🧬 Recombinant DNA Technology

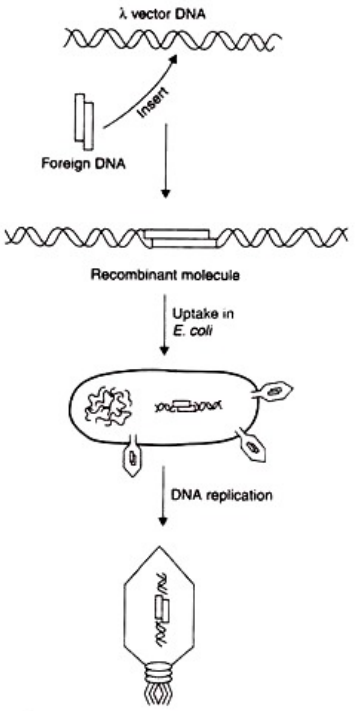


Fig. 23.5 Method for generation of a recombinant DNA molecule.

🔹 Definition

Recombinant DNA (rDNA) technology, also called genetic engineering, is a method to combine DNA from different sources to form a new genetic combination. It allows scientists to modify genes, produce proteins, and create genetically improved organisms. Recombinant DNA is formed when a gene of interest (donor DNA) is inserted into another DNA molecule called a vector (like a plasmid or bacteriophage DNA).
Steps to Create Recombinant DNA:

Isolation of Donor DNA:
The desired gene is isolated and purified from the donor organism.

2. Cutting DNA with Restriction Enzyme:
   Both donor DNA and vector DNA are cut using the same restriction enzyme.
   - These enzymes make staggered cuts, creating sticky ends — short single-stranded overhangs.

3. **Joining Donor DNA and Vector DNA:**
   The sticky ends of donor and vector DNA are complementary, allowing them to join (hybridize).

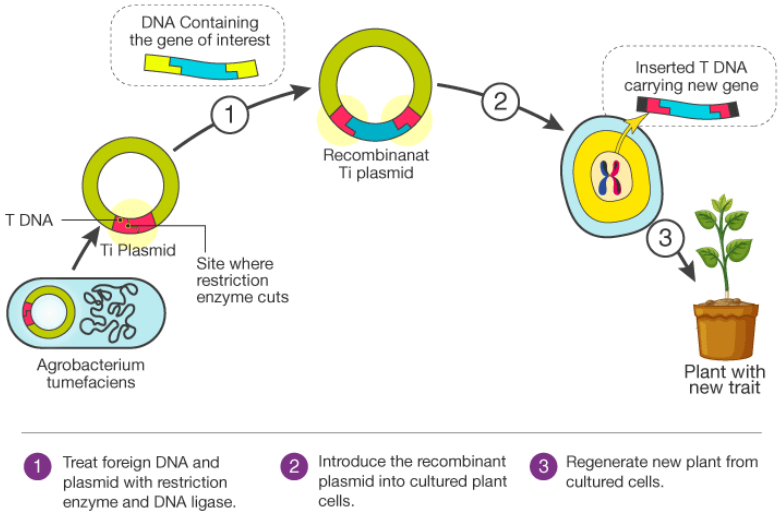
4. **Sealing with DNA Ligase:**
   The enzyme DNA ligase seals the sugar-phosphate backbone by forming phosphodiester bonds, creating a stable recombinant DNA molecule.

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 copes.

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.

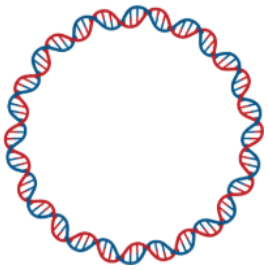


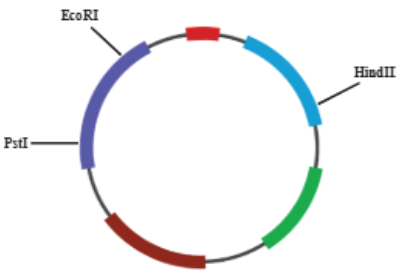
Features of a Good Cloning Vector

1. Origin of Replication (ori): Allows the vector to copy itself inside the host. Affects how many copies of the gene you get.

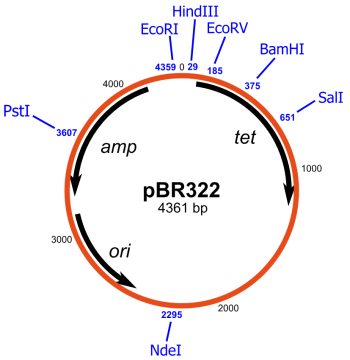
2. Selectable Marker Lets you find cells that took up the vector.Example: an antibiotic resistance gene. If a cell survives the antibiotic, it likely has the vector.

3. Cloning Site A specific site (like a restriction enzyme site) where you can insert foreign DNA. Often located inside a selectable marker so that if the gene is interrupted, it shows cloning worked.

