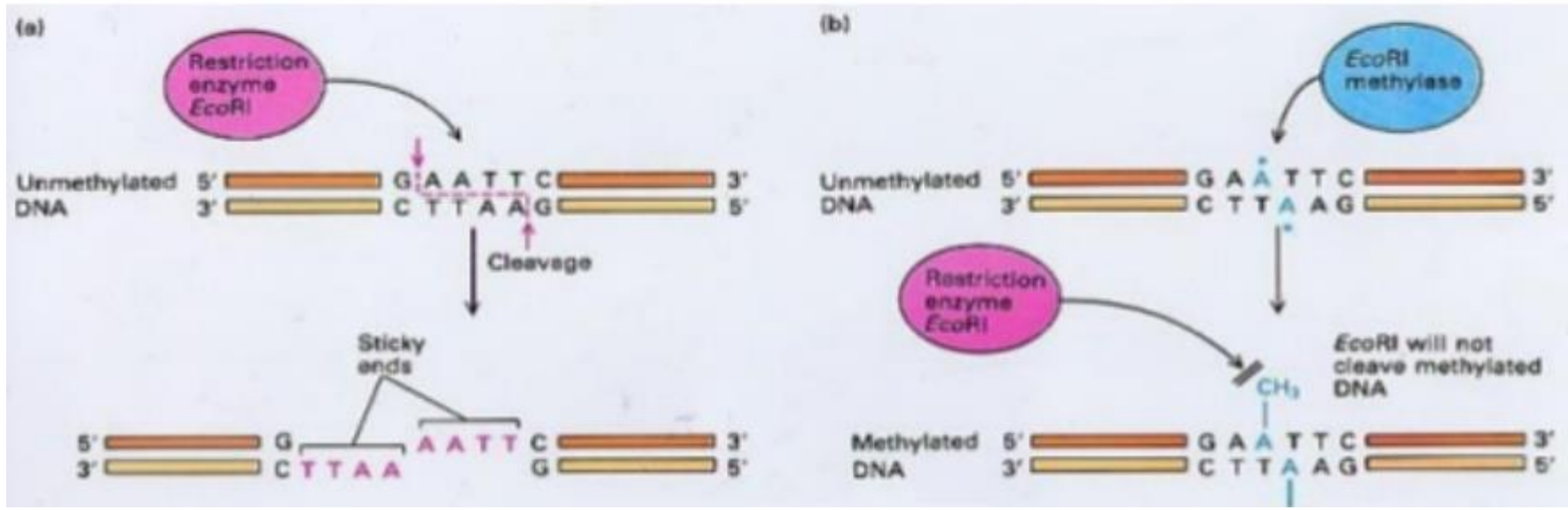
Genus: *Haemophilus*
Species: *influenzae*
Strain: D
Order discovered: II

Example: *EcoR*I

Genus: *Escherichia*
Species: *coli*
Strain: R
Order discovered: I

Biological Role:

- Most bacteria use RE as a defence against bacteriophages.
- REs prevent the replication of the phage by cleaving its DNA at specific sites.
- Restriction Modification System: REs are paired with methylases.
- Methylases are enzymes that add methyl groups to specific nucleotides within the recognition sequence. The methylation prevents recognition by the RE. Therefore, the RE within a cell doesn't destroy its own DNA.



