In The Name of God

Gene Cloning

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Institute of Pharmaceutical Sciences

Kerman University of Medical Sciences

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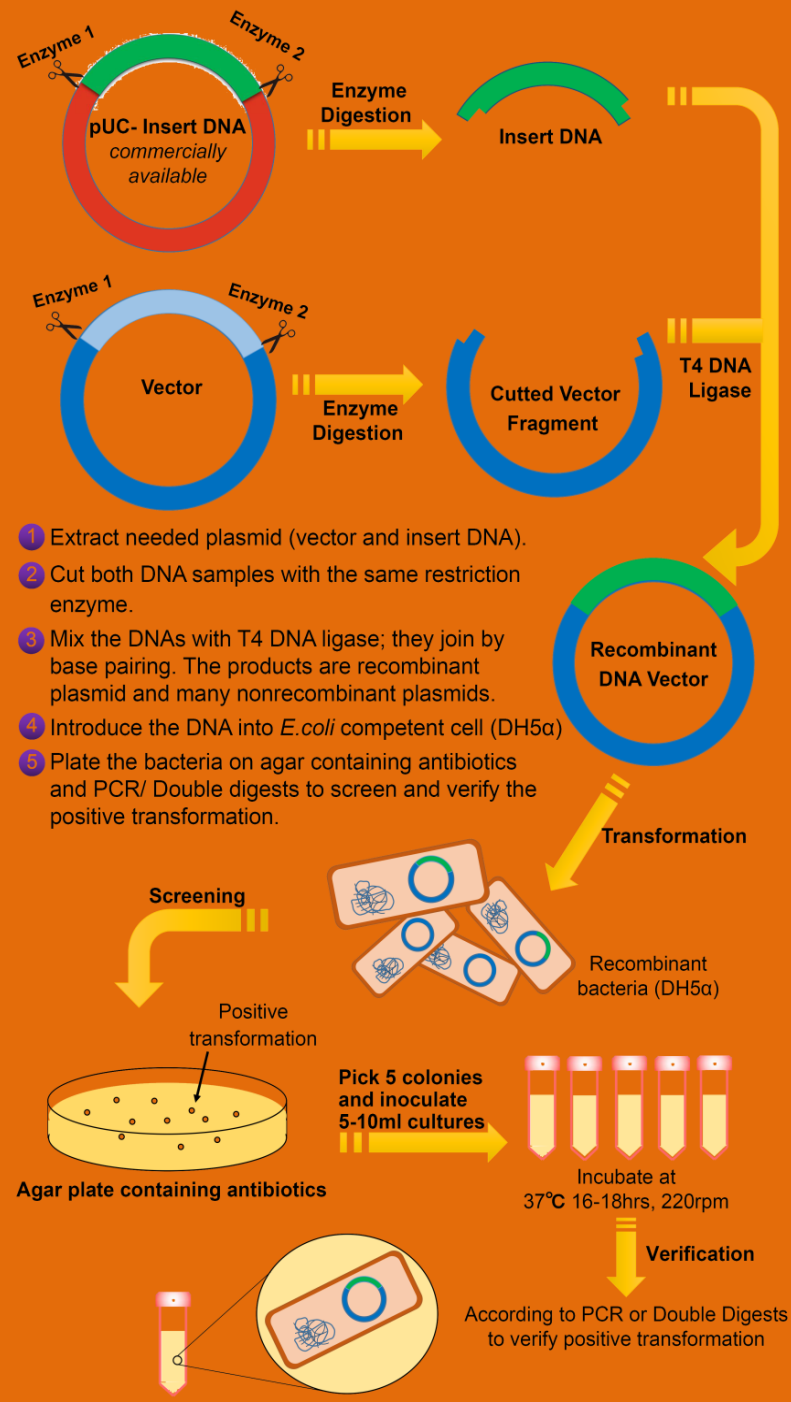
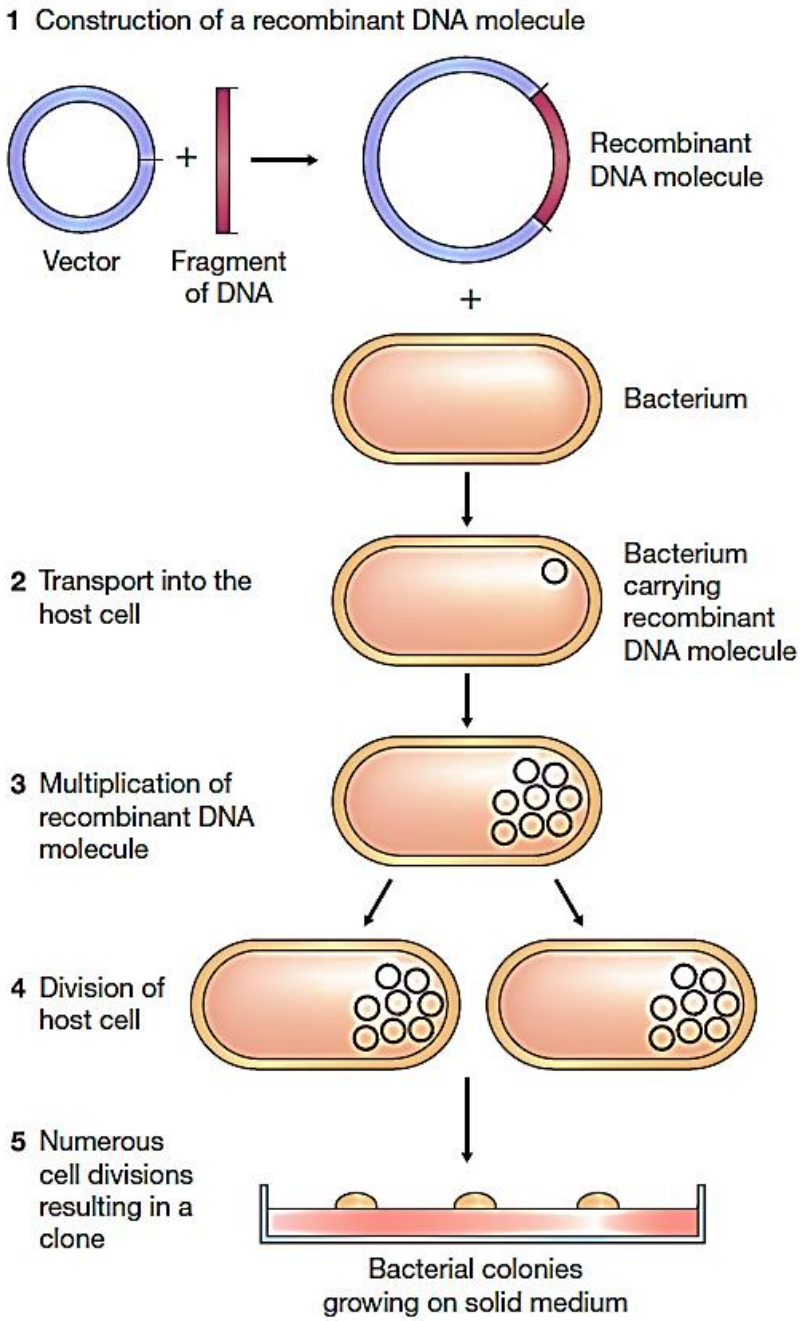


