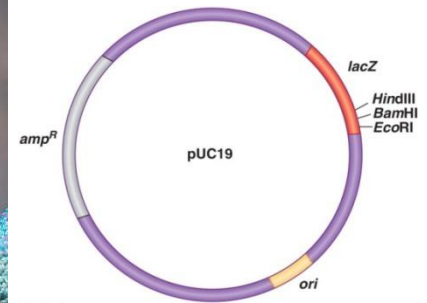


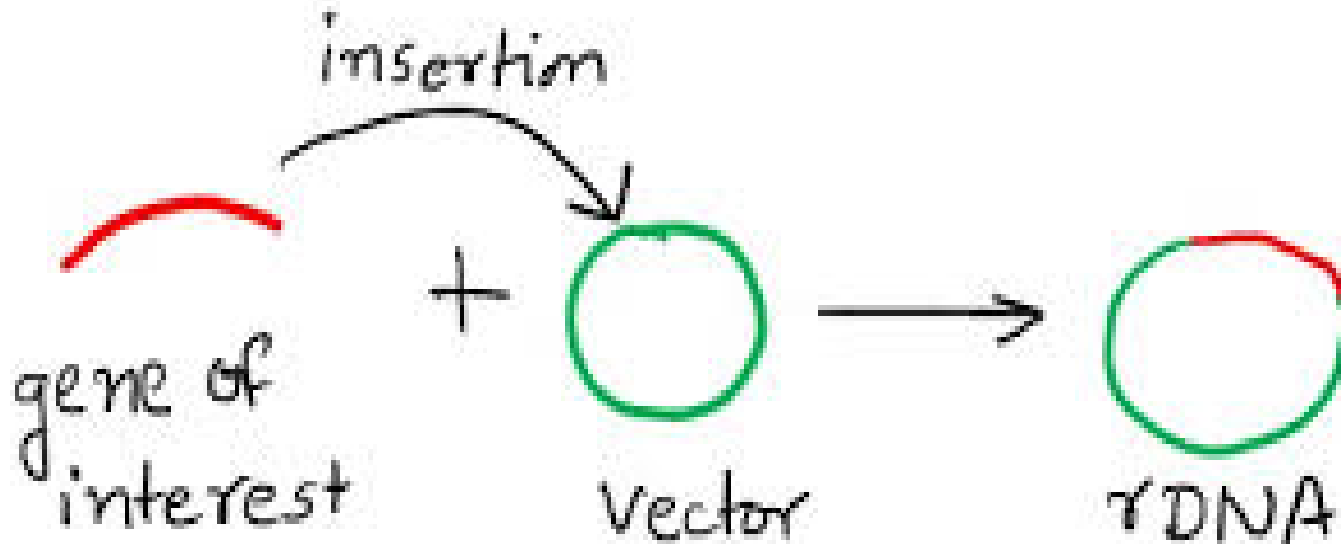
Recombinant DNA Technology

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Recombinant DNA Technology...

Definition: It is a technology of joining together of DNA molecules from two different species that are inserted into a host organism to produce new genetic combinations that are of value to science, medicine, agriculture, and industry.



Cont.....

- ▶ Since the focus of all genetics is the gene, the fundamental goal of laboratory geneticists is to isolate, characterize, and manipulate genes.
- ▶ Although it is relatively easy to isolate a sample of DNA
- ▶ Consider the fact that each cell contains approximately 2 meters (6 feet) of DNA.
- ▶ Therefore, a small tissue sample will contain many kilometers of DNA.
- ▶ However, recombinant DNA technology has made it possible to isolate one gene or any other segment of DNA, enabling researchers to determine its nucleotide sequence, study its transcripts, mutate it in highly specific ways, and reinsert the modified sequence into a living organism.

History of Genetic Engineering/Recombinant DNA technology

In conjunction with his studies of the tumor virus SV40, in **1972, Paul Berg succeeded in inserting DNA from a bacterium into the virus' DNA.** He thereby created the first DNA molecule made of parts from different organisms. This type of molecule became known as "hybrid DNA" or "recombinant DNA". Among other things, Paul Berg's method opened the way to creating bacteria that produce substances used in medicines.



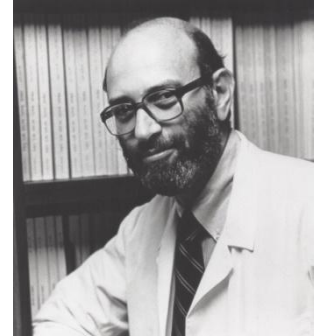
Paul Berg is the "father of genetic engineering".

History of Recombinant DNA technology

In 1973, **Herbert Boyer**, of the University of California at San Francisco, and **Stanley Cohen**, at Stanford University, reported the construction of functional organisms that combined and replicated genetic information from different species. Their experiments dramatically demonstrated the potential impact of DNA recombinant engineering on medicine and pharmacology, industry and agriculture.

Boyer and Cohen's achievement represented an advance upon the ingenious techniques developed by Paul Berg, in 1972, for inserting viral DNA into bacterial DNA. It was a creative synthesis of earlier research that made use of:

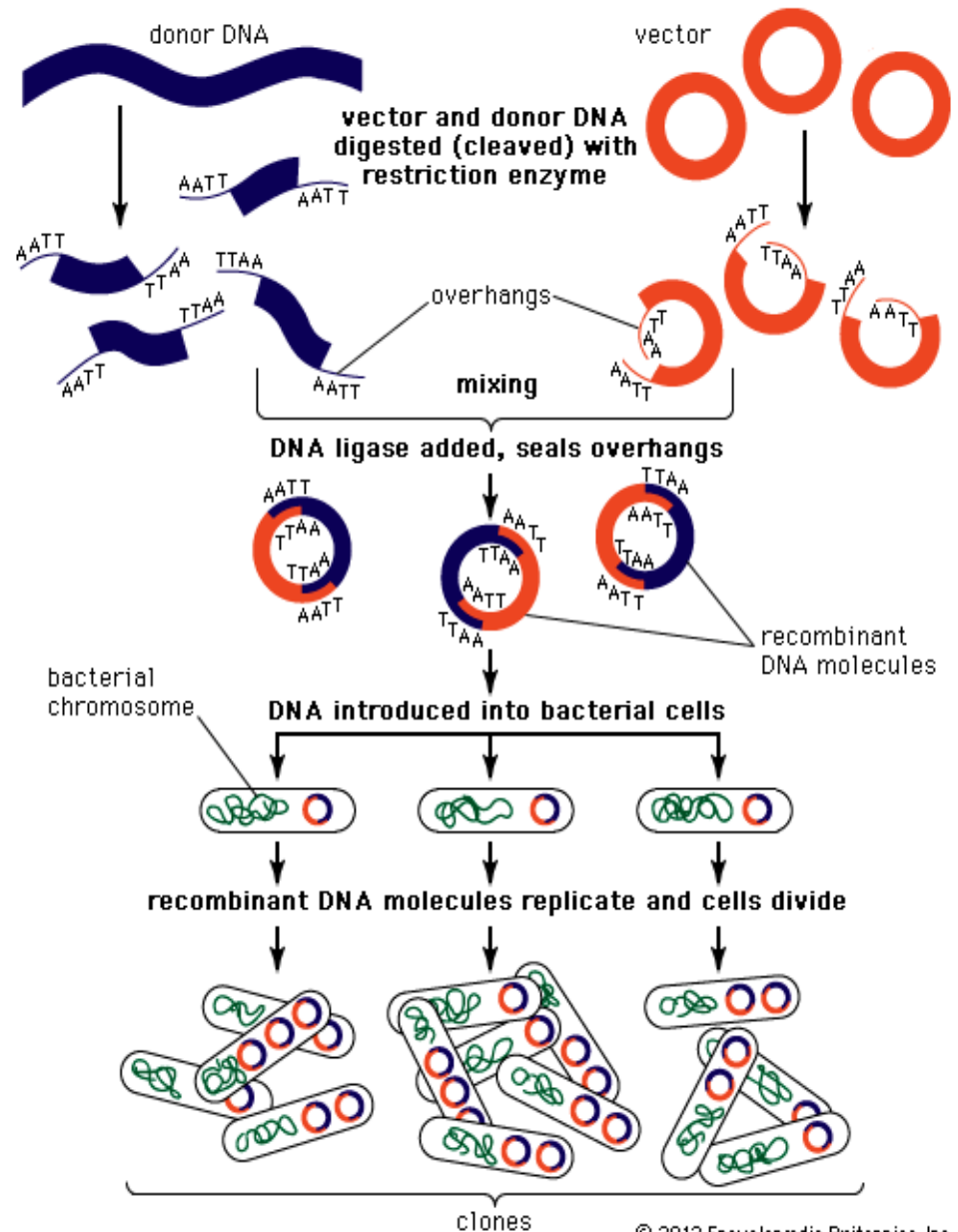
- ✓ **Living organisms able to serve as carriers for genes from another organism.**
- ✓ **Enzymes to cleave and rejoin DNA fragments that contain such genes.**
- ✓ **DNA molecules from one organism precisely targeted and manipulated for insertion into the DNA of another organism.**