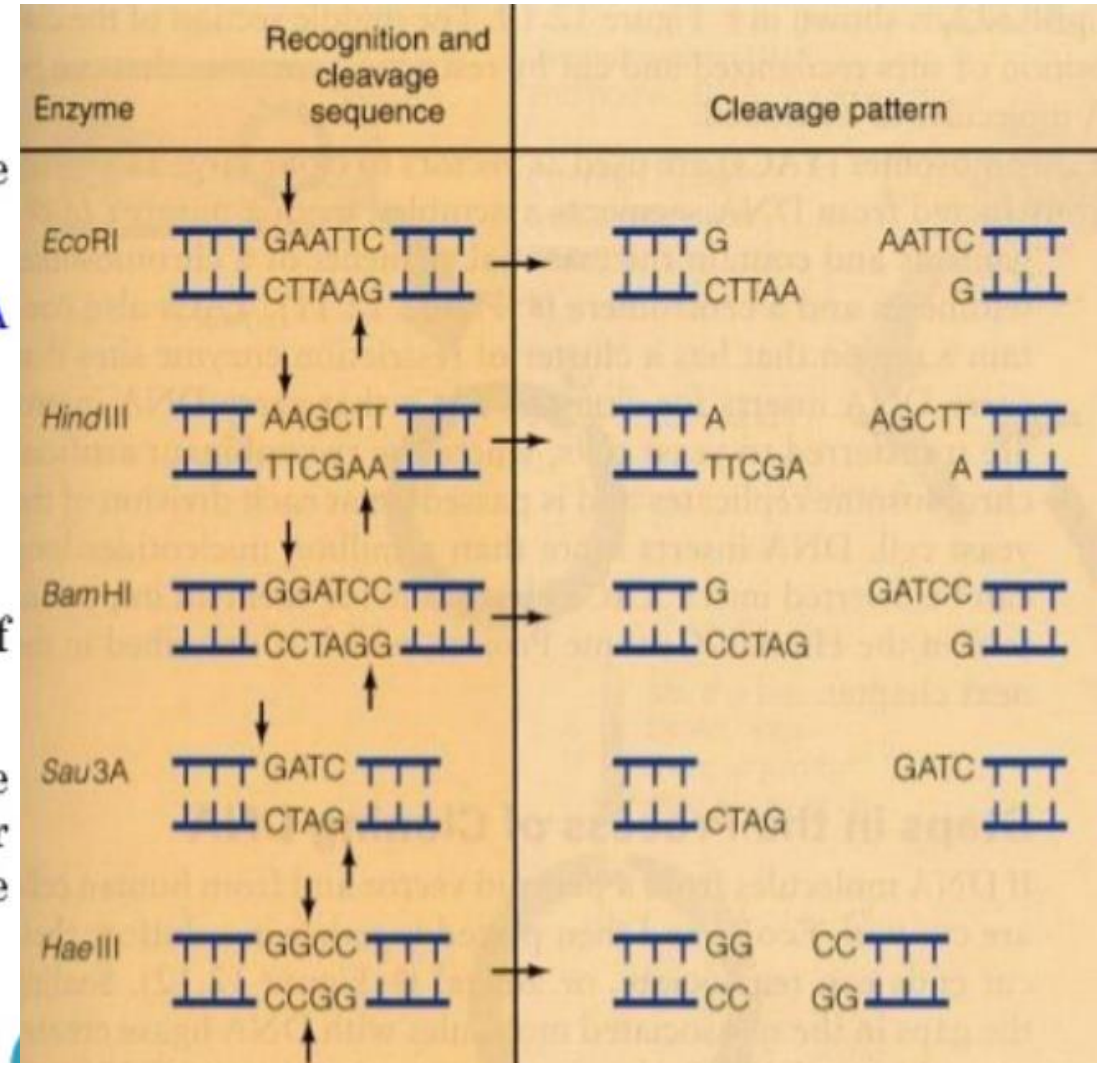
- RE are bacterial enzymes that recognize specific 4-8 bp sequences, called restriction sites and cleaves both the DNA strands at this site (site specific).

- They cleave the DNA within the molecule, hence, endonucleases.

- RE has 3 functions:
  - Recognition
  - Cleavage
  - Modification

Type II Restriction endonucleases:

- Simple enzymes having separate endonuclease and methylase activities
- Recognise a specific nucleotide sequence and cut a DNA molecule at this site and nowhere else
- Mostly recognise a hexanucleotide sequence
- Very stable and only require Mg2+ as cofactor
- Many REs make a simple double stranded cut in the middle of the sequence and result in 'blunt ends'
- Other REs do not cut both strands of the DNA at the same position and result in 'staggered end' or 'cohesive ends' or 'sticky ends'. Base pairing between these ends can stick the DNA fragments back together again.
- This type is used for gene cloning.



Digestion Conditions

- *Xba*I
  - Buffer 2: (10 mM Tris-HCl, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, pH 7.9).
  - 100 µg/ml BSA (optional)
  - 1 Unit digest 1 µg DNA
  - Incubate at 37°C for 1 hour
  - Heat inactivate 65° for 20 min

20 µl reaction:

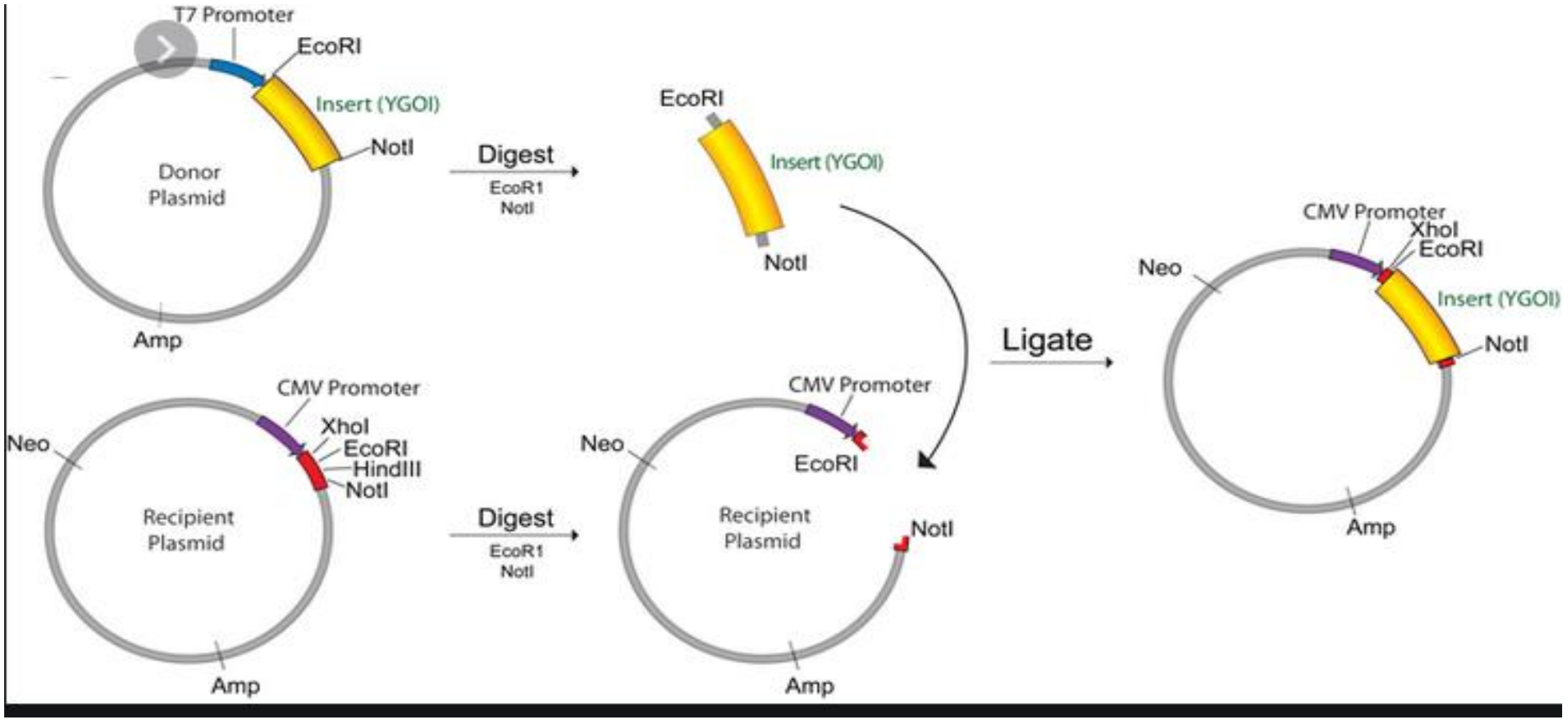
10 µl DNA (~1 µg total)
7 µl water
2 µl 10X reaction buffer
1 µl RE 10 units/µl