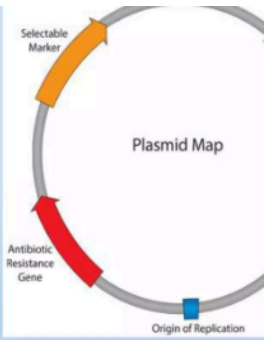




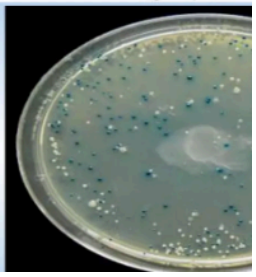
- Scientists (**Herbert Boyer, Keiichi Itakura** and **Arthur Riggs**) working in **Boyer's lab (University of California)** recognized a general cloning vector with unique restriction sites for cloning in foreign DNA and the expression of antibiotic resistance genes for selection of transformed bacteria.
- **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.



- **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.



Ideal Properties of a Vector

A good vector must:Replicate independently inside the host cell.Have restriction sites to insert foreign DNA.Allow easy identification of recombinant molecules (e.g., antibiotic resistance). Be small and stable inside the host.

Types of Common Vectors

a) Plasmids

- Small, circular, **double-stranded DNA molecules found in bacteria.**
  Replicate independently of chromosomal DNA.
  Passed accurately to daughter cells during cell division.

Types of Plasmid DNA

Type	Description
CCC DNA	Covalently Closed Circular DNA – both strands intact
OC DNA	Open Circular DNA – one strand broken
Linear Plasmids	Found in some species like *Streptomyces*, *Borrelia*

Features

- Size: 2–4 kb.
  Has an origin of replication (ori) — signal for replication.
  May carry antibiotic resistance genes (useful as selectable markers).

Example:To insert human gene X into a plasmid:

1. Cut both human DNA and plasmid DNA using the same restriction enzyme (e.g., *EcoRI*).

Their sticky ends pair up and join.
DNA ligase seals the bonds → forms recombinant plasmid DNA.
Many such plasmids form, each carrying a different fragment.
The one with gene X is identified through DNA cloning.

b) Bacteriophage λ (Lambda) Vectors

- **Bacteriophage λ DNA (virus that infects *E. coli*) is used as a vector.**
  Can carry larger DNA fragments — up to 15 kb.

Types of λ Vectors

Type	Description
Insertional Vector	Has a single restriction site where foreign DNA is inserted
Replacement Vector	A fragment of λ DNA is removed and replaced with foreign DNA

Modified Phage Vectors

- Wild-type phage λ has many restriction sites → unsuitable as a vector.
  Modified forms are created:
  Have fewer target sites.
  Contain deletions to make extra space for foreign DNA.
  Usually 25% shorter than normal λ DNA.

Source / Vector	Key Feature	Enzyme Used	Use / Advantage
Genomic DNA	Full gene (with introns)	Restriction enzyme	For studying entire gene
cDNA (from RNA)	No introns, only coding region	Reverse transcriptase	For studying expressed genes
Synthetic DNA	Chemically made fragments	—	For custom or artificial genes
Plasmid Vector	Small circular DNA, replicates independently	Restriction enzyme + DNA ligase	Cloning small DNA fragments
Phage λ Vector	Linear viral DNA, large insert capacity	Restriction enzyme + DNA ligase	Cloning large DNA fragments

- Cut vector DNA and gene of interest are mixed.
- DNA ligase joins them by forming phosphodiester bonds.
- The resulting circular DNA molecule = Recombinant DNA (rDNA).

Feature	λ (Lambda) Bacteriophage Vector	Cosmid Vector

Origin	Naturally occurring virus (*bacteriophage λ*) that infects *E. coli*.	**Artificially constructed vector combining plasmid and λ phage cos sites.**
DNA Type	Linear double-stranded DNA (~48.5 kb).	Circular double-stranded plasmid DNA with cos sites.
Insert Capacity	Can carry up to 15 kb of foreign DNA.	Can carry up to **45 kb** of foreign DNA.
Main Components	Has phage genes, origin of replication, and multiple cloning sites (in modified vectors).	Has plasmid origin of replication, antibiotic resistance gene, and cos sites for phage packaging.
Replication in Host	Replicates as phage DNA in *E. coli* during infection, forming phage particles.	Replicates as a plasmid inside *E. coli* (extra-chromosomal replication).
Packaging & Entry into Host	DNA is packaged into phage heads → injected into *E. coli* by infection process.	Recombinant cosmids are packaged into λ phage particles for transfer → then replicate as plasmids inside *E. coli*.
Use of cos sites	Natural cos sites are required for phage DNA packaging.	Cos sites are artificially inserted → enable packaging into phage heads.
Lytic/Lysogenic Cycle	Can follow lytic or lysogenic cycle in bacteria.	Does not form phage particles (lacks phage genes), remains as plasmid.
Identification/Selection	Plaque formation on bacterial lawn identifies recombinant phages.	Antibiotic resistance markers help select recombinant clones.
Advantages	Efficient infection and cloning for medium DNA fragments; high copy number.	Can clone larger fragments; combines easy plasmid maintenance with phage packaging efficiency.
Limitations	Limited insert size (~15 kb); complex life cycle.	Cannot produce phage particles; only replicates as plasmid after infection.