Basic Principle of Recombinant DNA Technology

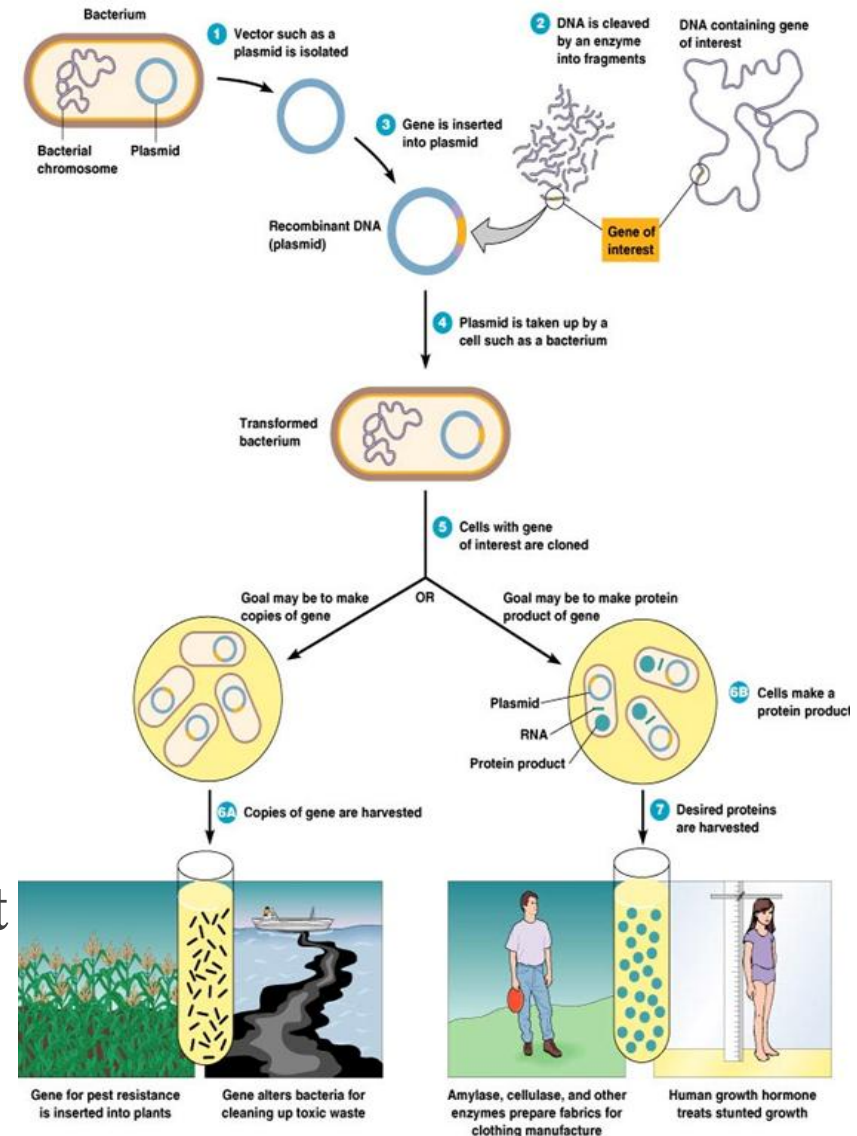
The DNA is inserted into another DNA molecule called vector.

The recombinant vector is then introduced into a host cell where it replicates itself and multiple copy of gene produced.



Recombinant DNA Technologies

1. **Gene of interest (DNA)** is isolated
(DNA fragment)
2. A **desired gene** is inserted into a DNA molecule - **vector**
(plasmid, bacteriophage or a viral genome)
3. After entering the host cell, vector grown/replicate to form a **clone**.
(bacteria, yeast, plant or animal cell)
4. Large quantities of the **gene product** can be harvested from the clone.



Important Tools for Genetic Engineering/ Recombinant Technology

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graph TD; A[Important Tools for Genetic Engineering/ Recombinant Technology] --- B[Restriction Enzymes]; A --- C[Ligase]; A --- D[Vectors]; A --- E[Suitable host]
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**Restriction
Enzymes**

Ligase

Vectors

**Suitable
host**

Restriction Enzymes

Naturally produced by bacteria – Restriction Endonucleases

- ▶ **Natural function** - destroy bacteriophage DNA in bacterial cells
- ▶ Cannot digest host DNA with methylated C (cytosine)

Restriction Endonucleases (RE): Endonucleases are enzymes that produce internal cuts called cleavages in DNA molecules. A class of endonucleases that cleaves/cut DNA only within or near those sites which have specific base sequences, such endonucleases are known as restriction endonucleases or restriction enzymes and sites recognized by them are called recognition sequences or recognition sites. There are three types of RE.

Type I Restriction Endonuclease

Type II Restriction Endonuclease

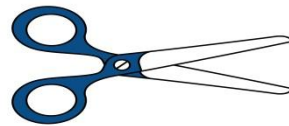
Type III Restriction Endonuclease

Type II RE

–They recognize a specific nucleotide sequence (recognition site) and cut a DNA molecule within that sequence (breaks down the bond between two nucleotides).

–Most of the enzymes recognize hexanucleotide (6bp) sequence but enzymes with 4, 5 and 8 bp recognition site are also present.

–Some enzymes produced blunt ends while some produce 5' or 3' staggered sticky ends.