Incubate 1 hour at appropriate temperature

Note:

1. 10 fold excess enzyme ensures complete digestion.
2. Enzyme should never exceed 1/10th of reaction volume.
3. BSA is often recommended because it stabilizes the enzyme.

Double Digestion for directional cloning



DNA LIGASE

- During replication DNA ligase catalyses the formation of 3' – 5' phosphodiester bonds between the short fragments of the lagging strand of DNA in the replication fork.
- In rDNA technology, purified DNA ligase is used to covalently join the ends of the restriction fragments *in vitro*.
- This enzyme catalyzes the formation of 3' – 5' phosphodiester bond between the 3'OH– end of one restriction fragment and the 5' phosphate end of another restriction fragment.
- The process is called ligation.

کاربرد DNA لیگاز در مهندسی ژنتیک

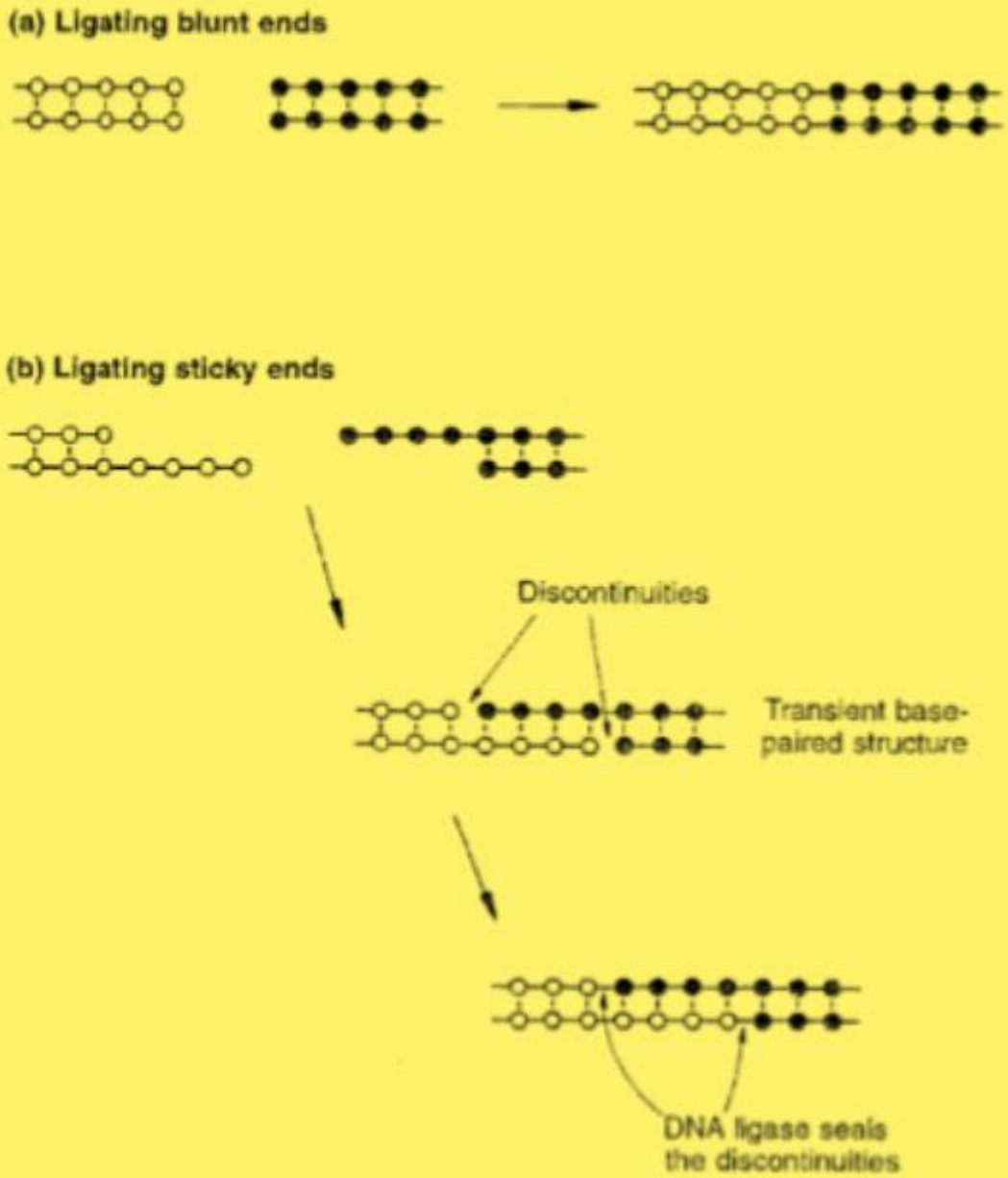
- 1 ligation of cohesive ends:

2 ligation of blunt ended termini:

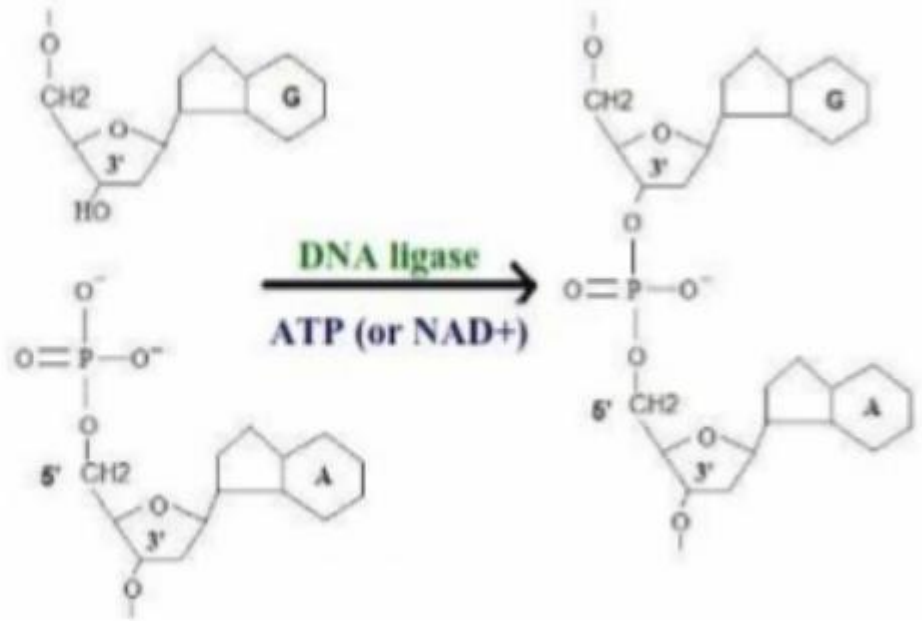
this reaction is much slower than ligation of sticky ends and the ligation is improved by addition of monovalent cation and low concentration of PEG

.3 Ligation of synthetic linkers or adapter

reactions catalysed by DNA ligase: (a) ligation of blunt-ended molecules; (b) ligation of sticky-ended molecules.



DNA Ligase – enzyme catalysing formation of phosphodiesteric bound between 3'-OH group of one end of DNA molecule and 5'-phosphate group of the second end of DNA



Ligase cofactors

1. ATP
   - DNA ligases of bacteriophages (phage T4, T7)
   - DNA ligases of mammals
2. NAD+
   - DNA ligases of bacteria (*Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*)

- T4 DNA ligase can ligate sticky as well as blunt ends
- *E. coli* DNA ligase can ligate only blunt ends