Gene Cloning

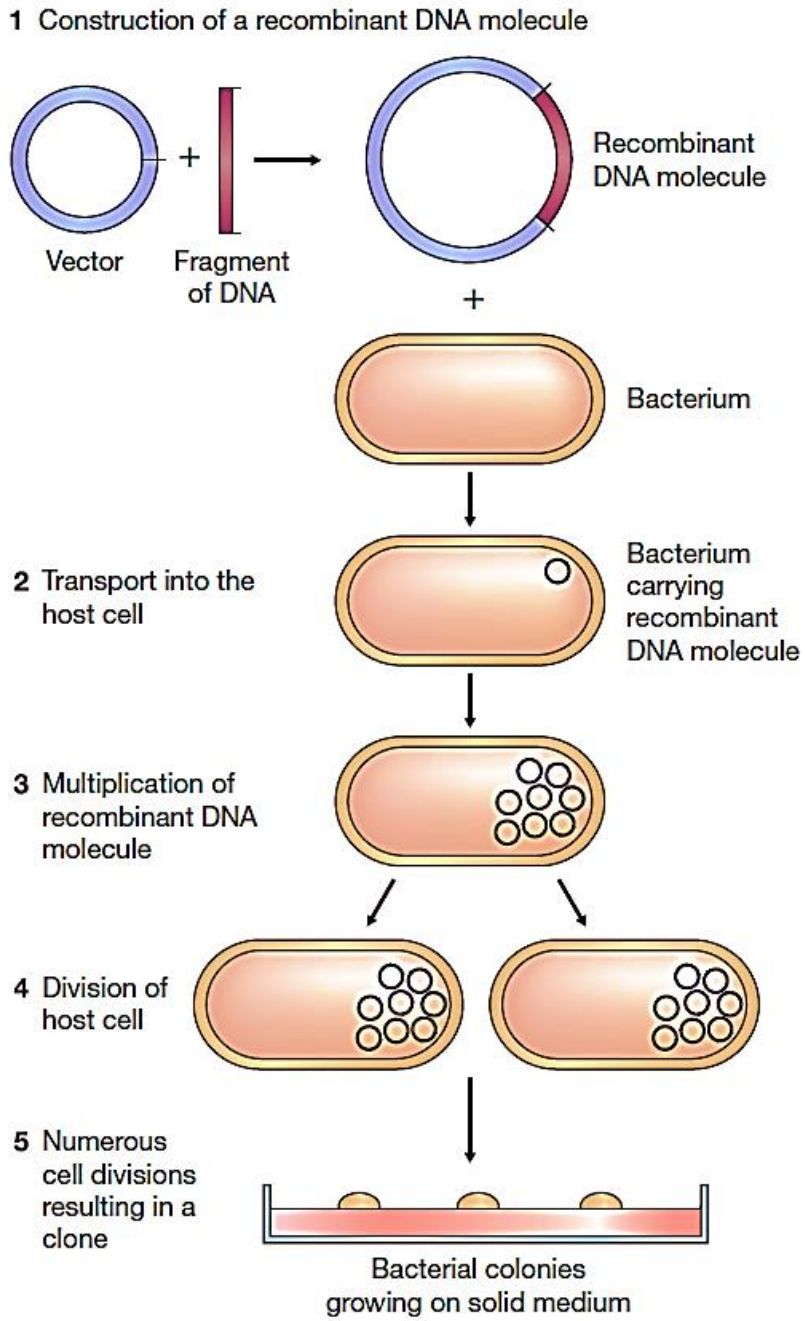
- **Gene cloning** is a process in which **a specific DNA sequence (typically a gene)** is isolated from a living organism and inserted into a vector, such as a plasmid or virus, to be replicated within a host (usually a bacterium like E. coli).

Gene cloning = Isolation + Amplification



Gene Cloning

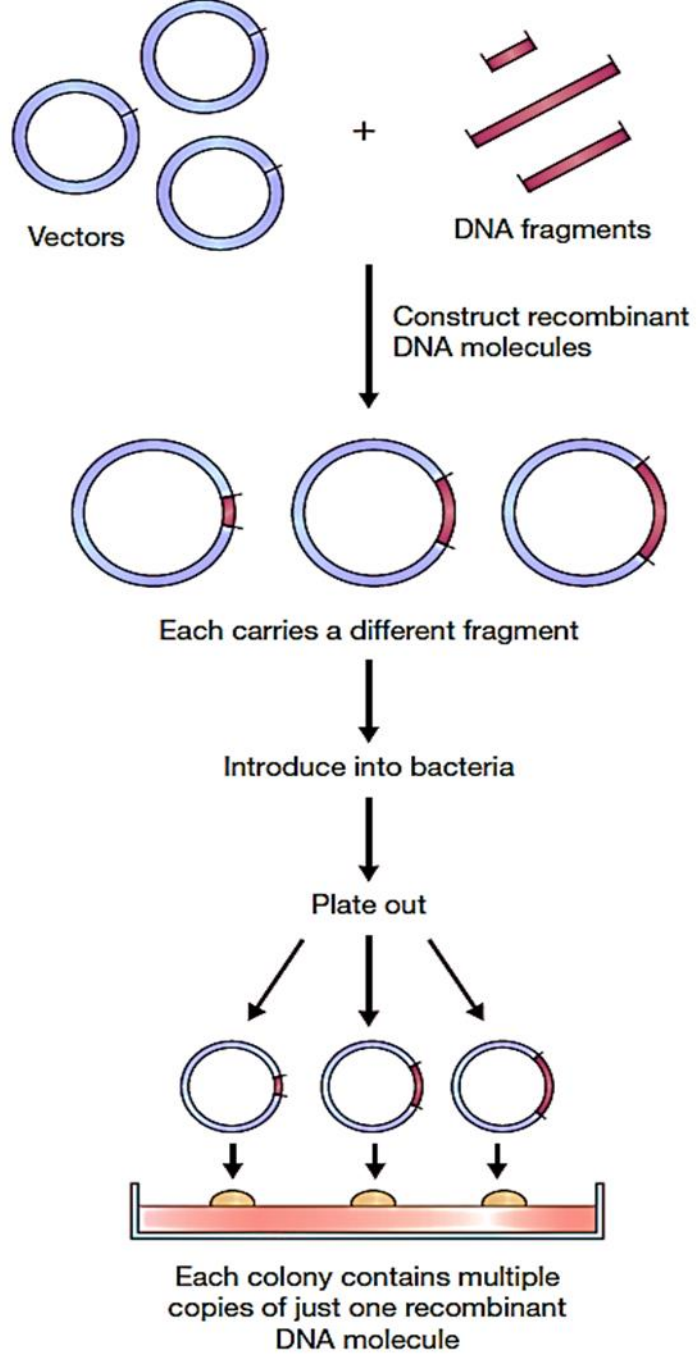
- The primary goal of this process is to generate **multiple copies of a specific gene** for research studies, production of recombinant proteins, or therapeutic interventions.



Gene Cloning

- Requirements for Gene Cloning (Cell-based):

1) DNA fragment containing the desired genes to be cloned (DNA, cDNA).
2) Restriction enzymes (EcoRI) and ligase enzymes (T4 DNA Ligase).
3) Vectors – to carry, maintain and replicate cloned gene in host cell (pUC19, pET).
4) Host cell– in which recombinant DNA can replicate (*Escherichia coli* DH5α, *E. coli* BL21).



Gene Cloning

Gene cloning involves following 7 essential steps:

1. Isolation of specific DNA fragment containing gene of interest
2. Selection of suitable cloning vector
3. Formation of Recombinant DNA (Ligation)
4. Transformation of recombinant vector into suitable host
5. Isolation of Recombinant Cells
6. Multiplication of Selected Host Cells
7. Isolation and Purification of the Product