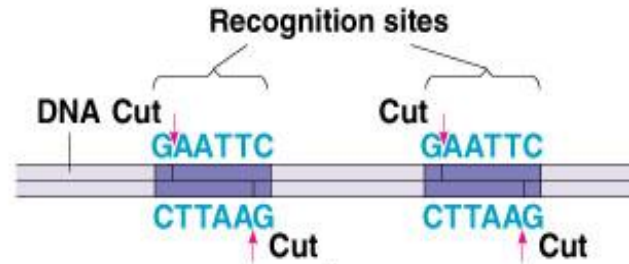


Selected Restriction enzymes used in rDNA technology

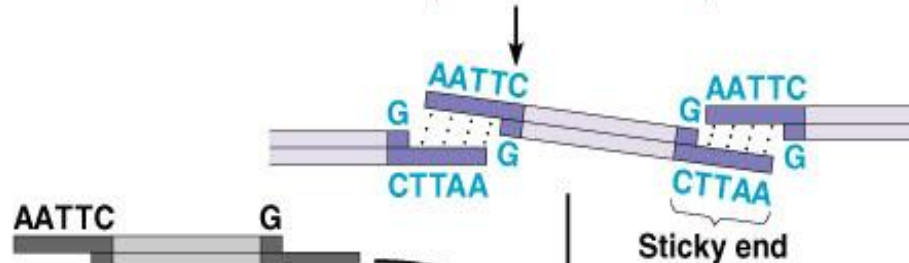
Enzyme	Bacterial Source	Recognition Sequence
<i>Bam</i> HI	<i>Bacillus amyloliquefaciens</i>	G↓G A T C C G C T A G↑G
<i>Eco</i> RI	<i>Escherichia coli</i>	G↓A A T T C C T T A A↑G
<i>Hae</i> III	<i>Haemophilus aegyptius</i>	G G↓C C C C↑G G
<i>Hind</i> III	<i>Haemophilus influenzae</i>	A↓A G C T T T T C G A↑A

How RE works in Recombinant DNA Technology?

- 1 Restriction enzyme cuts (magenta arrows) double-stranded DNA at its particular recognition sites, shown in blue.



- 2 These cuts produce a DNA fragment with two sticky ends.



- 3 When two such fragments of DNA cut by the same restriction enzyme come together, they can join by base pairing.

DNA from another source, perhaps a plasmid, cut with the same restriction enzyme



- 4 The joined fragments will usually form either a linear molecule or a circular one, as shown here for a plasmid. Other combinations of fragments can also occur.



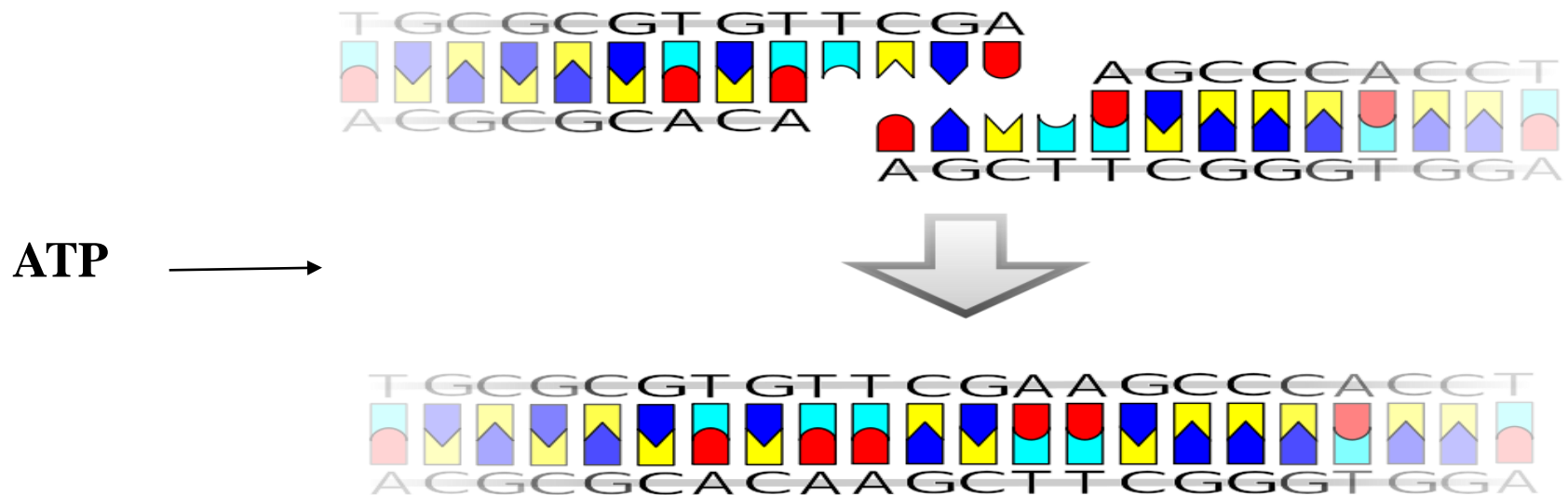
- 5 The enzyme DNA ligase is used to unite the backbones of the two DNA fragments, producing a molecule of recombinant DNA.



Recombinant DNA

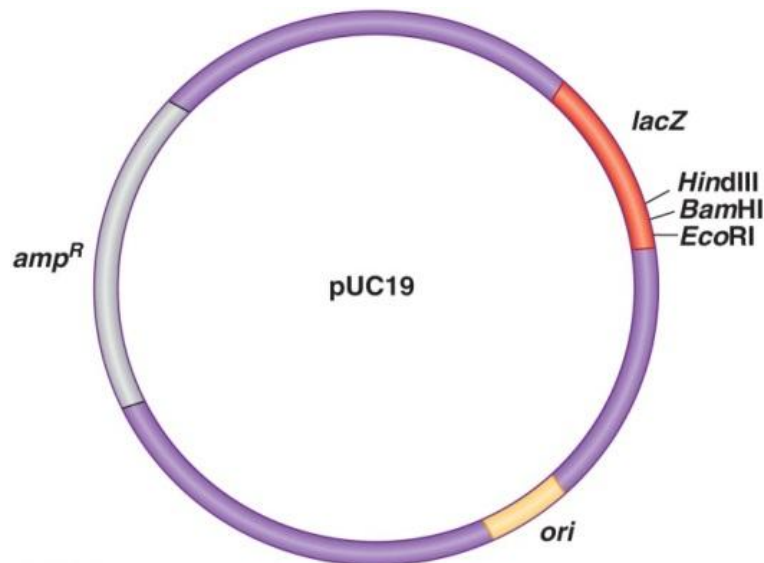
Ligase

- **DNA ligase** is an enzyme that can link together DNA strands that have double-strand breaks (a break in both complementary strands of DNA).
 - Naturally DNA ligase has applications in both **DNA replication** and **DNA repair**.
 - Needs ATP
- DNA ligase has extensive use in molecular biology laboratories for **genetic recombination experiments**



Vectors

“A vector is a DNA molecule that has the ability to replicate autonomously in an appropriate host cells and serve as a vehicle that carry DNA fragment or insert to be cloned.” Therefore, a vector must have an origin of DNA replication (*ori*) that functions in the host cell. Any extra-chromosomal small genome eg. Plasmid, Phage or virus may be used as a vector.



Vector Element	Description
Origin of Replication (ORI)	DNA sequence which allows initiation of replication within a plasmid by recruiting replication machinery proteins
Antibiotic Resistance Gene	Allows for selection of plasmid-containing bacteria.
Multiple Cloning Site (MCS)	Short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA. In expression plasmids, the MCS is often downstream from a promoter.
Insert	Gene, promoter or other DNA fragment cloned into the MCS for further study.
Promoter Region	Drives transcription of the target gene. Vital component for expression vectors: determines which cell types the gene is expressed in and amount of recombinant protein obtained.
Selectable Marker	The antibiotic resistance gene allows for selection in bacteria. However, many plasmids also have selectable markers for use in other cell types.
Primer Binding Site	A short single-stranded DNA sequence used as an initiation point for PCR amplification or sequencing. Primers can be exploited for sequence verification of plasmids.