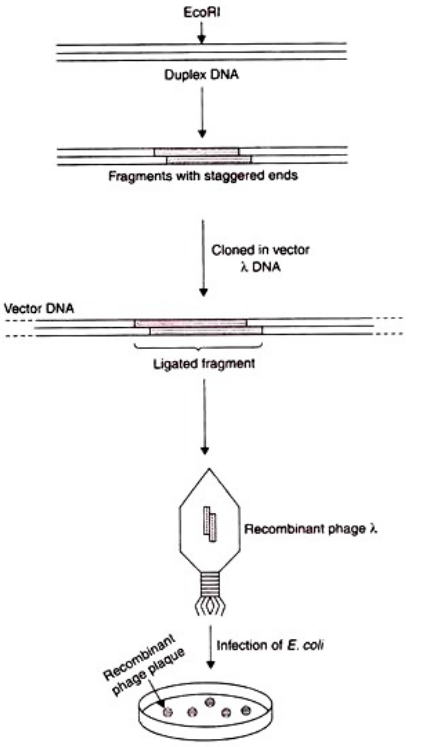


Fig. 23.6 Procedure for cloning in lambda bacteriophage vector.

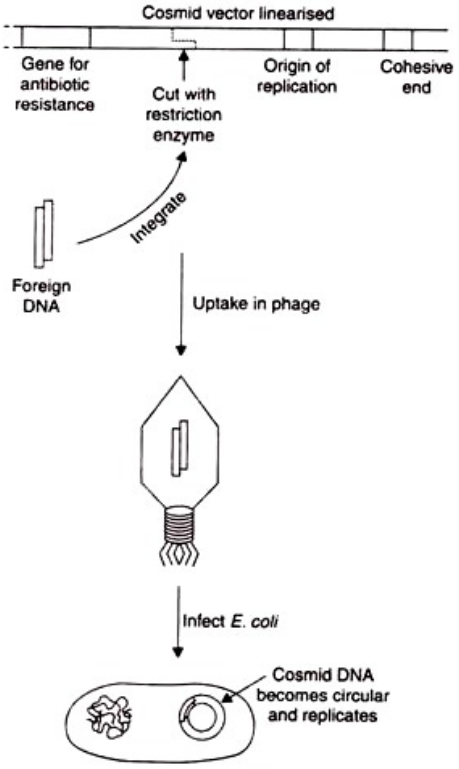


Fig. 23.7 Cloning in artificial cosmid vector.

DNA ligation is the process of joining two DNA fragments together using an enzyme called **DNA ligase**. This enzyme creates a bond between the DNA fragments by forming a **phosphodiester bond**. DNA ligase is essential for maintaining the integrity of genomes, playing a key role in **DNA replication** and **repair**.

DNA Ligation Mechanism

Ligase activation: DNA ligase binds to ATP (or NAD+) and forms an intermediate with AMP attached to it.
Transfer of AMP: The AMP is transferred to the 5' end of the DNA strand.
Joining: DNA ligase seals the DNA by connecting the fragments, releasing AMP in the process.

Types of DNA Ligase:

Mammalian DNA ligase: Involved in repairing DNA in animals; categorized into four types (I-IV).
T4 DNA ligase: From the T4 bacteriophage, commonly used in labs to join blunt-ended DNA fragments.
E. coli DNA ligase: Found in bacteria, it works well for cohesive (sticky) ends.
Thermostable DNA ligase: Stays stable at high temperatures, useful in **PCR**. Taq DNA ligase is an example.

DNA Ligation Reactions:

1. **Blunt-End Ligation**:
   Occurs when both DNA ends are straight (blunt).
   It's less efficient because the DNA ends don't have overhangs to pair up easily.

2. **Sticky-End Ligation**:
   DNA fragments cut by restriction enzymes create sticky ends (single-stranded overhangs).
   These overhangs must be complementary for ligation to work.

Steps:

1. Prepare the Ligation Reaction Mix:Combine the following in a PCR or Eppendorf tube:
   Vector DNA
   Insert DNA
   Ligase Buffer: 1µL per 10µL reaction for 10X buffer, or 2µL per 10µL reaction for 5X buffer.
   T4 DNA Ligase: 0.5–1µL (depending on scale).
   Water: Add to bring the total reaction volume to 10µL.

2. **Calculate DNA Amounts**:
   For standard cloning where the insert is smaller than the vector, a 3:1 molar ratio of insert to vector typically works well. Around 100ng of total DNA is recommended for a standard ligation.