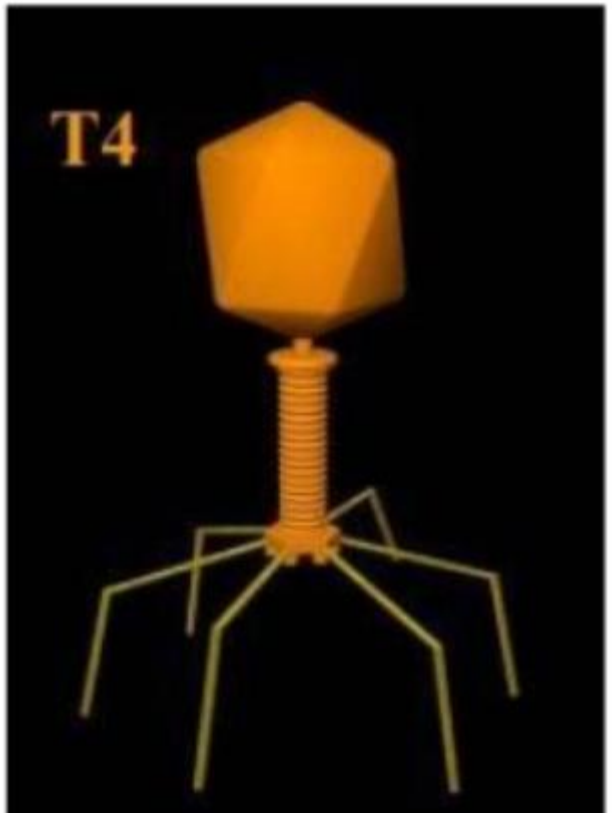
Ligase of phage T4

- Requires ATP as a co-factor
- Optimal pH (7.2-7.8)
- Requires bivalent ions (Mg2+, Mn2+) and reducing factors (ß mercaptoethanol or ditiotreitol)
- Inhibitors: polyamines (spermin, spermidine), high concentration of ions (Na+, K+, Li+, NH4+)
- Can connect both cohesive and blunt ends (but for blunt ends reaction is slower and requires higher concentrations of enzyme)

Typical ligation reaction:

COMPONENT	20 µl REACTION
T4 DNA Ligase Buffer (10X)*	2 µl
Digested Vector DNA (4 kb)	50 ng (0.020 pmol)
Digested Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

Incubate at 16°C overnight or room temperature for 2 hours



Inactivation of T4 ligase:
Heat to 70°C for 10 minutes.

What should be optimized for a successful ligation:

1. The ratio of the molar concentration of vector to insert.

- Optimum ratios may vary from 8:1 to as high as 1:16 vector:insert, though generally fall in the range of 3:1 to 1:3.

2. Amount of DNA.

- Usually 10-200 ng of plasmid is used for reaction.

3. Volume of reaction.

- Usually a minimal volume is recommended (e.g. 10 µl).

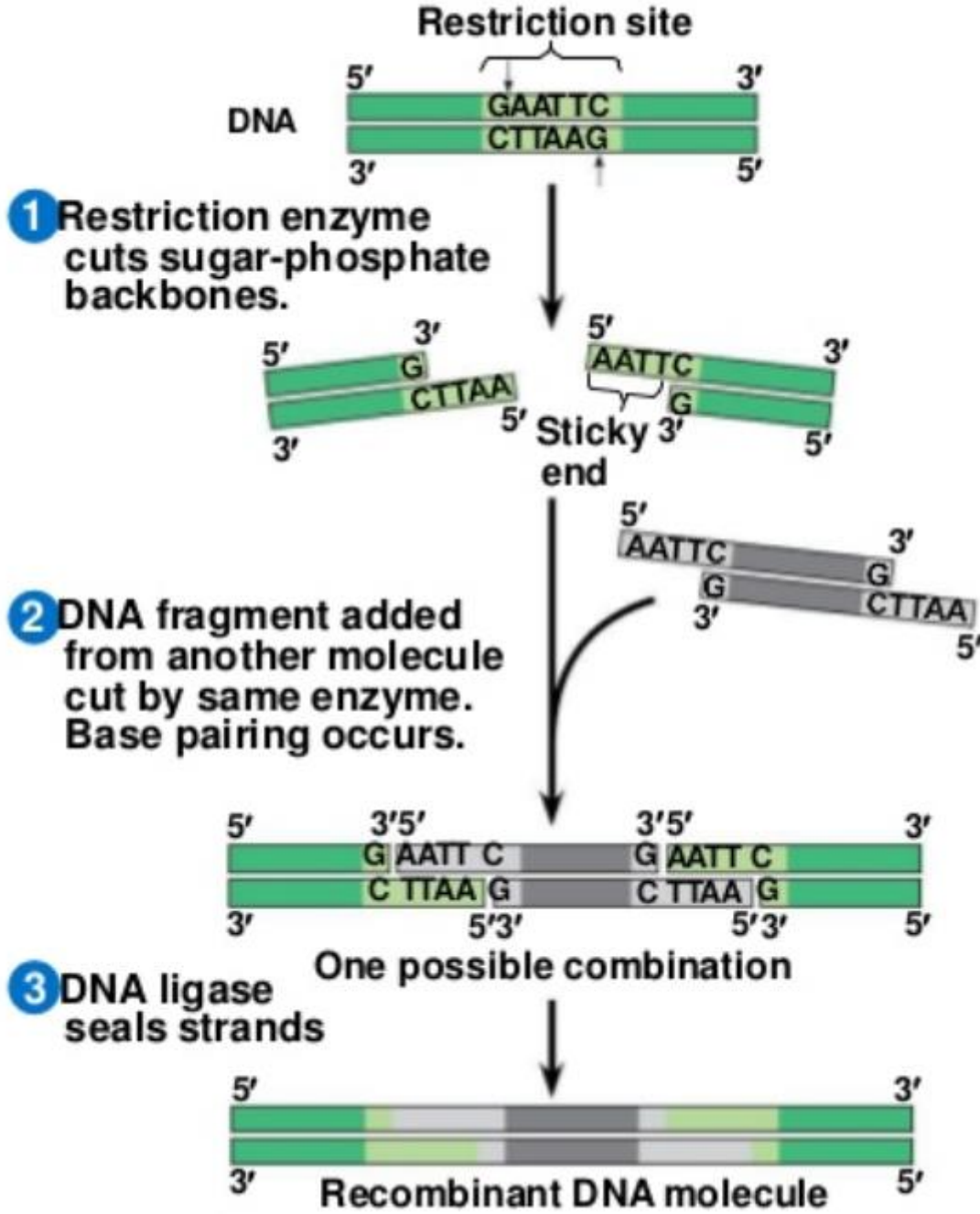
4. Amount of ligase.