Properties of Good Vectors

- Able to replicate autonomously
- Small in size
- Easy to isolate and purify
- Easy transformation into host cell
- Suitable marker gene to allow easy detection and selection
- Multiple cloning site
- **Gene Transfer:** integrate DNA insert in host genome
- **Expression:** Suitable control/regulatory elements

Cloning vs. Expression Vector

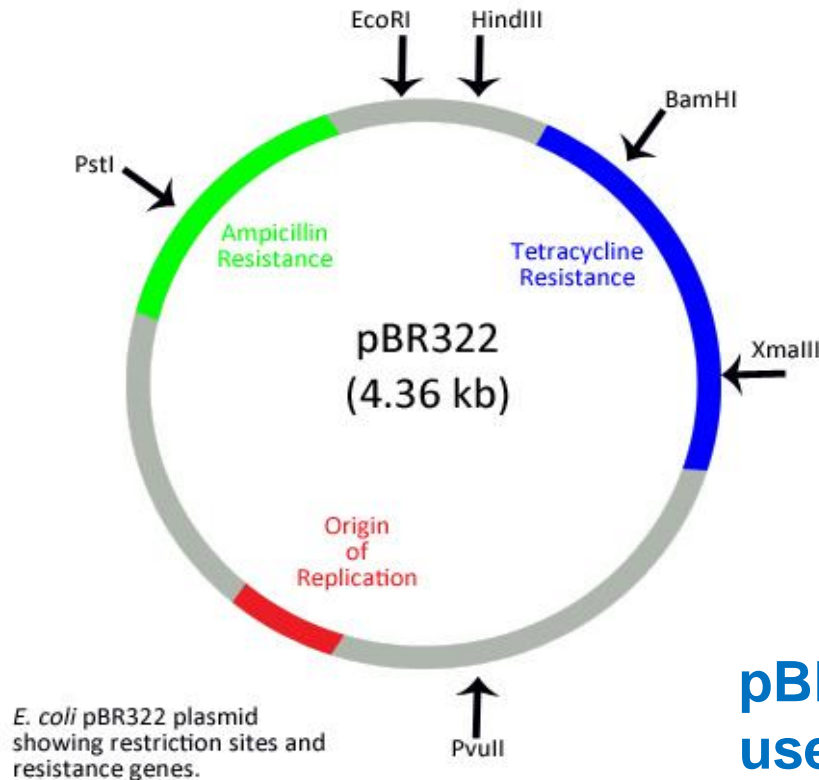
- **Cloning vector:** vector used for propagation of DNA inserts in a suitable host are called cloning vector.
- **Expression vector:** designed for expression of DNA insert i.e. production of protein specified by inserted DNA, it is termed as expression vector

Type of Vectors

- Plasmid vector
- Bacteriophage vector
- Cosmid vector
- Phagemid vector
- Phasmid vector
- BAC
- YAC
- PAC

Plasmid Vector

- Many different *E. coli* plasmids are used as vectors. The natural plasmid have been modified, shortened, reconstructed and recombined both in vitro and in vivo to create plasmid of enhance utility and specific functions.
- Examples: **pBR322**, **pUC18/19**, **pGEM3Z**, **pTZ57R/T**



pBR322: One of the first widely used *E. coli* cloning vectors

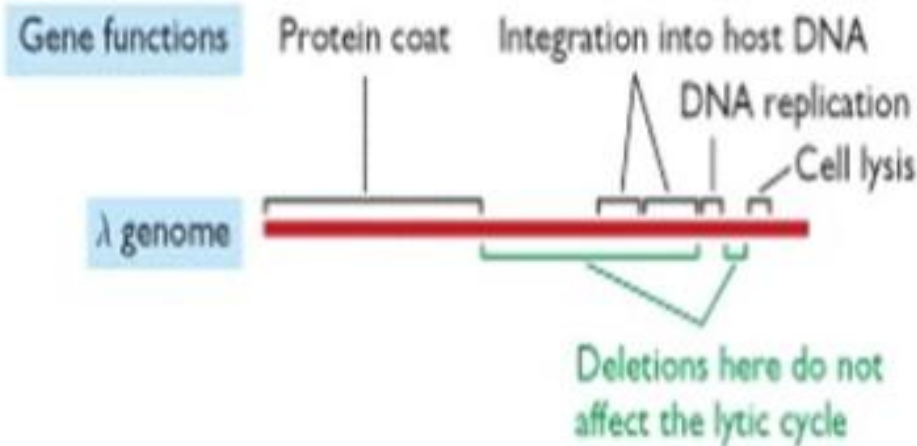
Bacteriophage vectors

- Bacteriophage are viruses that attack bacteria. Several bacteriophage are used as cloning vectors. The most commonly used E. coli phages are λ (lambda) and M13 phage.
- **Advantage over plasmid**
 - **More efficient for cloning large DNA fragment (approx. 24 kb)**
 - **Transformation of host cell is easier with phage particle**
- Examples: λ gt10, λ gt11, λ EMBL4, M13mp8, M13m9