3. **Incubate**: Room temperature for 2 hours (standard).
   Alternatively, you can incubate at 16°C overnight for better efficiency.
   For high-efficiency ligation, sometimes 5 minutes at room temperature works if using high-concentration ligase. 37°C incubation may be used for difficult ligations (like oligo ligation).
   Scale Reaction:
   If you cannot achieve the 100ng total DNA concentration, scale the reaction accordingly. Ensure to adjust the buffer and ligase amounts proportionally.

Applications in Molecular Biology:

1. **Molecular Cloning**:
   Creating recombinant DNA by cutting and joining plasmids and target genes.
   The recombinant plasmid is inserted into a host organism (like E. coli) to multiply.

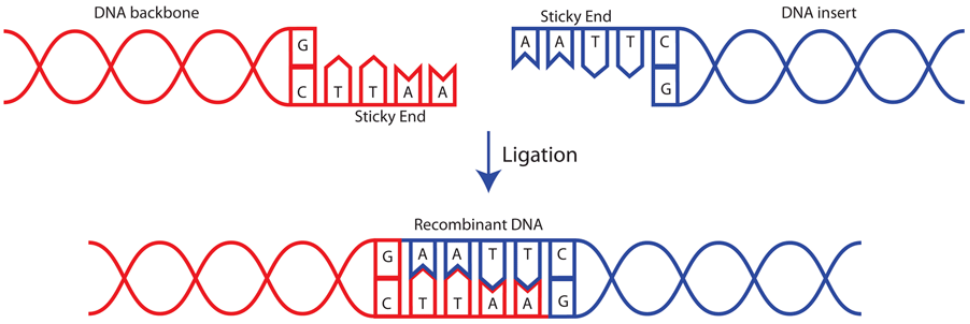
2. **Gene Synthesis and Assembly**:
   DNA ligase joins small DNA fragments to form longer pieces for applications like gene expression or gene silencing.

Troubleshooting DNA Ligation:

1. **Check DNA Fragment Quality**: Ensure the DNA fragments are clean and at the right concentration.
   Optimize DNA Ligase: Use the correct amount of ligase enzyme for the reaction.
   Adjust Conditions: Optimize temperature, time, and buffer for better ligation efficiency.
   Handle Reagents Carefully: Properly store and handle ligase, ATP, and DNA samples to avoid losing enzyme activity.

Verifying Ligation Success:

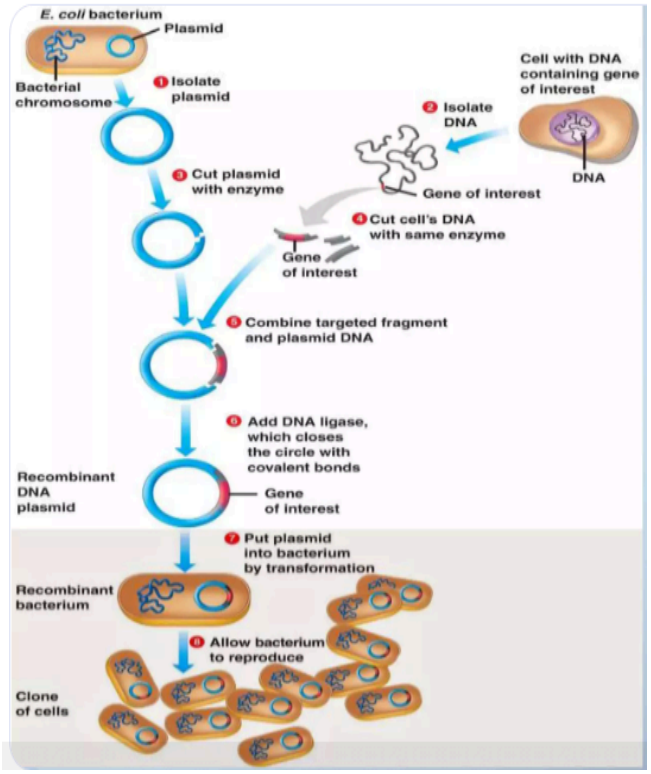
- **Gel Electrophoresis**: Separates DNA fragments to confirm if ligation worked.
  PCR: Amplifies the ligated DNA to check if the target gene is present.
  Sequencing: Techniques like **Sanger sequencing** or **NGS** can confirm if the ligation is accurate.



The final step in constructing a recombinant plasmid involves ligating (joining) the **insert DNA** (gene or DNA fragment of interest) into a **vector backbone** (the plasmid). This is done by covalently linking the two DNA fragments through phosphodiester bonds formed by the enzyme T4 DNA ligase.

Objective

- To isolate a specific gene of interest.
  To insert it into a suitable vector.
  To transfer it into a host organism.
  To express and produce the desired gene product (protein).