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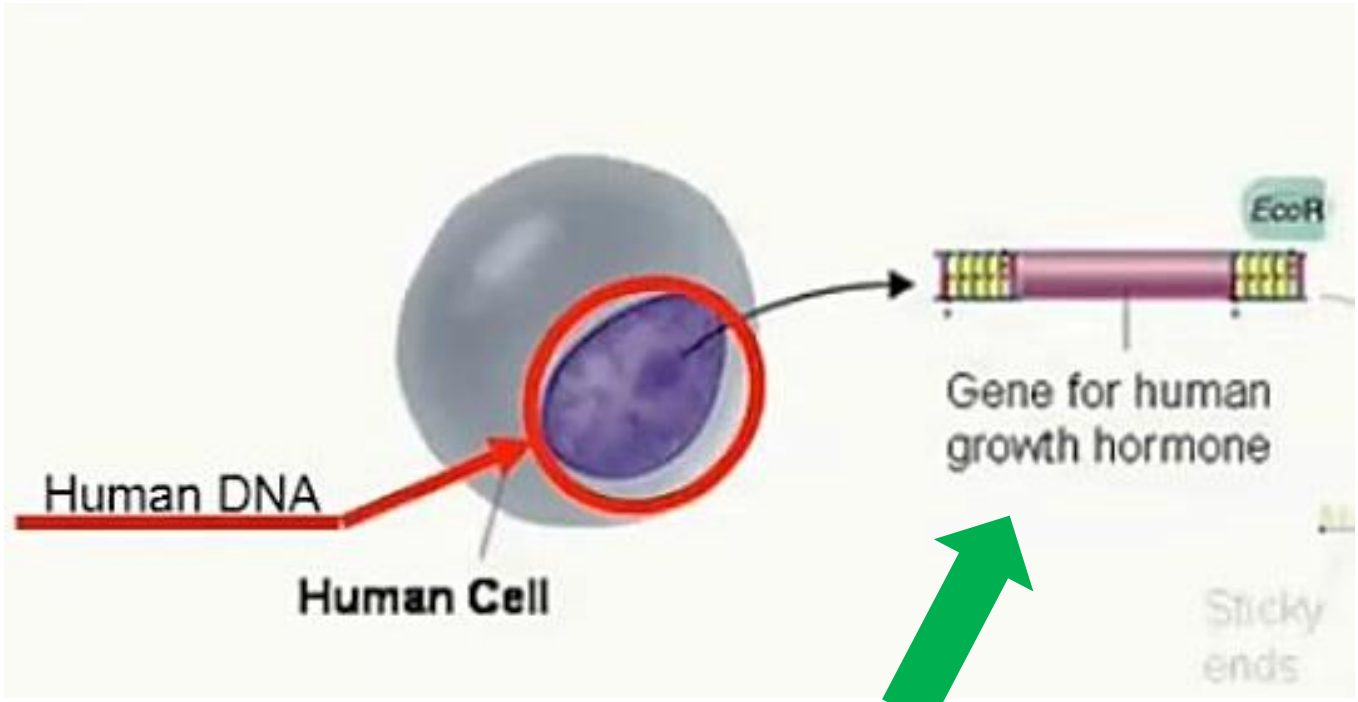
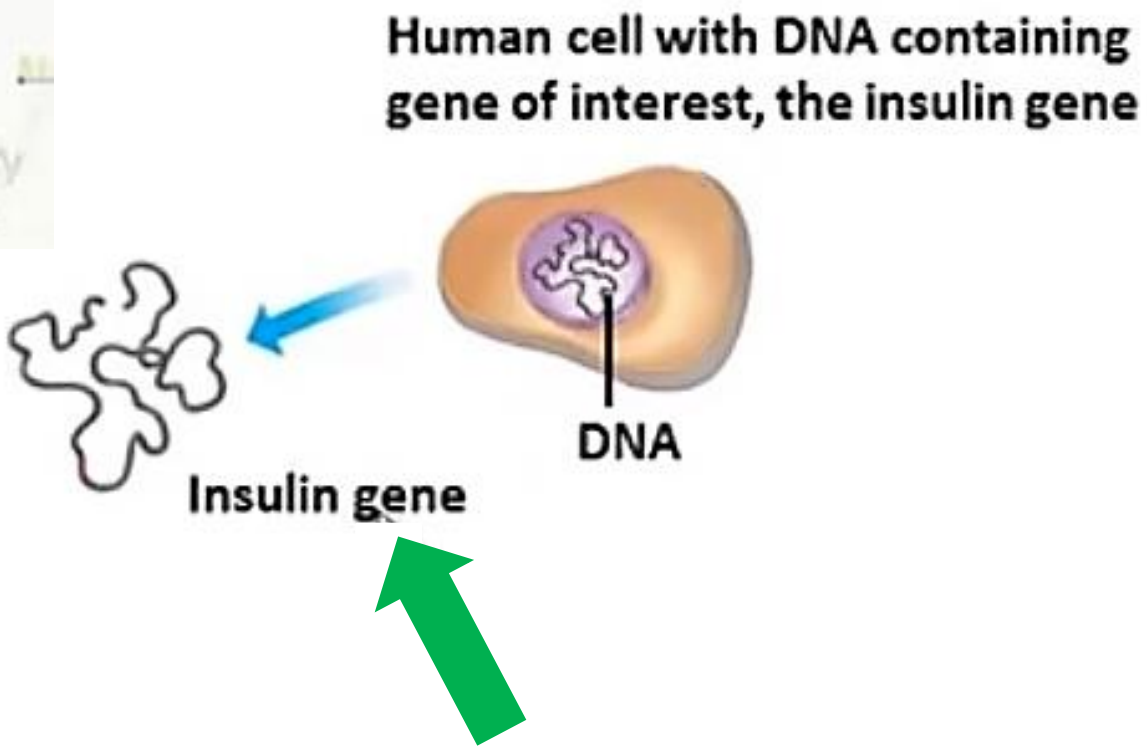
Gene Cloning

1. Isolation of the DNA fragment or gene

- ✓ The very first step is isolation of target DNA or gene fragment to be cloned.
- ✓ A gene of interest is a fragment of gene whose product (a protein, enzyme or a hormone) interests us.

A. Isolation of the DNA fragment or gene

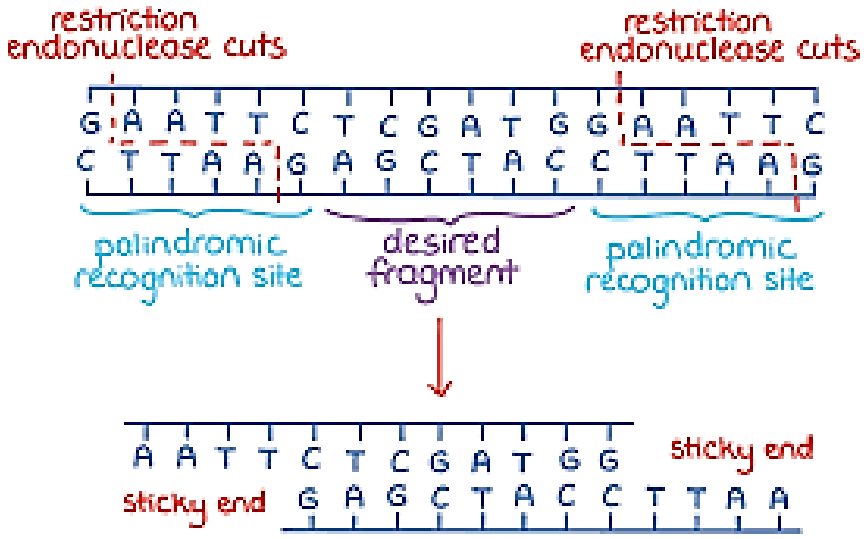




Gene Cloning

1. Isolation of the DNA fragment or gene

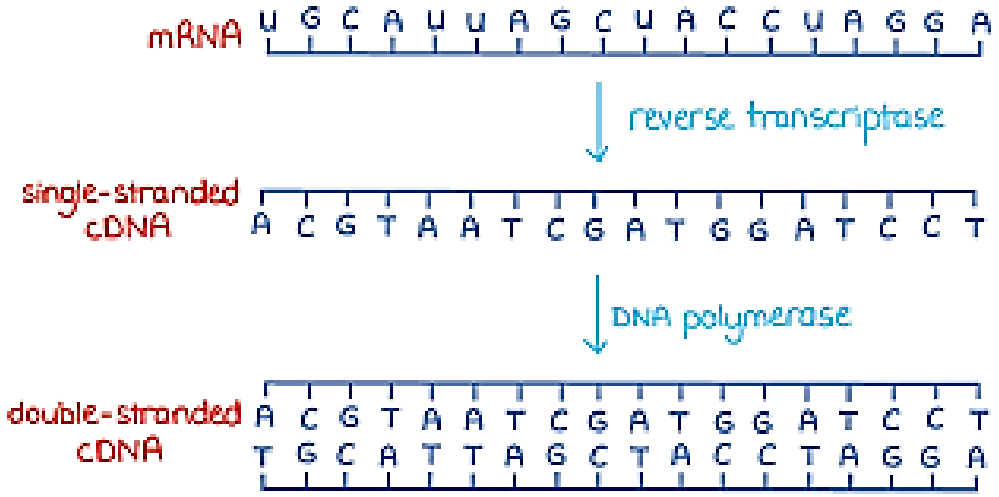
✓ The desired gene may be isolated by using restriction endonuclease enzyme, which cut DNA at specific recognition nucleotide sequences known as restriction sites towards the inner region producing blunt or sticky ends.