Insertion & Replacement λ Vector

(A) The λ genome contains 'optional' DNA



(B) Insertion and replacement vectors



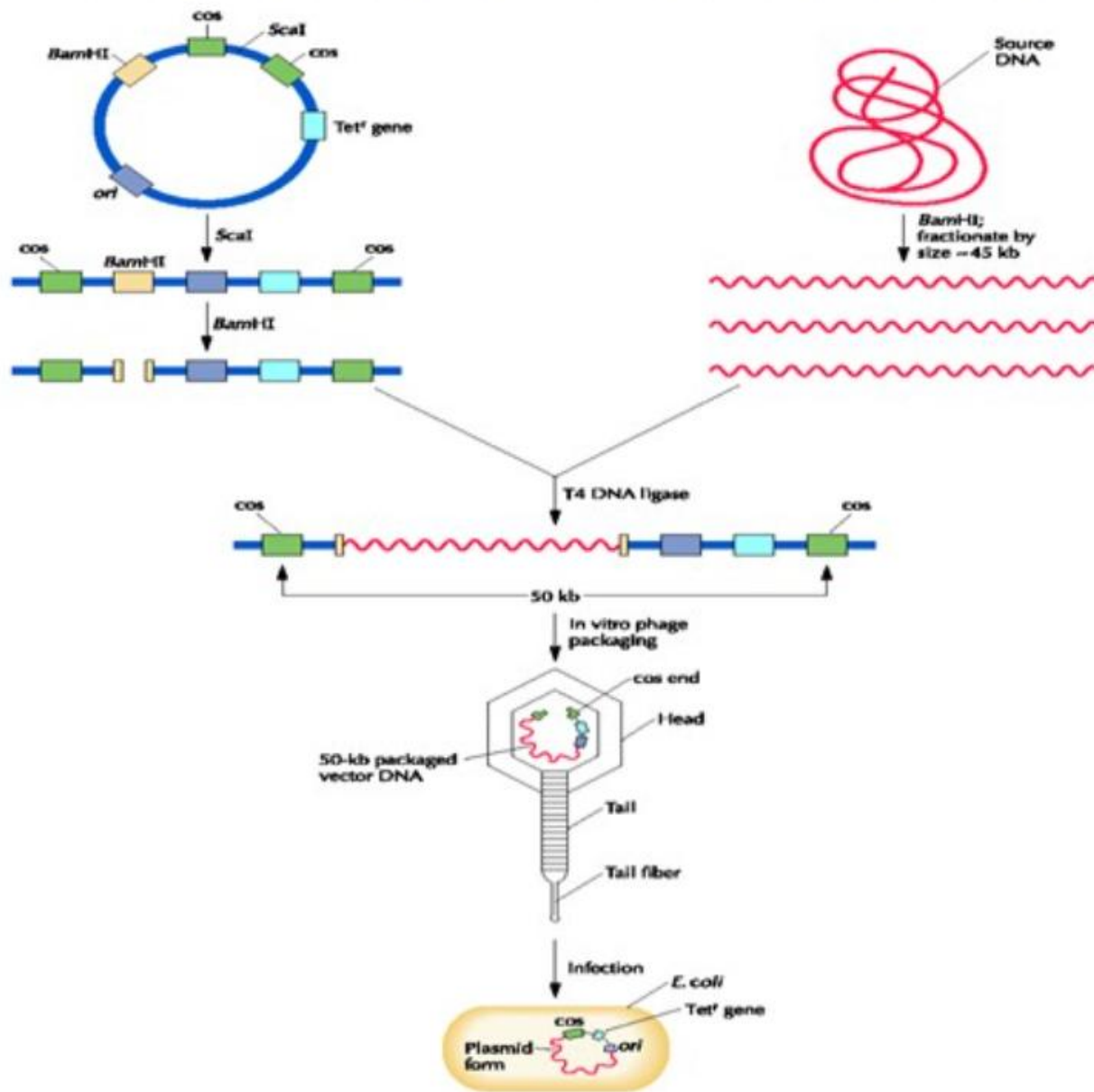
Cosmid

- ◆ Hybrid of plasmid/Phage vectors
- ◆ contain E coli **ori** that allow it to be maintained as a plasmid in the cell and carries a λ **cos site**.
- ◆ a plasmid that carries a λ **cos site**.
- ◆ **cos sites**, act as substrates for *in vitro* packaging
- ◆ because the **cos site is the only sequence that a DNA molecule needs** in order to be recognized as a ' λ genome' by the proteins that package DNA into λ phage particles.

Example: SupercosI, pJB8, pWEB,

Cosmid can accommodate upto 40 kb DNA insert

Cosmid Cloning System



λ *cos* sites inserted into a small plasmid

Target DNA ligated between two *cos* DNA molecules

Recombinant DNA packaged and *E. coli* Infected as before

Can clone DNAs up to 45 kb

Cosmid vectors

◆ *Advantages:*

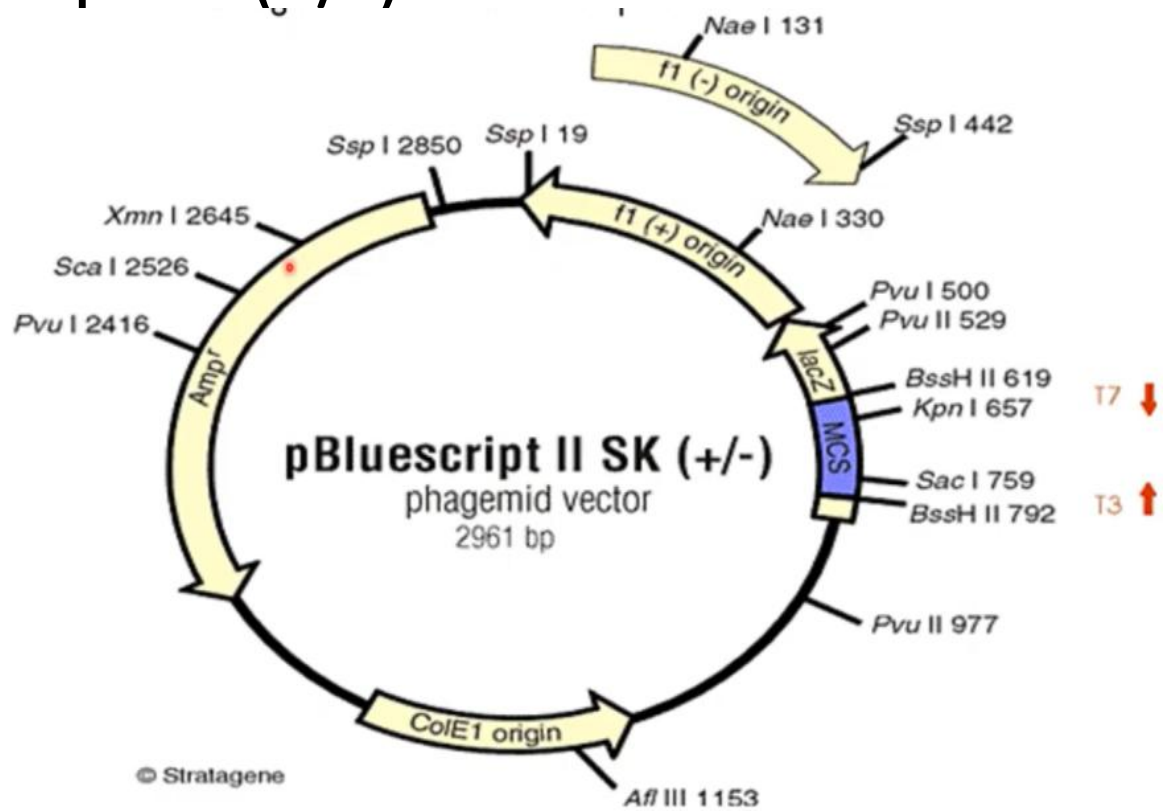
- Useful for cloning very large DNA fragments (32 - 47 kbp)
- Inherent size selection for large inserts
- Handle like plasmids

◆ *Disadvantages:*

- Not easy to handle very large plasmids (~ 50 kbp)

Phagemid Vectors

- A plasmid vector that contain origin of replication from a phage virus, in addition to that of the plasmid is called phagemid.
- Example: pBluescript SK(+/-)



Phagemid vectors

- ◆ **plasmids** which contain a small segment of the genome of a **filamentous phage**, such as M13, fd or f1.
- ◆ contain all the ***cis*-acting elements** required for DNA replication and assembly into phage particles.
- ◆ They permit successful cloning of inserts **several kilobases long**.

- ◆ Following transformation of a suitable *E. coli* strain with a recombinant phagemid,
- ◆ the bacterial cells are *superinfected* with a filamentous **helper phage**, such as f1, **which is required to provide the coat protein.**
- ◆ Phage particles secreted from the superinfected cells will be a mixture of helper phage and recombinant phagemids.

- ◆ The mixed single-stranded DNA population can be used directly for DNA sequencing.
- ◆ because the primer for initiating DNA strand synthesis is designed to bind specifically to a sequence of the phagemid vector adjacent to the cloning site.
- ◆ Commonly used phagemid vectors include the **pEMBL series** of plasmids and the **pBluescript** family.