- Each ligation reaction generally requires 1-10 units of high quality ligase.

5. Incubation time and temperature.

The ligation incubation time and temperature may also need to be optimized. In general:

- blunt-ended ligations are performed at 4°C overnight;
- - sticky-end ligations are performed for 1-3 hours (at 22°C or 16°C) or overnight at 4°C.
- In general, ligation reactions performed at lower temperatures require longer incubation times.



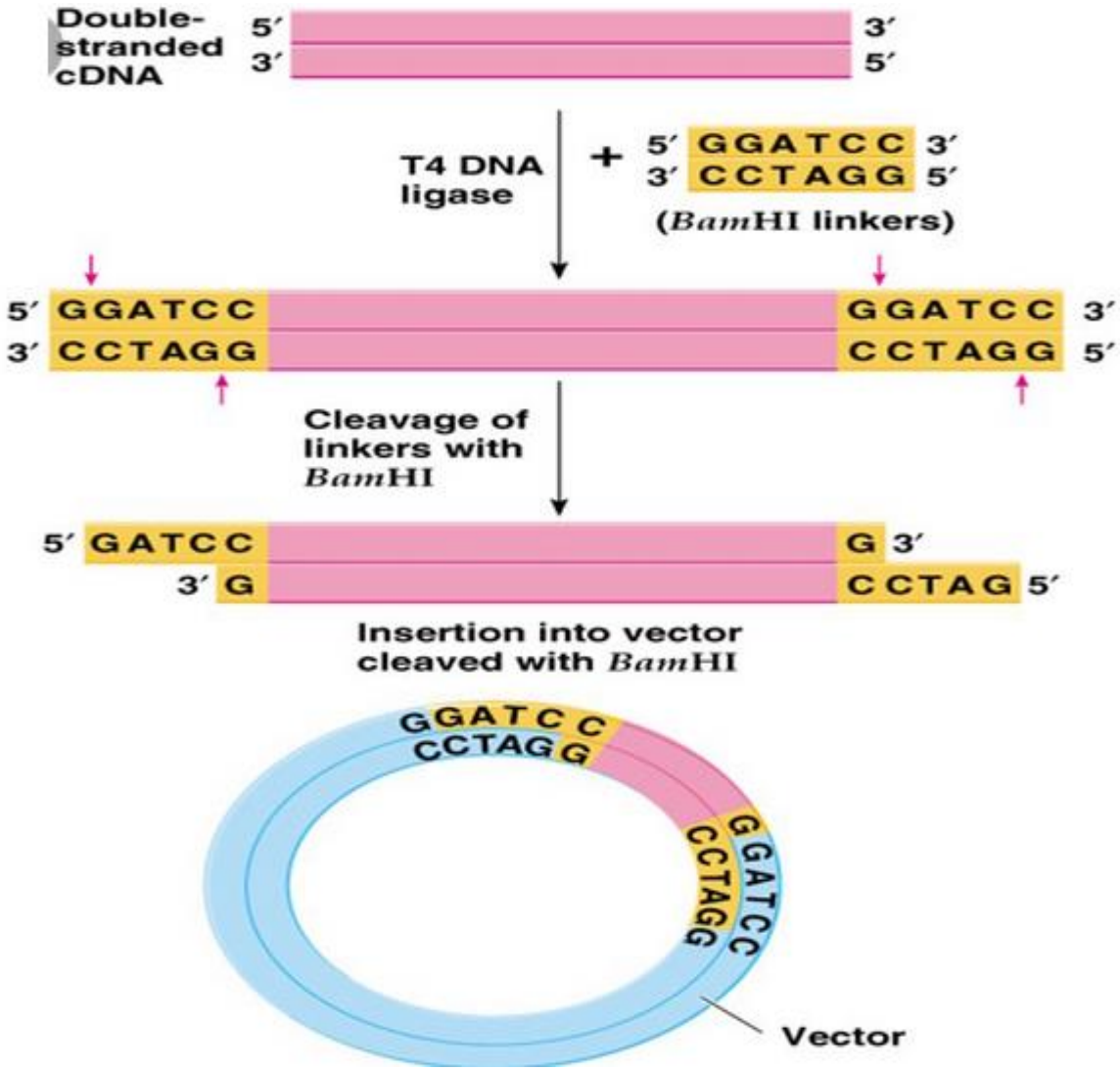
Blunt end ligation

- Mainly three methods can be used to put the correct sticky ends onto the DNA fragments-

1. Cloning foreign DNA by adding linkers
2. Cloning foreign DNA by adding adaptors
3. Homopolymeric tail adding by using Terminal transferase enzyme.