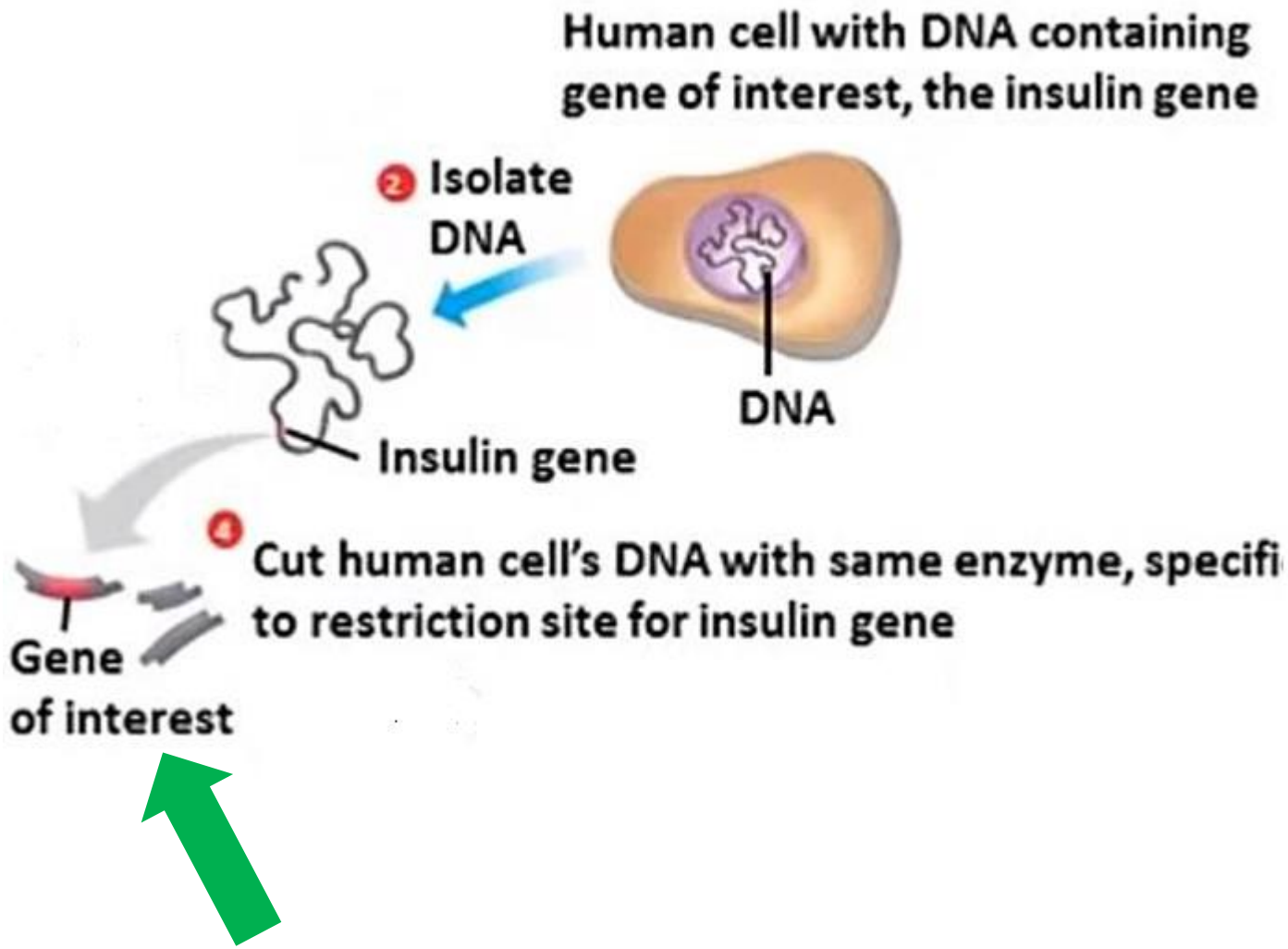


Gene Cloning

1. Isolation of the DNA fragment or gene

✓ Sometimes, reverse transcriptase enzyme may also be used which synthesizes complementary DNA strand of the desired gene using its mRNA.




Human cell with DNA containing gene of interest, the insulin gene
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DNA
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Gene of interest

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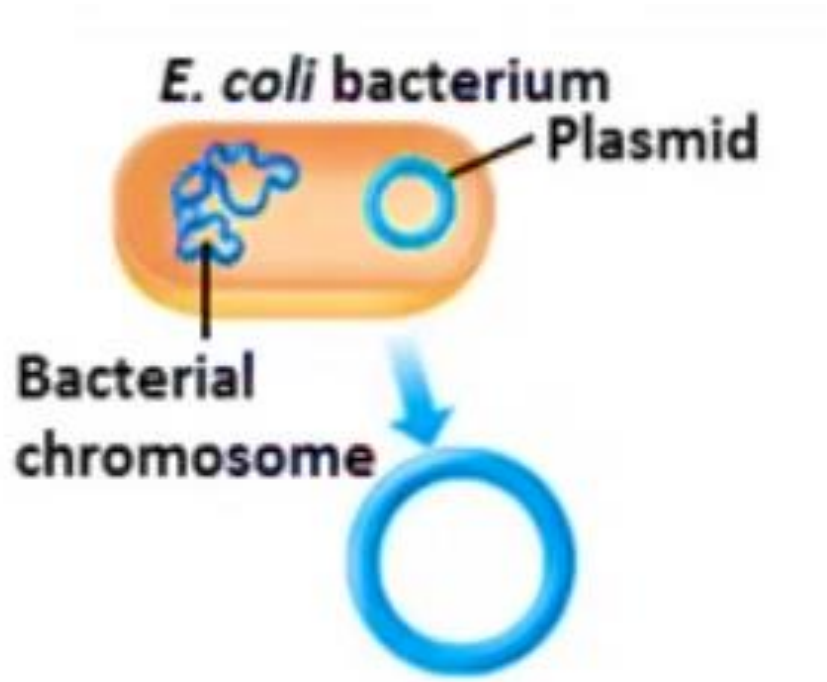
Gene Cloning

2. Selection of suitable cloning vector

- ✓ The vector is a carrier molecule which can carry the gene of interest into a host, replicate there along with the gene of interest making its multiple copies.



Gene Cloning

2. Selection of suitable cloning vector

- ✓ Plasmids
- ✓ Bacteriophages
- ✓ Bacterial Artificial Chromosomes (Bacs)
- ✓ Yeast Artificial Chromosomes (Yacs)
- ✓ Mammalian Artificial Chromosomes (Macs).

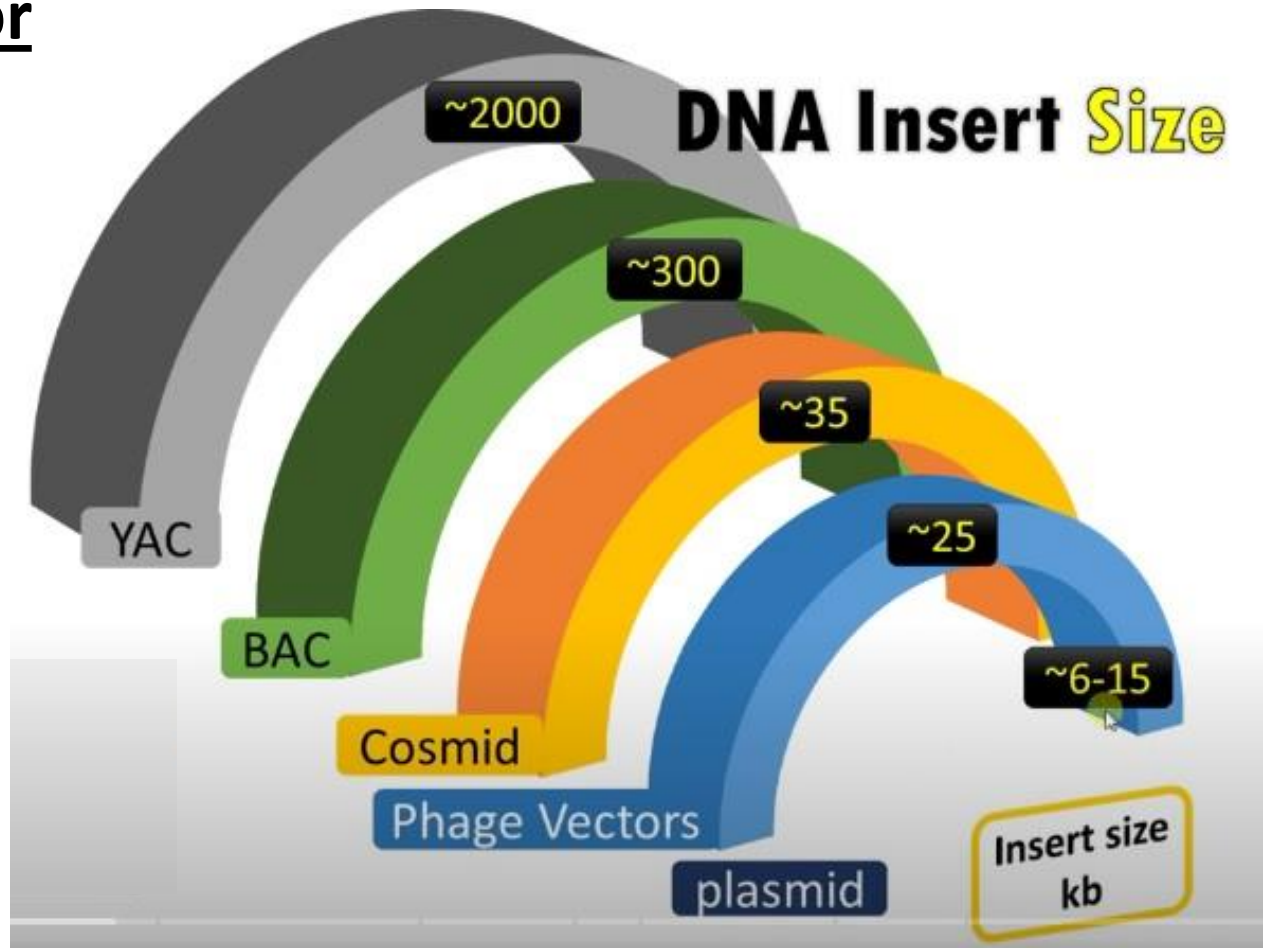
- ✓ However, the most commonly used cloning vectors include plasmids and bacteriophages (phage λ) beside all the other available vectors.