Phasmid Vector

- λ bacteriophage insertion vector in which their middle non essential segment is replaced by linear plasmid with intact replication module and lac Z gene.
- Examples: λ ZAPII, λ ZAP Express

Bacterial Artificial Chromosome (BAC) & Yeast Artificial Chromosome (YAC)

YAC vector means Yeast Artificial Chromosome vectors.	BAC vector means Bacterial Artificial Chromosome Vectors.
Developed by Burke and Olson in 1987	Developed Melsimon et al 1992
<ul style="list-style-type: none"> • Can replicate inside yeast • Used to clone DNA sequences in yeast cells 	<ul style="list-style-type: none"> • Can replicate inside bacteria • used to clone DNA sequences in bacterial cells
<p>Basic Components of YAC vector</p> <ul style="list-style-type: none"> • ARS (Autonomously Replicating Sequence) – Ori; origin of replication for Yeast • CEN (Centromeric) –yeast centromere for movement of YAC vectors to daughter cells • 2 TEL (Telomeric) -Telomeric region for behaving as yeast chromosome on linearization 	<ul style="list-style-type: none"> • Basic components of BAC vector(pBAC108L first developed BAC vector) • OriS – ori for bacteria <i>repE</i> – initiation & assembly of the replication complex • <i>parA</i> and <i>parB</i> – movement of BAC to daughter cells • Selectable marker –Chloramphenicol Resistance for selection of transformed cells

Bacterial Artificial Chromosome (BAC) & Yeast Artificial Chromosome (YAC)

<p>Its construction is based on regions of yeast chromosome like ARS</p>	<p>BAC is a DNA construct, based on functional fertility plasmid (or F-plasmid), of <i>E. coli</i>.</p>
<ul style="list-style-type: none"> • Linear vector inside yeast cell with 2 telomeric ends • just functions as yeast chromosomes without disturbing original chromosomes 	<p>Circular vector inside the bacterium</p>
<p>Clone DNA fragments larger than 100 kb and up to 3,000 kb</p>	<p>Insert size is 150–350 kb.</p>
<ul style="list-style-type: none"> • It is used for physical mapping and sequencing of genomes. YAC libraries of human and <i>Caenorhabditis elegans</i> and many genomes • First used in Human genome project later shifted to BAC vectors due to stability issues with YAC 	<ul style="list-style-type: none"> • It is used for physical mapping and sequencing of genomes. • Used in HGP and in physical genetic maps of including <i>Mus musculus</i>, <i>Arabidopsis thaliana</i>, and <i>Homo sapiens</i>.
<p>Advantage of YAC: large insert size</p>	<p>Advantage of BAC: More stable than YACs, and easy to manipulate compared to YAC</p>

Hosts for DNA Recombinant Technology

1. Bacteria

- *E. coli* - used because is easily grown and its genomics are well understood. Gene product is purified from host cells

2. Yeasts - *Saccharomyces cerevisiae*

- ▶ Used because it is easily grown and its genomics are known
- ▶ May express eukaryotic genes easily
- ▶ Easily collected and purified

3. Plant cells and whole plants

- ▶ May express eukaryotic genes easily
- ▶ Plants are easily grown - produce plants with new properties.

4. Mammalian cells

- ▶ May express eukaryotic genes easily
- ▶ Harder to grow
- ▶ Medical use.

5. Fish Cell Lines

Processes of Recombinant DNA Technology

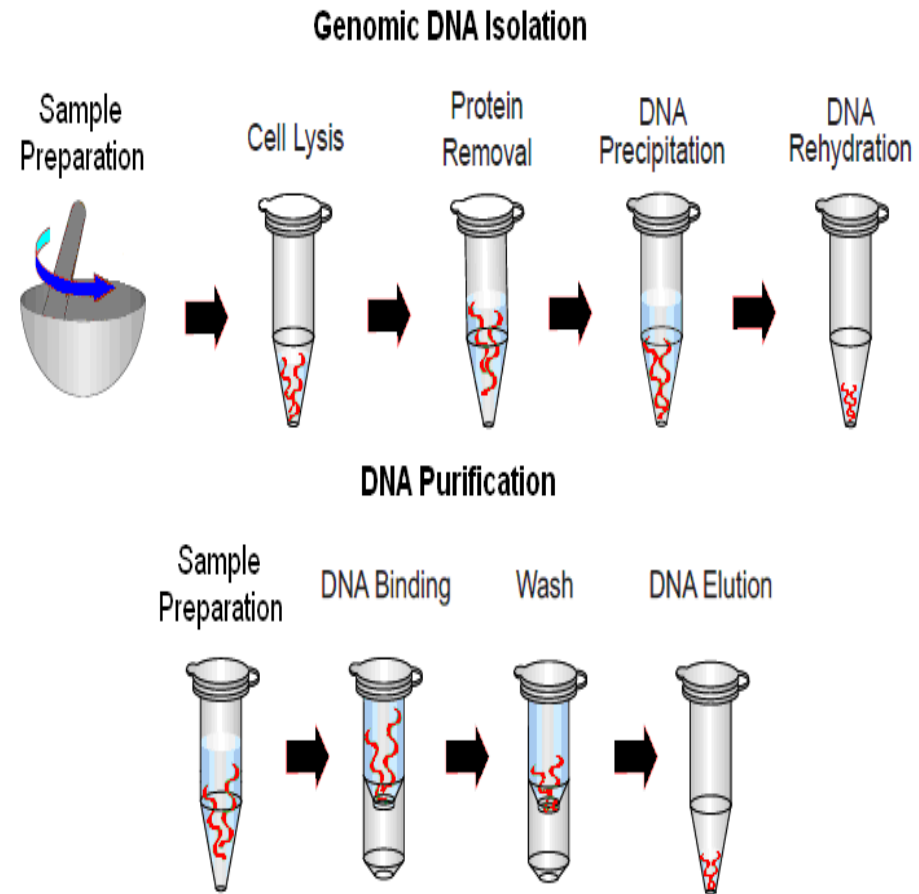
(Has five steps)

- Isolation of DNA
- Cutting of DNA at specific location
- Amplification of gene of interest using PCR
- Insertion of Recombinant DNA into Host
- Obtaining the foreign gene product

Isolation of DNA

DNA isolation is a process of purification of DNA from sample using a combination of physical and chemical methods. The basic steps in a DNA extraction are as below:

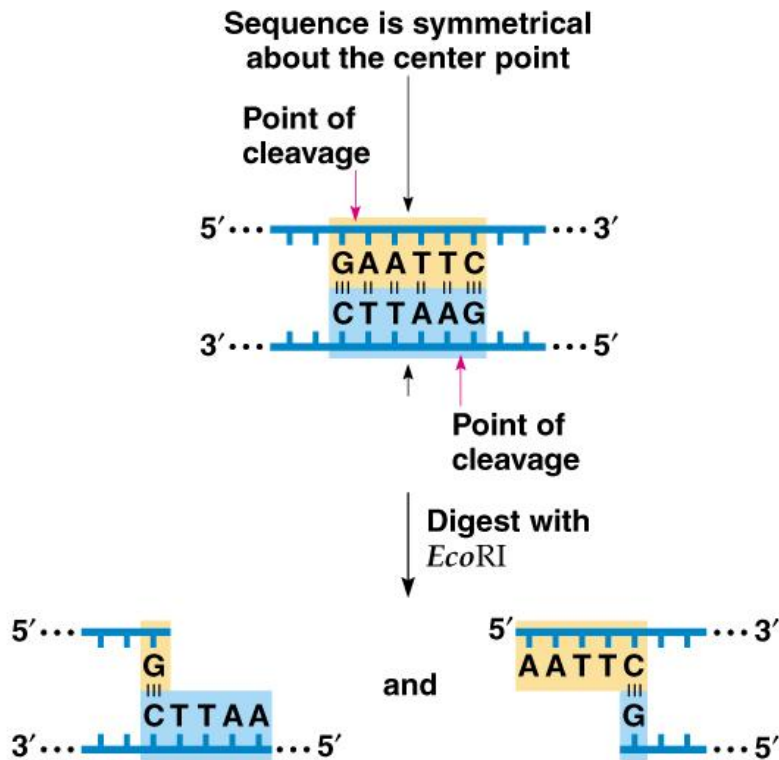
- Cells which are to be studied need to be collected.
- Breaking the cell membranes to expose the DNA along with the cytoplasm - Cell Lysis.
- Lipids from the cell membrane and the nuclear membranes are broken down with detergents.
- Breaking proteins by adding an Proteinase
- After centrifugation of the sample, denaturated proteins stay in the organic phase while aqueous phase containing nucleic acid