1. Linker

- Linkers are the chemically synthesized double stranded DNA oligonucleotides containing on it one or more restriction sites for cleavage by restriction enzymes, e.g. Eco RI, Hind III, Ban HI, etc.
- Linkers are ligated to blunt end DNA by using DNA ligase.
- Both the vector and DNA are treated with restriction enzyme to develop sticky ends.
- The staggered cuts i.e. sticky ends are then ligated with T4 DNA ligase with very high efficiency to the termini of the vector and recombinant plasmid DNA molecules are produced.

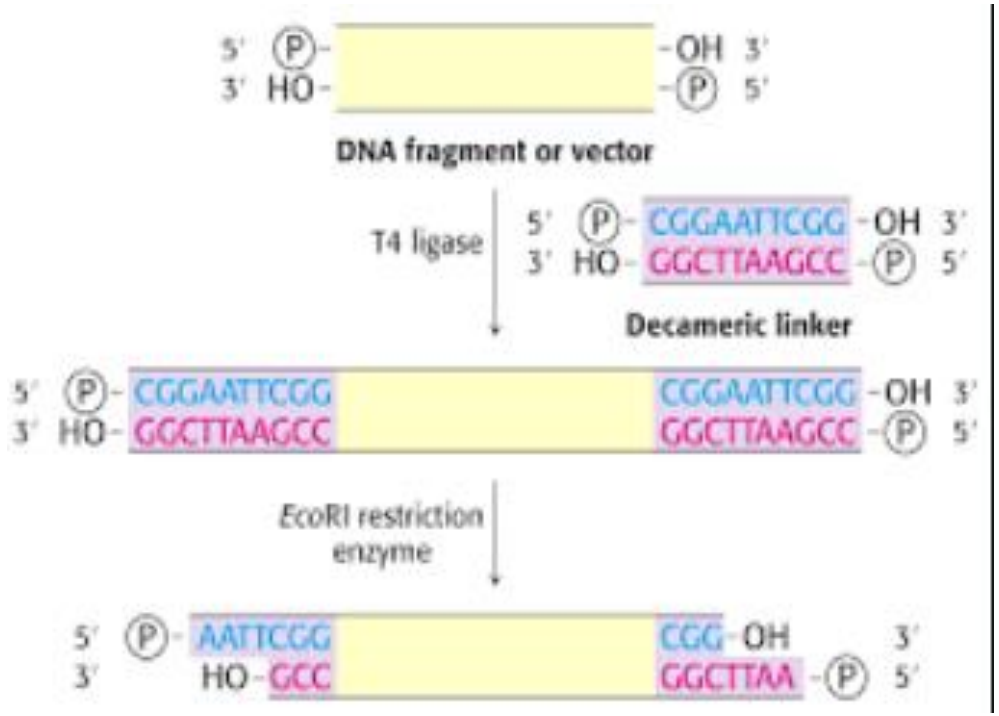


Limitations

- It may be the case that the restriction enzyme used to generate the cohesive ends in the linker will also cut the foreign DNA at internal sites.

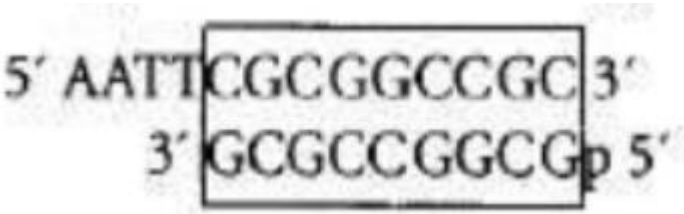
Solution: CHOOSE ANOTHER RESTRICTION ENZYME

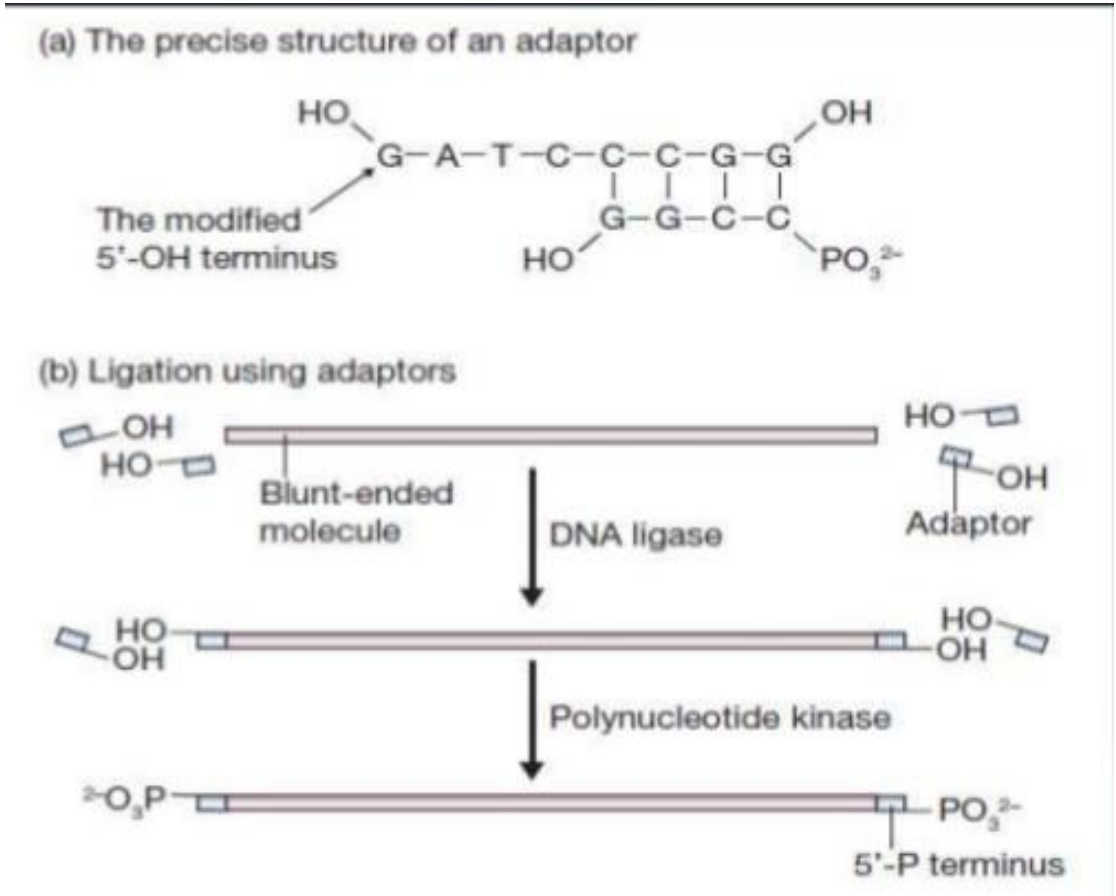
But there may not be a suitable choice if the foreign DNA is large and has sites for several restriction enzymes.