Gene Cloning

2. Selection of suitable cloning vector

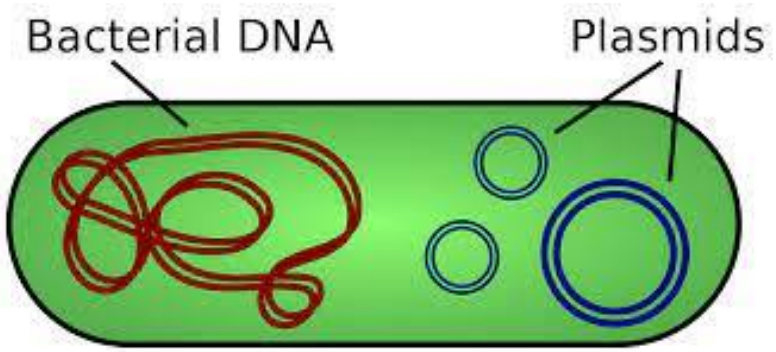
The cloning vectors are limited to:

- Size of insert that they can carry
- Application (cloning vector and expression vector)



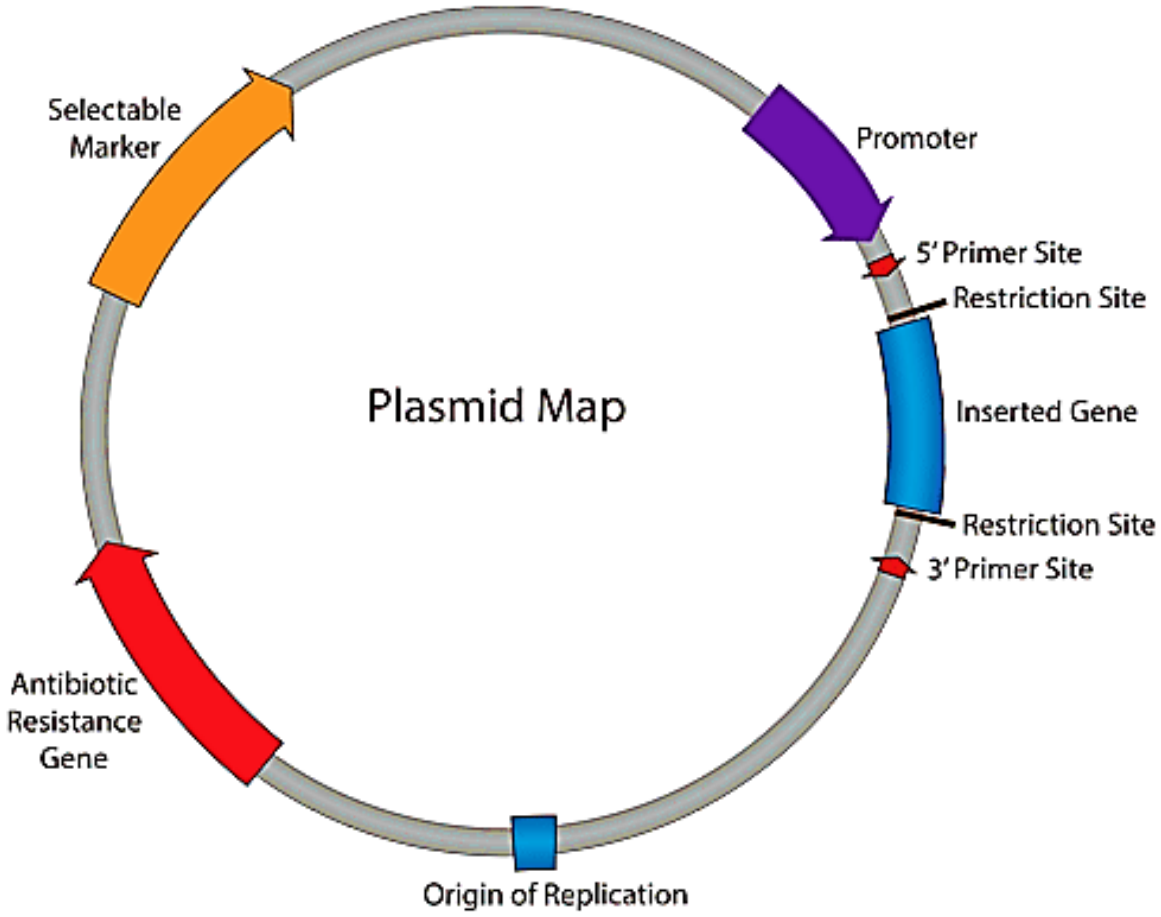
Plasmids

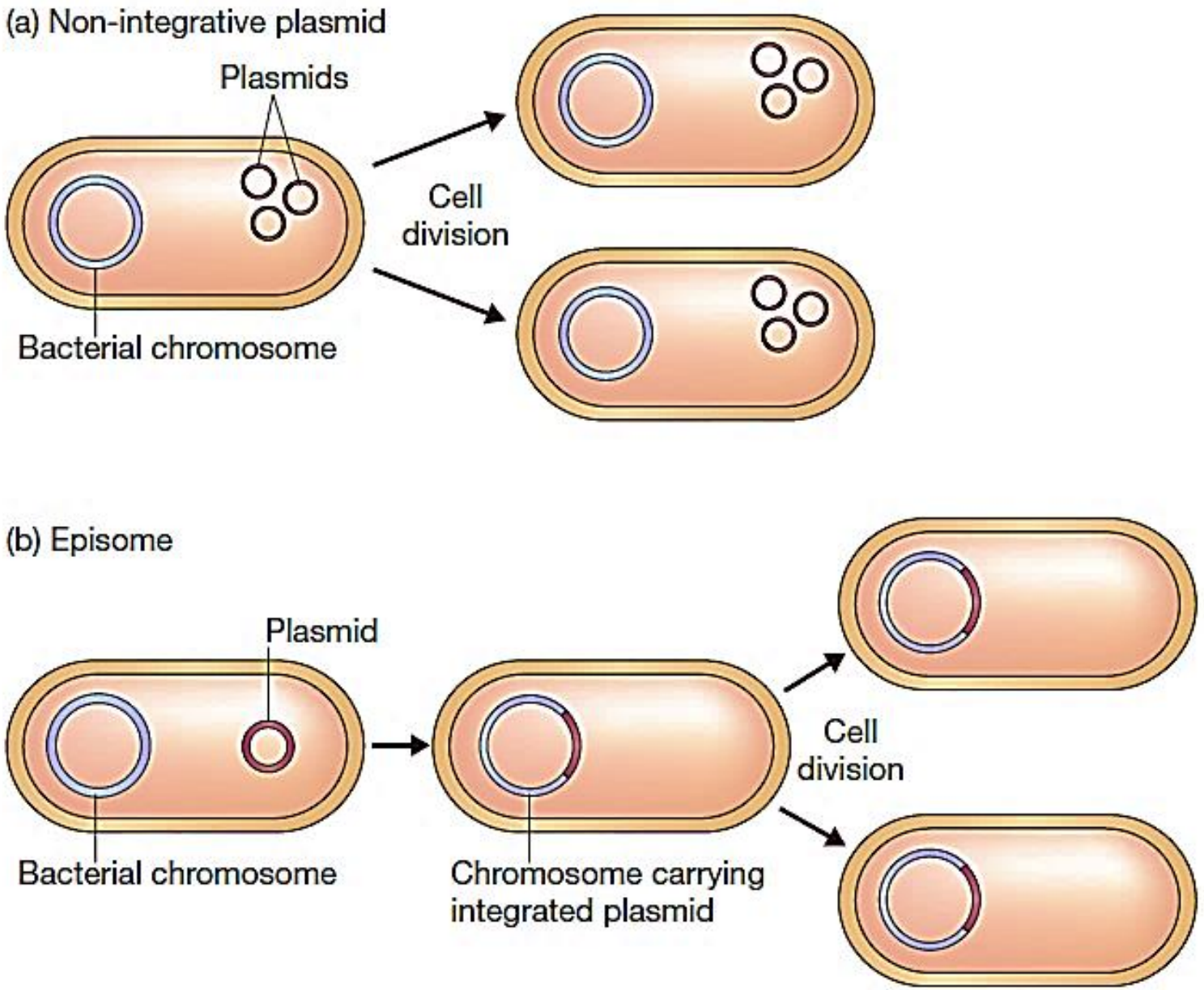
- A plasmid is a small, extrachromosomal DNA molecule within a cell that is physically separated from chromosomal DNA and can replicate independently.
- They are most commonly found as small circular, double-stranded DNA molecules in bacteria.