🔹 Key Molecular Tools

Tool	Function	Examples
Restriction Enzymes	Cut DNA at specific sequences (act as molecular scissors)	EcoRI, BamHI
DNA Ligase	Joins DNA fragments by forming phosphodiester bonds	T4 DNA Ligase
Vectors	Carry foreign DNA into host cells	Plasmids, Bacteriophages, Cosmids
Host Organism	Receives and replicates recombinant DNA	*E. coli*, Yeast, Animal/Plant cells
Polymerase Chain Reaction (PCR)	Amplifies DNA sequence in vitro	Uses Taq polymerase
Selectable Marker	Helps identify transformed cells	Antibiotic resistance genes (e.g., Ampicillin resistance)

Seven Main Stages

1️⃣ Isolation of Genetic Material (DNA) 🔹 Sources of Donor DNA

a) Genomic DNA

- Comes directly from the chromosomal DNA of the donor organism.
  Isolation of cDNA: Extract **mRNA** from human pancreatic cells.
- Contains both coding (gene) and non-coding regions.
  Used when you want the complete gene, including promoters or introns.

b) Complementary DNA (cDNA) from RNA

- Sometimes the required sequence is available only as RNA (like mRNA).
  Reverse transcriptase enzyme is used to make a DNA copy from RNA.
  This copy is called cDNA (complementary DNA).

- Steps:
  Take mRNA from a cell.
  Use reverse transcriptase to make cDNA.
  Use cDNA as donor DNA to insert into a vector.

- Useful because:
  mRNA is short-lived and difficult to isolate directly.
  cDNA represents only the expressed genes (no introns).

🧠 *Example:* Making cDNA libraries helps study which genes are active in a specific tissue.

c) Chemically Synthesised DNA

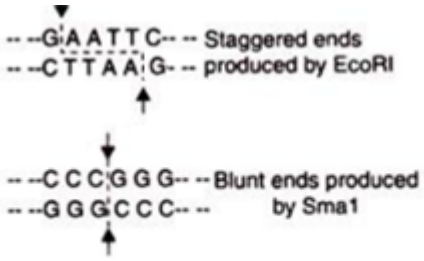
- If a gene sequence is known but not available naturally, it can be chemically synthesised in the lab.
  Oligonucleotides (short DNA fragments, 15–100 bases long) are built using automated machines.
  The fragments can be joined to form complete synthetic genes for use in recombinant DNA.

- DNA is isolated from the donor organism.
  Cell wall breakdown enzymes:
  Lysozyme → Bacteria
  Cellulase → Plants
  Chitinase → Fungi
  Other macromolecules removed:

  - RNA → by *Ribonuclease (RNase)*
    Proteins → by *Protease*
    DNA is precipitated with chilled ethanol and collected as fine threads.

2️⃣ Cutting of DNA at Specific Sites (Restriction Digestion)

- DNA is cut using restriction endonucleases at specific recognition sites. Both donor DNA and vector DNA are cut with the same enzyme to create complementary sticky ends.
  Example: *EcoRI* cuts between G/AATTC → sticky ends form.



Staggered and blunt ends produced by cleavage using restriction enzymes.

Table. Some restriction enzymes and their recognition sites.

Enzyme	Source	Recognition site
*Eco*RI	*E. coli*	GAATTC CTTAAG
*Sma*I	*Serratia marcescens*	CCCGGG GGGCCC
*Pst*I	*Providencia stuartii*	CTGCAG GACGTC
*Hind*III	*Haemophilus influenzae*	AAGCTT TTCGAA
*Taq*I	*Thermus aquaticus*	TCGA AGCT

3 Isolation of Desired DNA Fragment

- Use agarose gel electrophoresis to separate DNA fragments by size. DNA (negatively charged) moves toward the anode.

- The desired DNA band is cut out (eluted) from the gel.

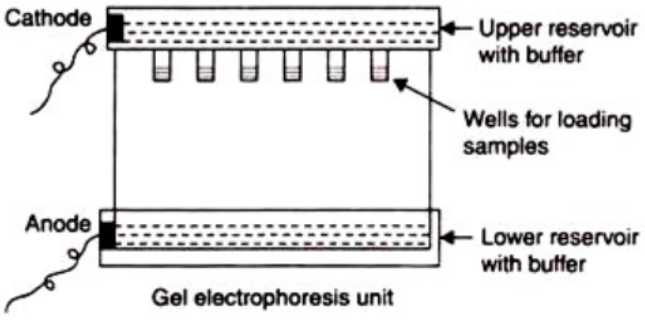


Diagram showing main components of the gel electrophoresis unit.