2. Adaptors

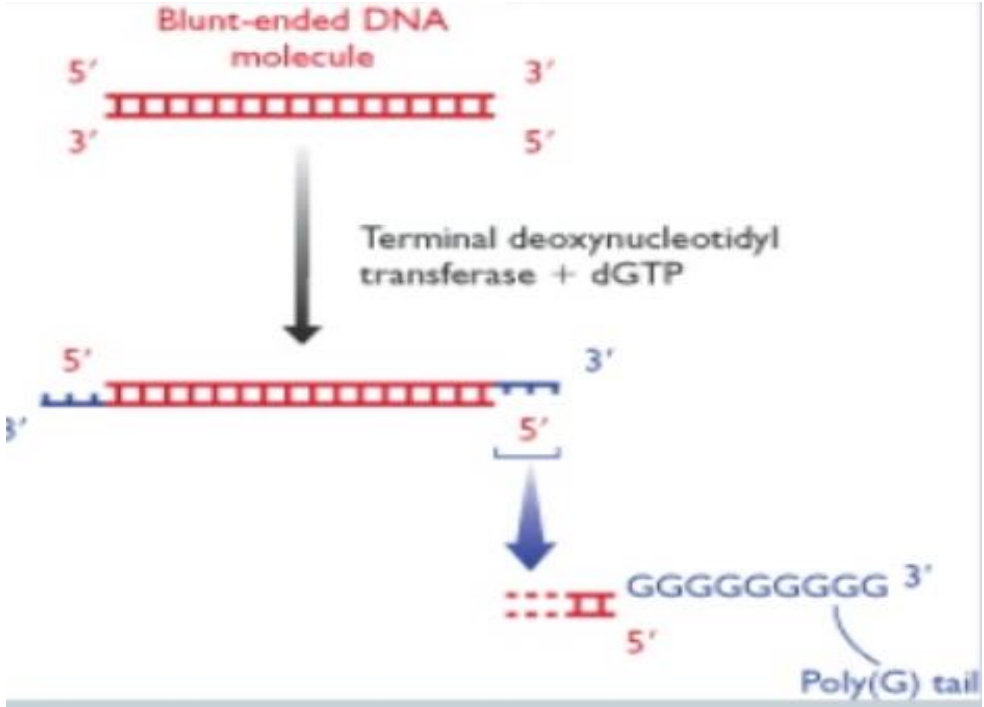
- They are also short double stranded oligonucleotides that carry an internal RE sites and single stranded tails at one or both ends.
- This protruding sequences can be ligated to DNA fragments containing a complementary single stranded terminus.
- After ligation, the DNA can be cleaved with appropriate RE to create new protruding terminus.





3. Homopolymeric tailing

- It is a technique by which sticky ends can be produced on a blunt-ended DNA molecule.
- In a homopolymer, all the subunits are same. A DNA strand made up entirely of deoxyguanosine is an example of homopolymer, and is referred to as polydeoxyguanosine or poly(dG).
- Tailing involves using the enzyme terminal deoxynucleotidyl transferase to add a series of nucleotides on to the 3'-OH termini of a double-stranded DNA molecule.
- The reaction when carried out in the presence of just one deoxynucleotide, then a homopolymer tail will be produced.



- TdT catalyses the addition of nucleotides to the 3' terminus of a DNA molecule. Unlike most DNA polymerases, it does not require a template.
- The preferred substrate of this enzyme is a 3'-overhang but it can also add nucleotides to blunt or recessed 3' ends.
- Cobalt is a necessary cofactor, however the enzyme catalyzes reaction upon Mg and Mn administration *in vitro*.

- For ligation of two tailed molecules, the homopolymers must be complementary. Frequently poly(dc) tails are attached to the vector and poly(dg) to the DNA to be cloned.