A typical cloning/expression plasmid vector

Origin of Replication (ori): Site where DNA replication begins — essential for plasmid copying in host cells.
Antibiotic Resistance Gene: Allows selection of cells that have taken up the plasmid (e.g., by growing on antibiotic-containing media).
Selectable Marker: General term for genes (like antibiotic resistance) used to identify and select transformed cells.
Promoter: DNA sequence that initiates transcription of the inserted gene — controls when and how much protein is made.
Restriction Sites: Specific DNA sequences cut by restriction enzymes — used to insert foreign DNA into the plasmid.
Inserted Gene: The target gene cloned into the plasmid for expression or study.
5' & 3' Primer Sites: Binding sites for PCR primers — used to amplify or verify the inserted gene.




(a) Non-integrative plasmid
Plasmids
Cell division
Bacterial chromosome
(b) Episome
Plasmid
Cell division
Bacterial chromosome
Chromosome carrying integrated plasmid

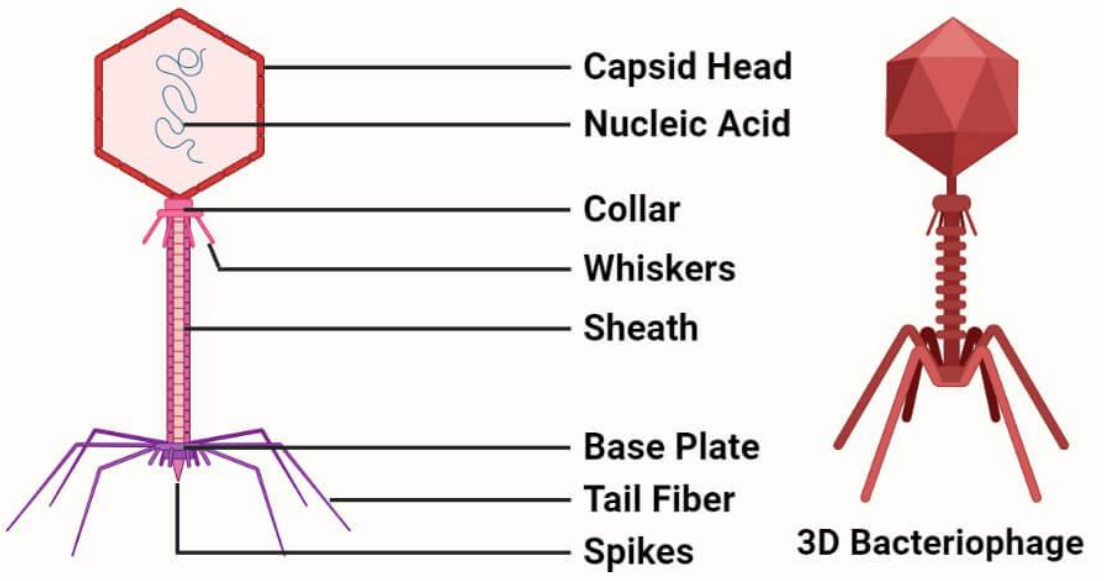
Aim	Key features	Plasmid
Mammalian protein expression	CMV promoter;Flag and HA tags.	pCDNA3 Flag HA
Transgenic mice generation	Neomycin resistance gene.	pBigT
Retroviral-mediated mammalian protein expression	Retroviral packaging sequences	pBABE
Lentiviral-mediated mammalian shRNA expression	Lentiviral packaging sequences; U6 promoter	pLKO.1
Recombinant protein production and purification from bacteria	Glutathione S-transferase (GST) tag	pGEX
Recombinant protein production and purification from bacteria	Maltose binding protein (MBP) tag	pMAL
Simple cloning experiment	-	pUC19