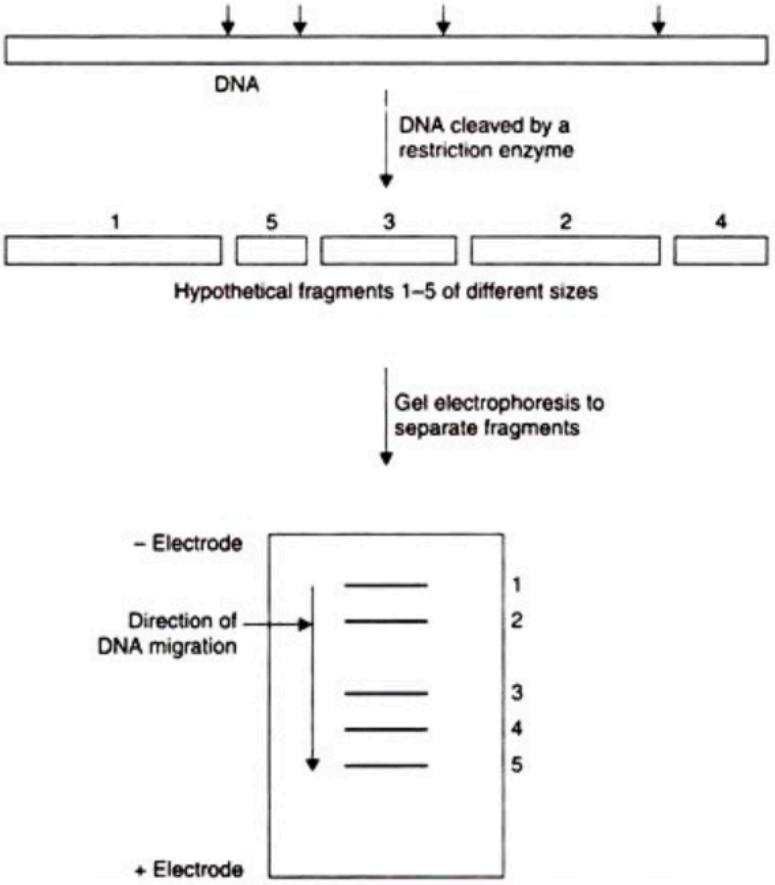


Fig. 23.3 Digestion of DNA by a restriction enzyme followed by separation of fragments on gel electrophoresis.

4 Amplification of Gene of Interest (PCR)

- Polymerase Chain Reaction (PCR) produces millions of copies of DNA in vitro.
  Developed by: *Kary Mullis (1985)*
  Enzyme used: *Taq polymerase* (from *Thermus aquaticus*)

- Steps:
  Denaturation (95°C): DNA strands separate.
  Annealing (40–60°C): Primers attach to each strand.
  Extension (72°C): Taq polymerase synthesizes new DNA strands.
  Repeated for 25–35 cycles → exponential amplification.

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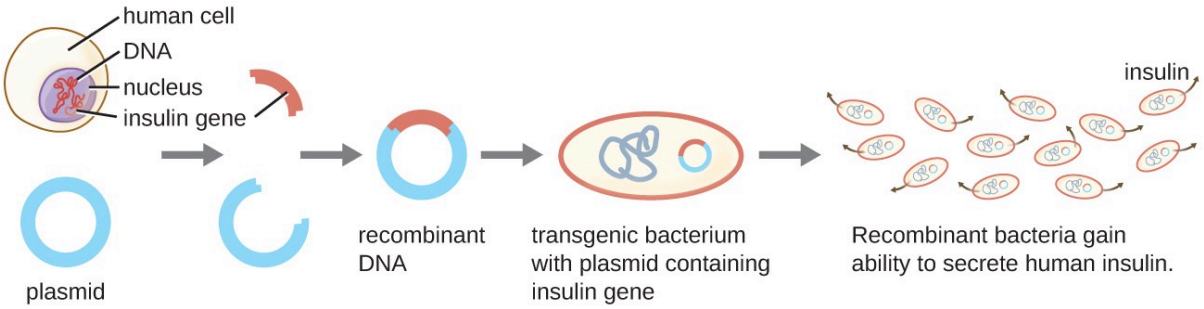
5 Ligation of DNA Fragment into a Vector

- Insert the insulin gene into a **vector** (such as a plasmid) for easier transfer into a host.
  Steps:
- Take the **cut insulin gene** and **cut plasmid/** λ DNA **vector** and mix them.
  Use **DNA ligase** (like **T4 DNA Ligase**) to seal the insulin gene into the vector,

forming **recombinant DNA**.
The vector now carries the insulin gene and can be transferred into a host cell.



6 Insertion of Recombinant DNA into Host Cells (Transformation)

- The recombinant DNA is transferred into a host organism (e.g., *E. coli*).

- Methods:
  Heat shock ($CaCl_2$ treatment)
  Electroporation (electric pulse)
  Microinjection (for animal cells)
  Gene gun (for plant cells)
  The host cells are made competent to accept DNA.

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7 Selection and Culturing of Transformed Cells

🧬 What is Screening?

- Screening is the process of identifying the clone in a DNA library that contains the desired gene or sequence.
  A probe (short, labeled DNA or protein fragment) is used to locate the correct clone.

Types of Probes

1. DNA Probes:
   Single-stranded DNA molecules that can hybridize (bind) to complementary DNA sequences in the library.
   Used when the DNA sequence of the gene is known.
2. Protein Probes:
   Used when only the protein product of a gene is known (not covered in detail here).