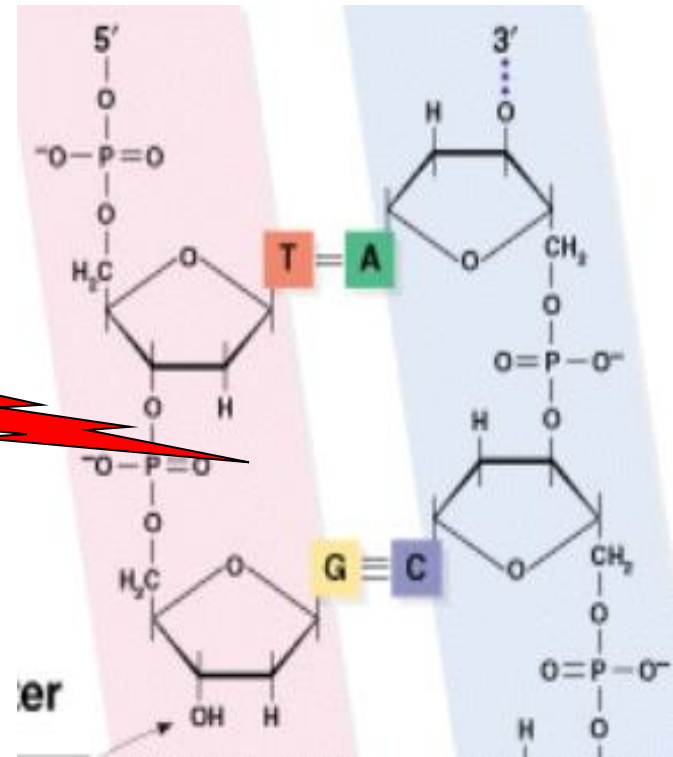
Cut DNA with restriction enzymes

Restriction enzymes (endonuclease) recognize specific bases pair sequences in DNA called restriction sites and cleave the DNA by hydrolyzing the phosphodiester bond.

- ✓ Restriction fragment ends have 5' phosphates & 3' hydroxyls.