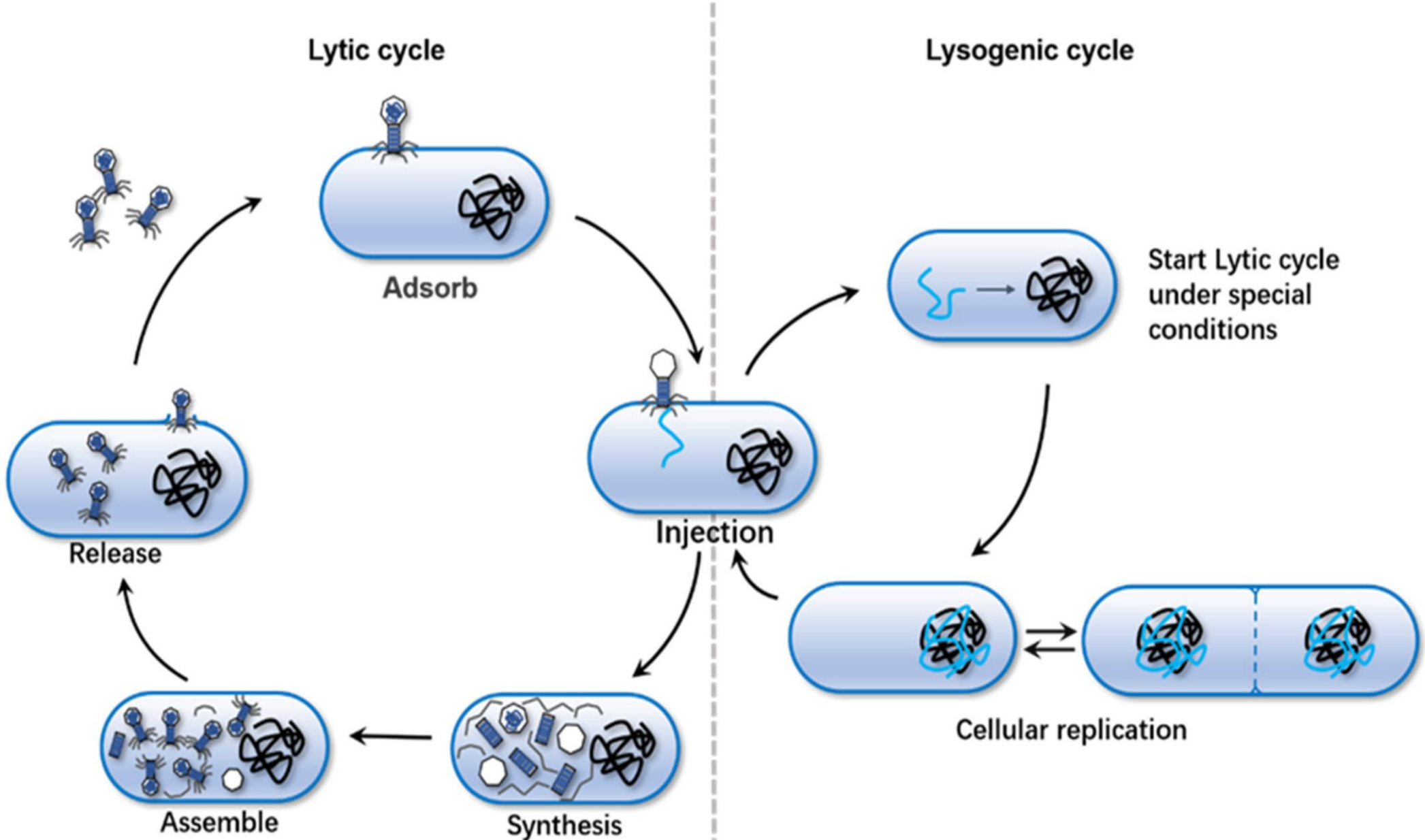
Bacteriophages

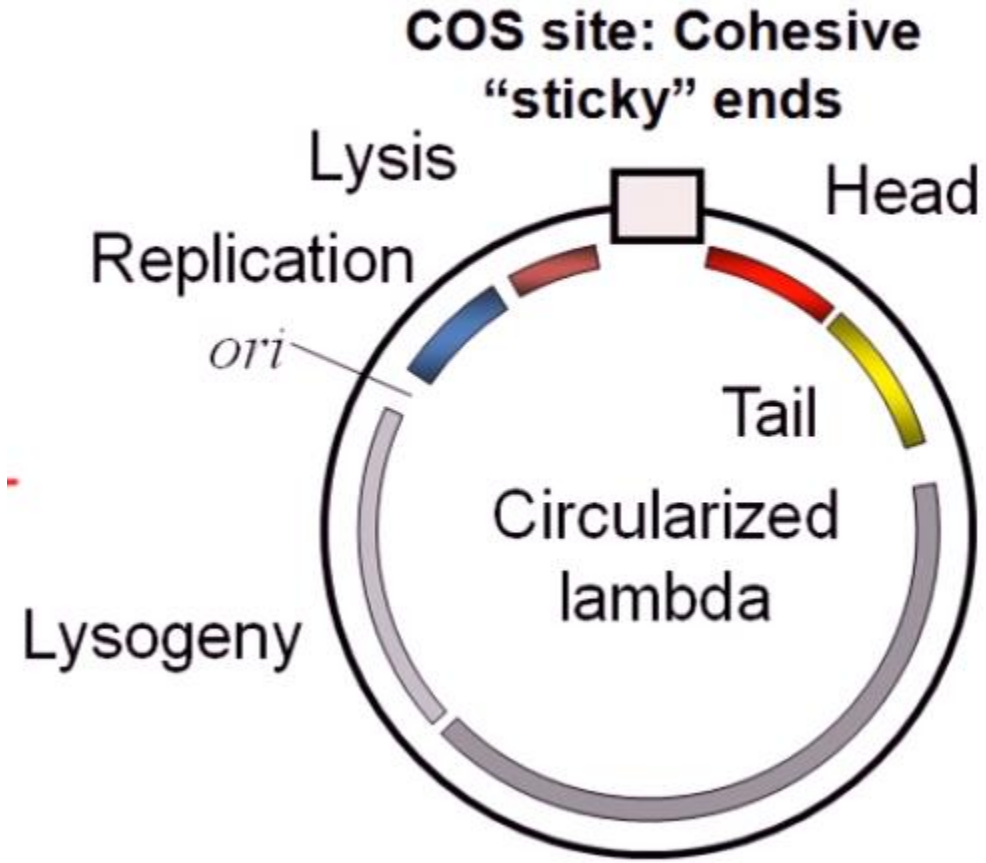
A bacteriophage, also known informally as a phage, is a virus that infects and replicates within bacteria.

Specific host: Each phage infects only one or a few specific bacterial species (e.g., λ phage infects only E. coli).

Simple structure:
Composed of a capsid (protein coat) containing DNA or RNA, and sometimes a tail used for attachment to the bacterial cell.




Lytic cycle
Lysogenic cycle
Adsorb
Start Lytic cycle
under special
conditions
Injection
Release
Cellular replication
Assemble
Synthesis



Circularized lambda: The circular form of λ phage DNA after infection, ready for replication or integration.

COS site: Cohesive "sticky" ends — allows linear phage DNA to circularize upon entering the host.
Head: Encapsulates the phage DNA (genome).
Tail: Structure used for attachment to and injection of DNA into the bacterial host.
Lysis: Genes involved in breaking open (lysing) the host cell to release new phage particles.
Replication: Region containing the origin of replication (ori) for copying the phage DNA.
Lysogeny: Genes that allow the phage to integrate into the host chromosome and enter a dormant (lysogenic) state.

Applications of phages in gene cloning:

Although plasmids are now the most common vectors, bacteriophages (e.g., λ phage) have been widely used in classical cloning, especially for:

1. **Genomic libraries**: λ phage can carry larger DNA fragments (10–20 kb) than plasmids (~5–10 kb), making it ideal for whole-genome cloning.

2. **cDNA libraries**: Used to clone cDNA copies of mRNA from specific tissues.

3. **Phage display expression systems**: a powerful molecular biology technique that allows the expression of peptides, proteins, or antibody fragments on the surface of bacteriophages (viruses that infect bacteria), while the genetic information encoding them remains inside the phage particle.
   - This technique is crucial for the development of biomedical applications, such as monoclonal antibodies.

Comparison: Plasmid vs. Lambda Phage Vectors

Feature	Plasmid	Lambda Phage (λ)
Max DNA Insert Capacity	~10 kb	~20 kb
Entry Method into Cell	Transformation	Infection
Transfer Efficiency	Moderate	Very High
Primary Application	Small-scale cloning / Expression	Genomic libraries

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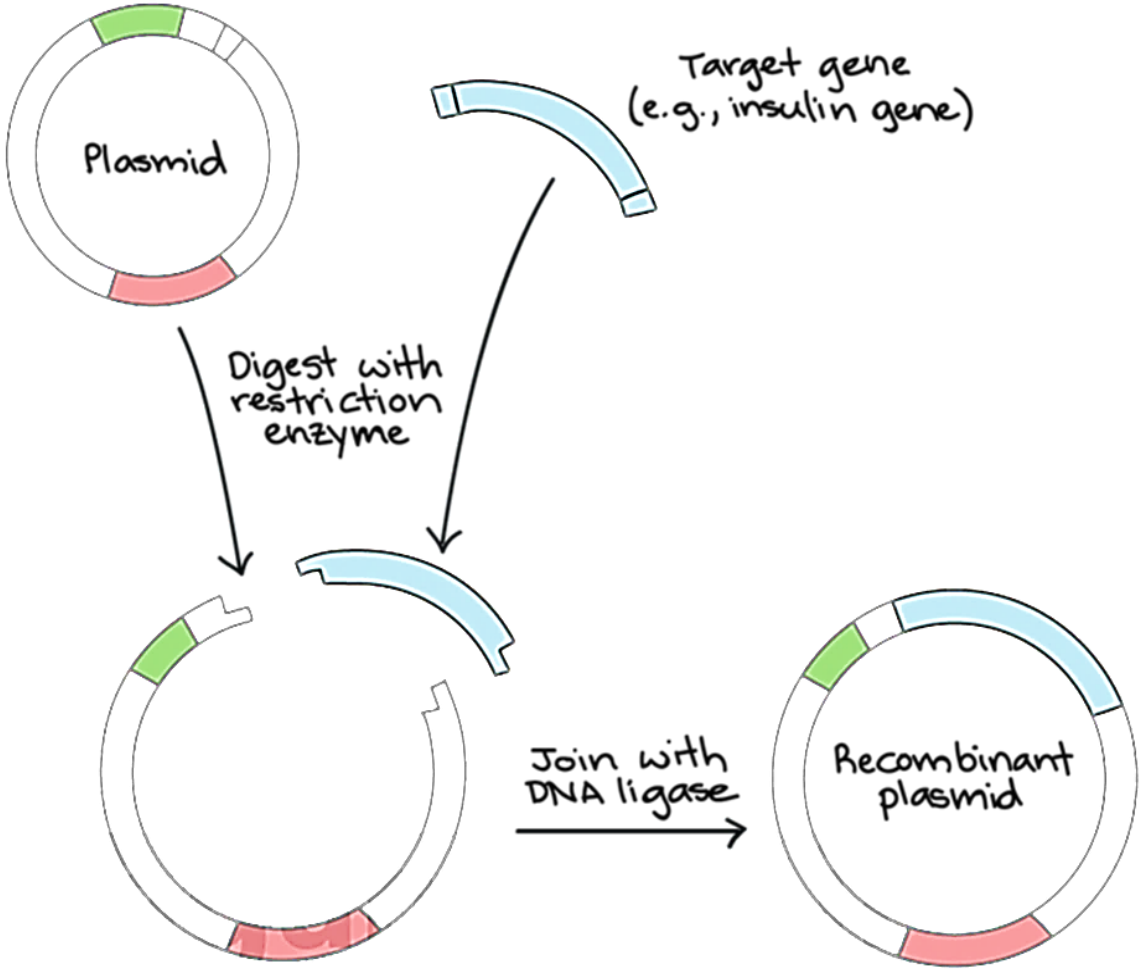
Gene Cloning

3. Formation of Recombinant DNA

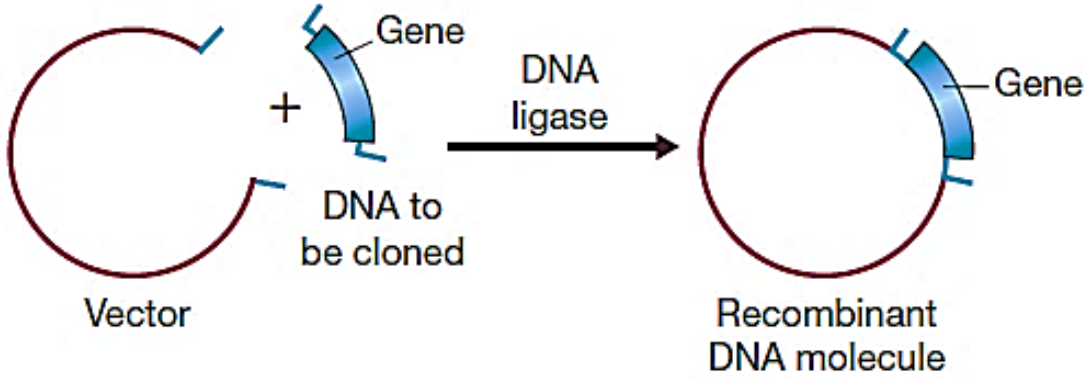
- ✓ The plasmid vector should cut open by the same restriction enzyme that is used for isolation of the gene of interest form DNA fragment.
- ✓ The gene of interest (DNA fragment) and plasmid vector should mixed together.
- ✓ In the presence of DNA ligase, base pairing of DNA fragment and plasmid vector should take place.