Steps in DNA Library Screening (Using a DNA Probe)

- Plating the Library:
  Recombinant phages are spread on *E. coli* lawn.

Each phage infects bacteria and forms a plaque (clear zone).

- Transfer to Membrane:
  A nitrocellulose or nylon membrane is gently placed on the plate.
  The pattern of plaques is copied onto the membrane.

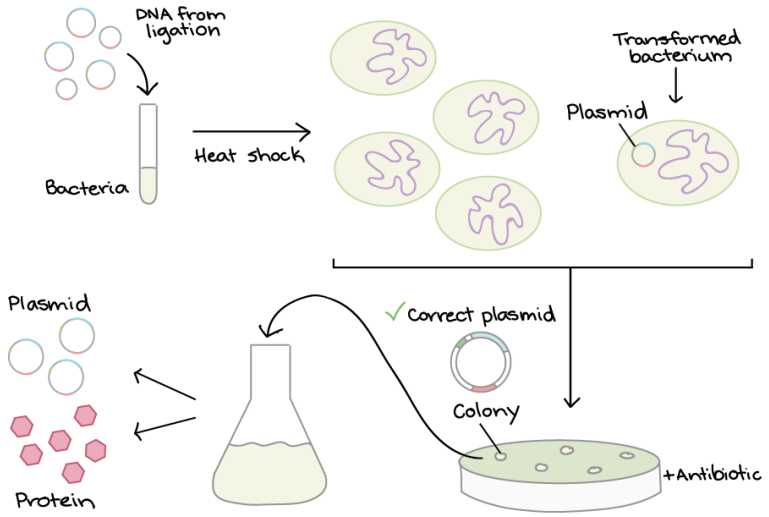
- Lysis and DNA Denaturation:
  The phages on the membrane are lysed.
  Their DNA is denatured (made single-stranded).

- Hybridization with Probe:
  The membrane is soaked in a solution containing a radiolabeled or fluorescent DNA probe specific to the target gene.
  The probe binds (hybridizes) to the complementary sequence on the membrane.

- Detection:
  The membrane is washed to remove unbound probes.
  The location of bound probe is detected by autoradiography (for radioactive probes) or fluorescence.
  The corresponding plaque on the original plate contains the desired recombinant clone.
  Only some host cells take up recombinant DNA.

- Selectable marker gene (e.g., antibiotic resistance) helps identify them.
  Example: Cells with *ampicillin resistance gene* survive on ampicillin plates.

- Transformed colonies are cultured in bioreactors to multiply and express the foreign gene.



- Downstream processing: Purification and extraction of the desired protein.

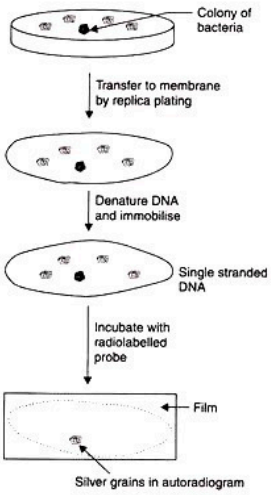
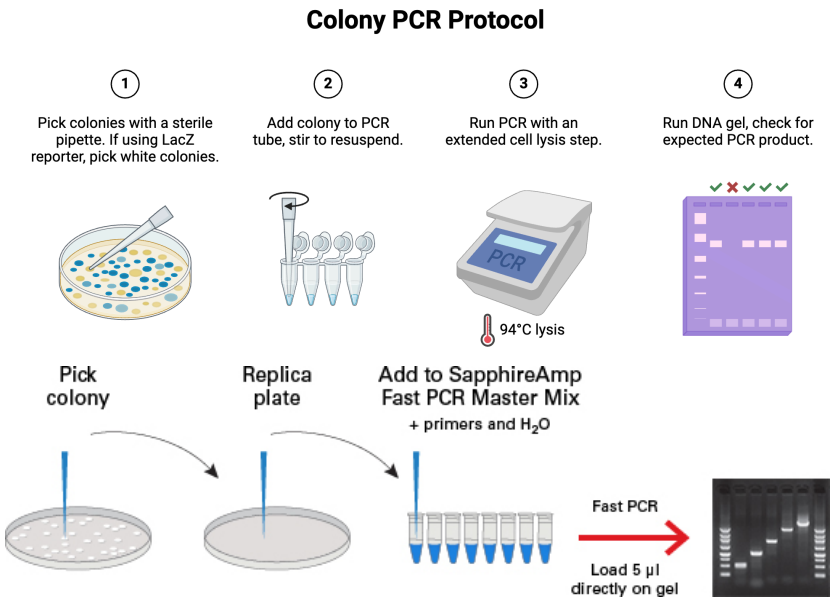


Fig. 23.9 Locating a desired gene in a bacterial colony. In the first step, when the bacterial cells in culture dish are growing into colonies they are replica plated on a filter paper, from where they are inoculated into new culture dishes (replica plating not shown in this figure). The cells in culture dish are then screened for those colonies that contain the recombinant DNA of interest. Such colonies are indentified, their cells removed and grown separately to yield large quantities of the DNA fragment of interest.