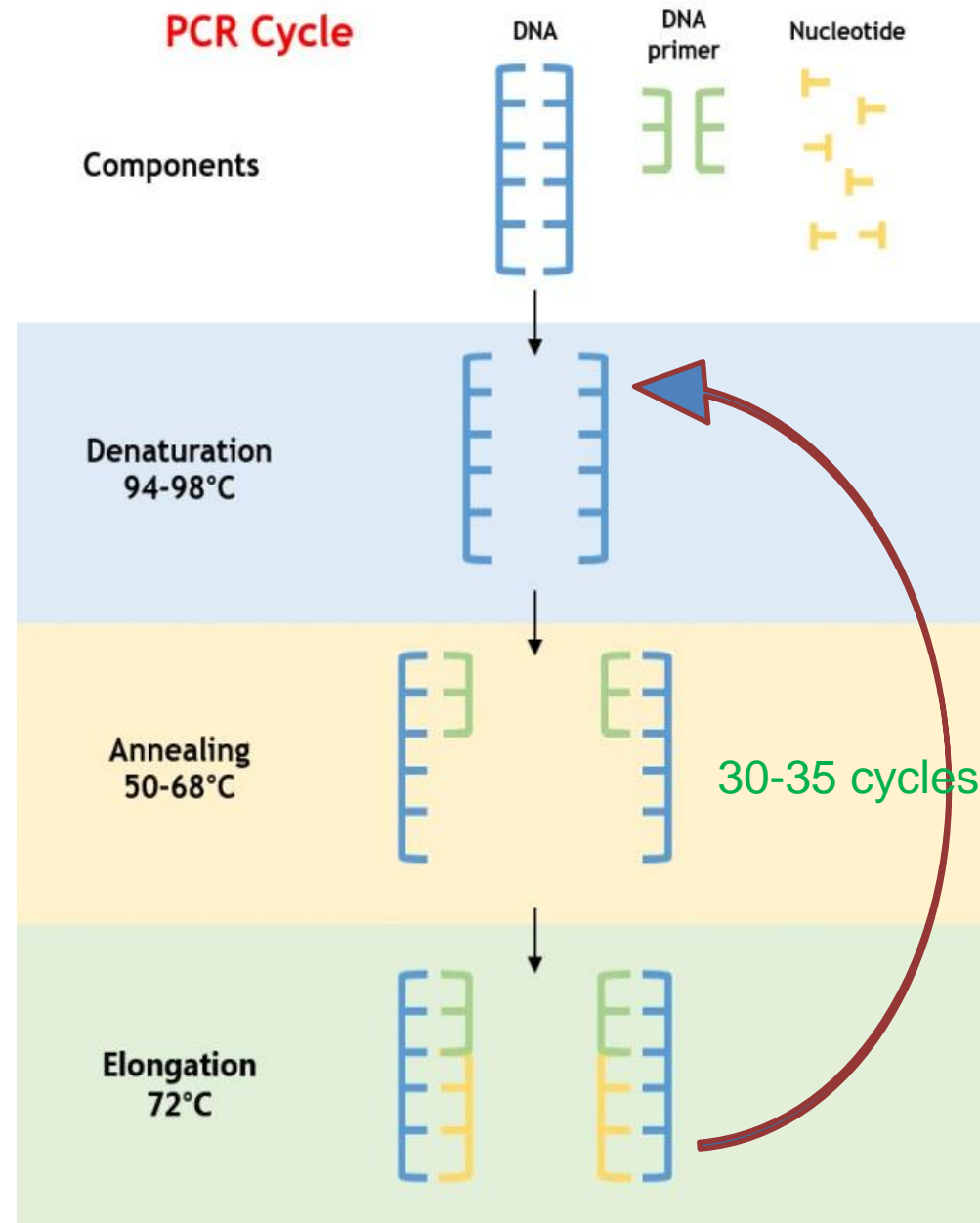


restriction enzyme



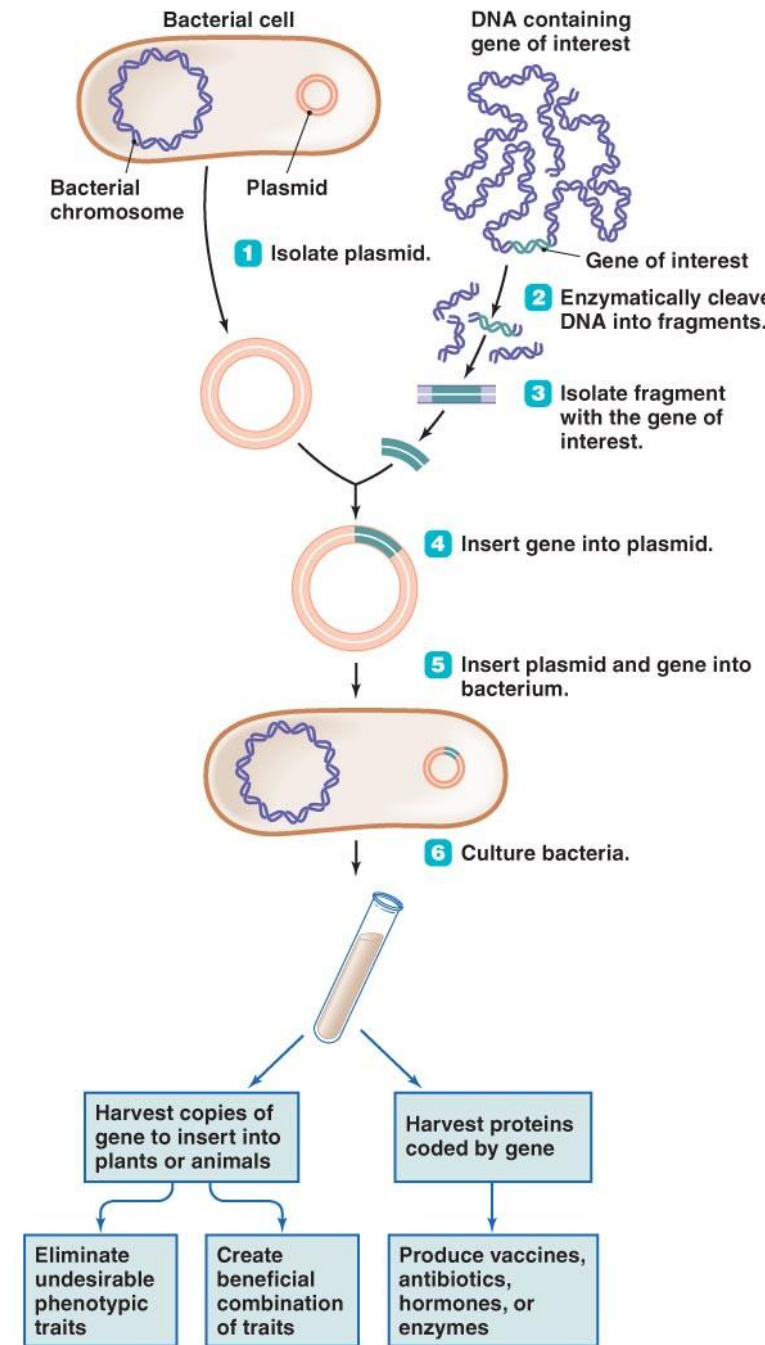
Step 3: Amplification of gene of interest using PCR

Polymerase chain reaction – It is a process to amplify the gene once the proper gene of interest has been cut using the restriction enzymes.



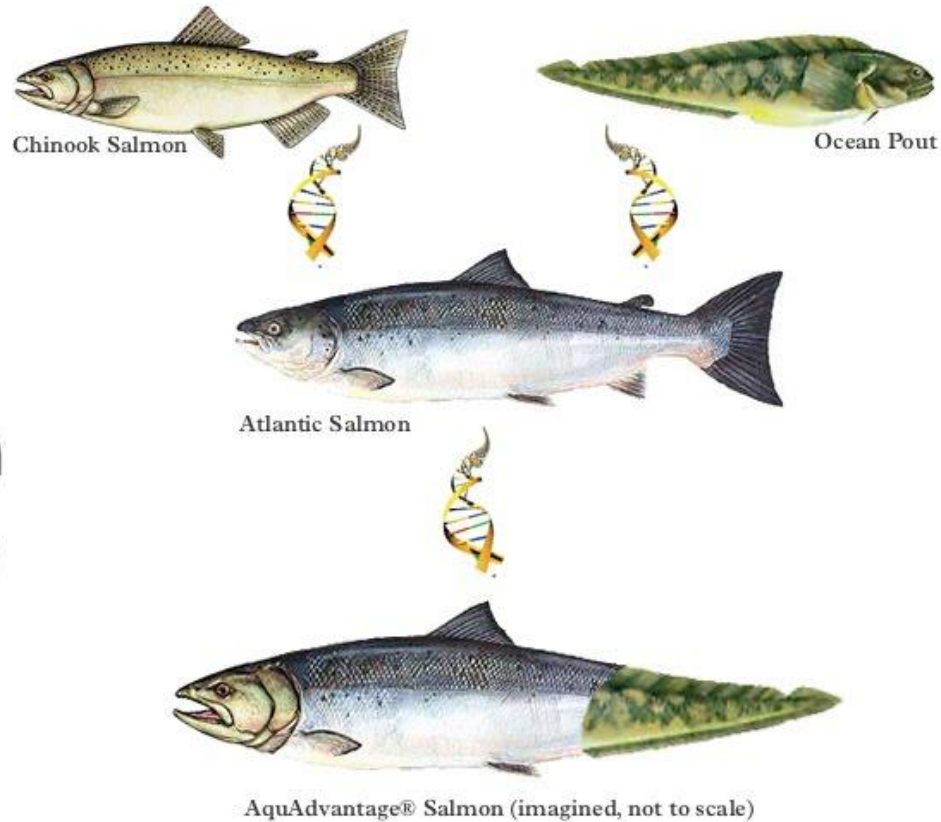
Insertion of DNA into the Host/vector.

- By digesting both the plasmid and DNA with the same restriction enzyme we can create thousands of DNA fragments, one fragment with the gene that we want, and with compatible sticky ends on bacterial plasmids.
- After mixing, the desired fragments and cut plasmids form complementary pairs that are then joined by DNA ligase.
- This creates a mixture of recombinant DNA molecules.



Obtaining the foreign gene product

- When you insert a piece of alien DNA into a cloning vector and transfer it into a bacterial, plant or animal cell, the alien DNA gets multiplied.
- In almost all recombinant technologies, the ultimate aim is to produce a desirable protein
- If any protein encoding gene is expressed in a heterologous host, it is called a recombinant protein.



Applications of Recombinant DNA Technology

1. Scientific applications

- ▶ Many copies of DNA can be produced
- ▶ Increase understanding of DNA
- ▶ Identify mutations in DNA
- ▶ Alter the phenotype of an organism

2. Diagnose genetic disease

3. Recombinant DNA techniques can be used to for genetic fingerprinting identification

- DNA fingerprinting to identify the source of bacterial or viral pathogens.
- bioterrorism attacks (Anthrax in U.S. Mail)
- medical negligence (Tracing HIV to a physician who injected it)
- outbreaks of foodborne diseases

4. Agricultural Applications

Cells from plants with desirable characteristics can be cloned to produce many identical cells, then can be used to produce whole plants from which seeds can be harvested.

5. Nanotechnology

- ▶ Bacteria can make molecule-sized particles
- ▶ *Bacillus* cells growing on selenium form chains of elemental selenium

6. Therapeutic Applications

- ▶ Produce human proteins – hormones and enzymes (Insulin, hGH, $\text{INF}\alpha$, $\text{INF}\beta$ and $\text{INF}\gamma$)
- ▶ Vaccines : Cells and viruses can be modified to produce a pathogen's surface protein (Influenza; Hepatitis B; Cervical cancer vaccine)

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