Transformation: You insert your gene of interest into a plasmid and introduce it into ***E. coli*** via **heat shock**.

Plate the bacteria: The bacteria are plated on agar with an antibiotic that selects for cells with the plasmid.
Pick colonies: After overnight incubation, you pick several individual colonies.

Perform Colony PCR: PCR is done on each colony to check if they contain the recombinant plasmid with the gene of interest.
If **colony 1** gives the correct PCR product size, you know that colony contains the recombinant plasmid.
Culture positive colonies: After colony PCR, you can pick the positive colonies to culture them for larger-scale plasmid extraction and further analysis.



Summary Table – Steps & Enzymes

Step	Process	Main Enzymes/Tools	Purpose
1	Isolation of DNA	Lysozyme, Cellulase, Chitinase	Get pure DNA
2	Cutting DNA	Restriction enzymes	Create sticky ends
3	Fragment Isolation	Gel electrophoresis	Obtain desired DNA piece
4	Amplification	Taq Polymerase (PCR)	Multiply DNA
5	Ligation	DNA Ligase	Join DNA + Vector
6	Transformation	$CaCl_2$, Electroporation	Transfer into host
7	Selection & Culture	Antibiotic marker, Bioreactor	Grow and express gene

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🔹 Applications of rDNA Technology

- Medicine: Production of insulin, growth hormone, vaccines (e.g., Hepatitis-B vaccine).
- Agriculture: Creation of pest-resistant crops (e.g., Bt-cotton).
  Drought or herbicide-tolerant plants.
- Industry: Production of enzymes, biofuels, and vitamins.
- Research: Gene cloning, DNA sequencing, and gene therapy studies.

🔹 Example: rDNA Use in Rumen Bacteria

- Goal: Improve digestion of cellulose in ruminant animals.
  Steps:
  Identify and clone cellulase genes.
  Insert them into rumen bacteria using rDNA techniques.
  Transform bacteria to enhance lignocellulose breakdown.
  Observe improved digestibility in animals.
- Rumen microflora include:
  Ruminococcus albus, *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes* (cellulose degraders)

Methanobrevibacter ruminantium (methanogen)

- Note: Balance in rumen microbes is essential; imbalance (e.g., excess *Streptococcus bovis*) causes acidosis.

- The same restriction enzyme must be used on both donor and vector DNA.
  Sticky ends ensure correct base pairing during ligation.
  Selectable marker confirms successful transformation.
  Taq polymerase is heat-resistant and key to PCR.
  Downstream processing is necessary for product purification.
  The final product may be a recombinant protein or a genetically modified organism (GMO).

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https://www.youtube.com/watch?v=OpU_CQOpFyQ

https://www.youtube.com/watch?v=evQPGxiG9hs

1. The main purpose of screening a DNA library is to:
A) Amplify the DNA fragments
B) Identify the clone containing the desired gene
C) Cut the DNA into smaller pieces
D) Insert DNA into the vector

2. A DNA probe is:
A) A restriction enzyme
B) A labeled single-stranded DNA molecule
C) A bacterial plasmid
D) A double-stranded RNA molecule

3. During screening, DNA probes bind to:
A) Proteins
B) Single-stranded complementary DNA sequences
C) Double-stranded DNA sequences
D) RNA transcripts

4. What type of membrane is commonly used to transfer plaques from agar plates during screening?
A) Agarose membrane
B) Nitrocellulose or nylon membrane
C) Polyacrylamide membrane
D) Cellulose acetate membrane

5. The plaques formed on a bacterial lawn represent:
A) Bacterial colonies
B) Phage-infected zones containing recombinant DNA
C) Uninfected bacterial regions
D) Hybridized probes

6. The process of breaking phage particles and separating DNA strands on the membrane is called:
A) Hybridization
B) Lysis and denaturation
C) Replication
D) Transformation

7. Which technique is used to detect radioactive probes after hybridization?
A) Gel electrophoresis
B) Autoradiography
C) Polymerase Chain Reaction (PCR)

D) ELISA

8. What is the advantage of using labeled probes in screening?
A) They act as restriction enzymes
B) They help visualize the specific DNA fragment bound to the probe
C) They increase DNA replication rate
D) They make DNA double-stranded

9. If the sequence of a gene is unknown but the protein is known, which type of probe would be used?
A) DNA probe
B) RNA probe
C) Protein (antibody) probe
D) Enzyme probe

10. The complementary binding between the probe and target DNA is called:
A) Transformation
B) Transcription
C) Hybridization
D) Translation

1(B), 2(B), 3(B), 4(B), 5(B), 6(B), 7(B),8(B), 9(C), 10(C)