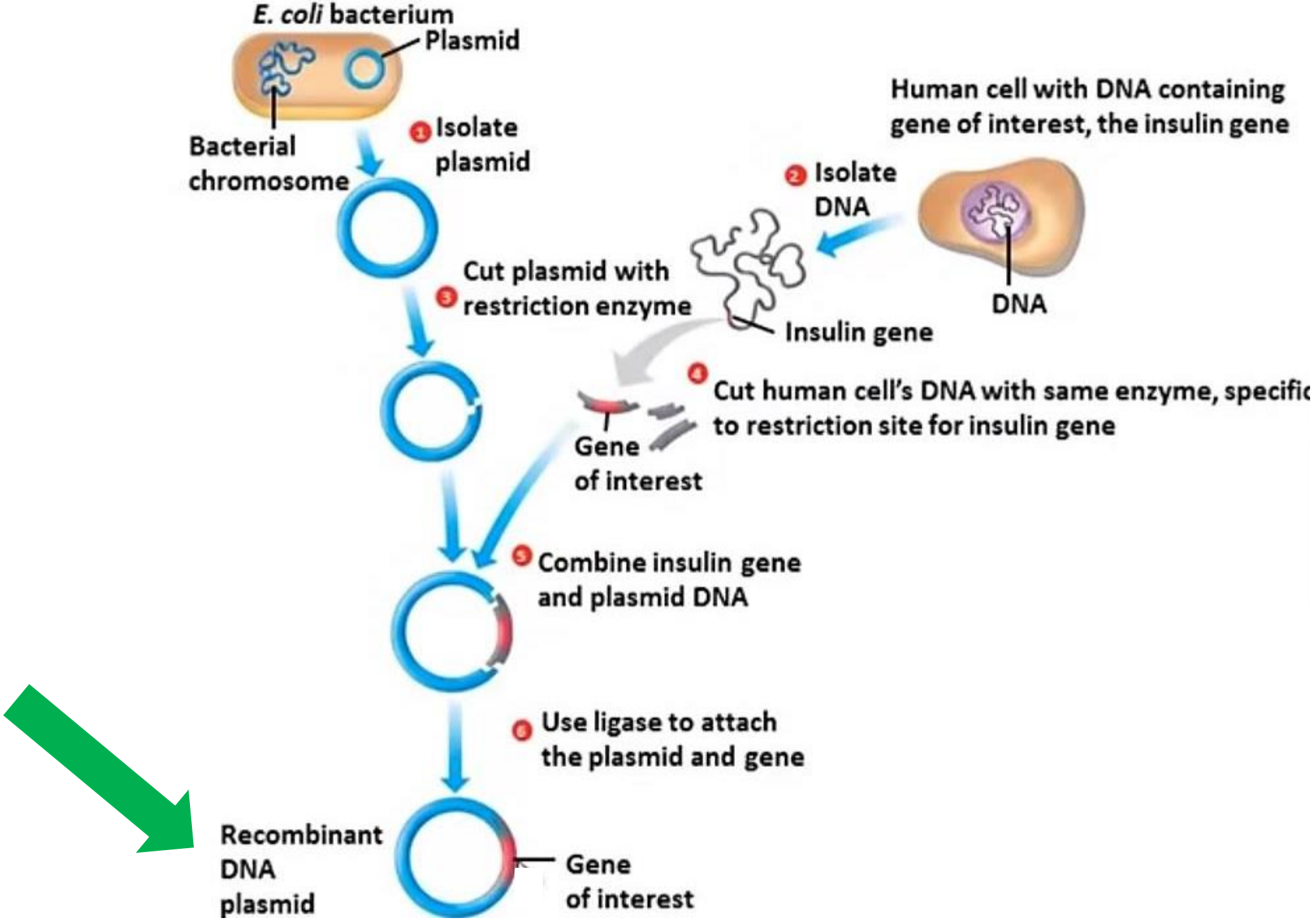


Gene Cloning

3. Formation of Recombinant DNA

✓ The resulting DNA molecule is a hybrid of two DNA molecules – the gene of interest and the vector. In the terminology of genetics this is called recombination.




E. coli bacterium
Plasmid
Bacterial chromosome
1 Isolate plasmid
Human cell with DNA containing gene of interest, the insulin gene
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5 Combine insulin gene and plasmid DNA
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Recombinant DNA plasmid
Gene of interest

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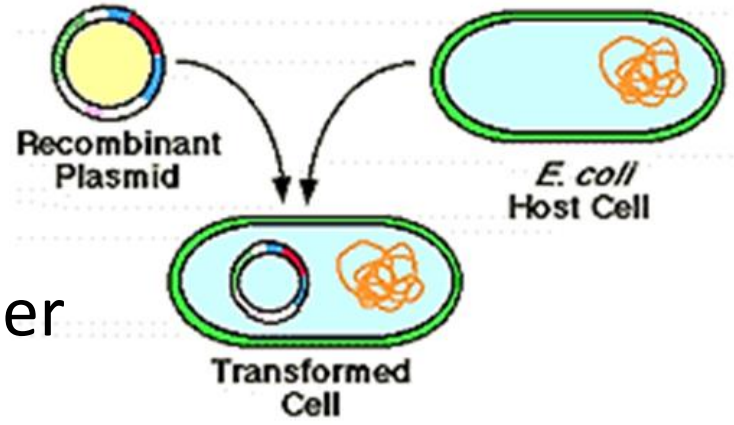
Gene Cloning

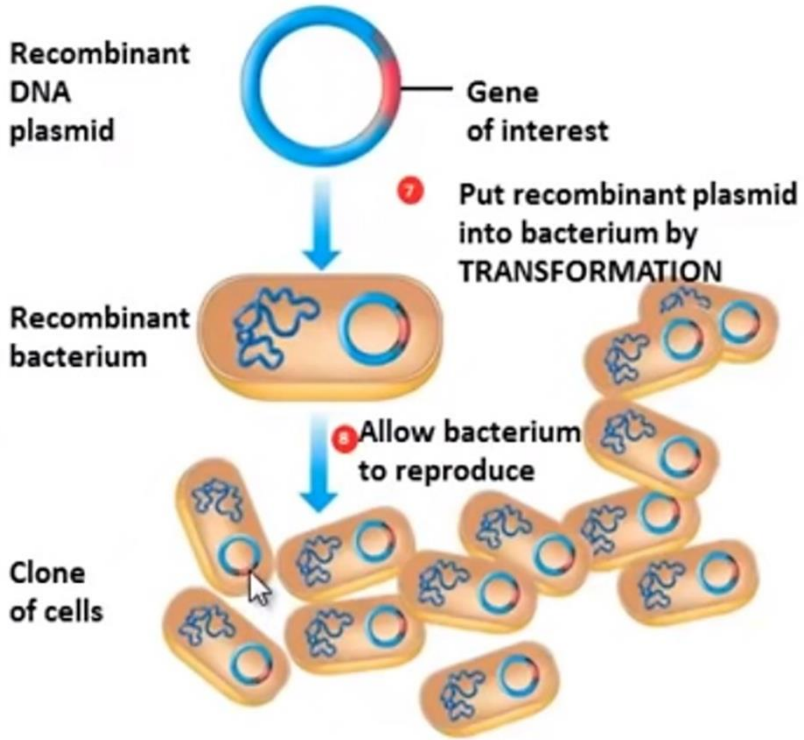
4. Transformation of recombinant vector into suitable host

- ✓ The recombinant generated is transformed into suitable host cell, bacterial cell generally.



- ✓ This is done may be for the following reasons:
  - To replicate the recombinant DNA molecule in order to get the multiple copies of the gene of interest .
  - To allow the expression of the gene of interest such that it produces its needed protein product.

Examples of the most common *E. coli* strains used for molecular cloning.

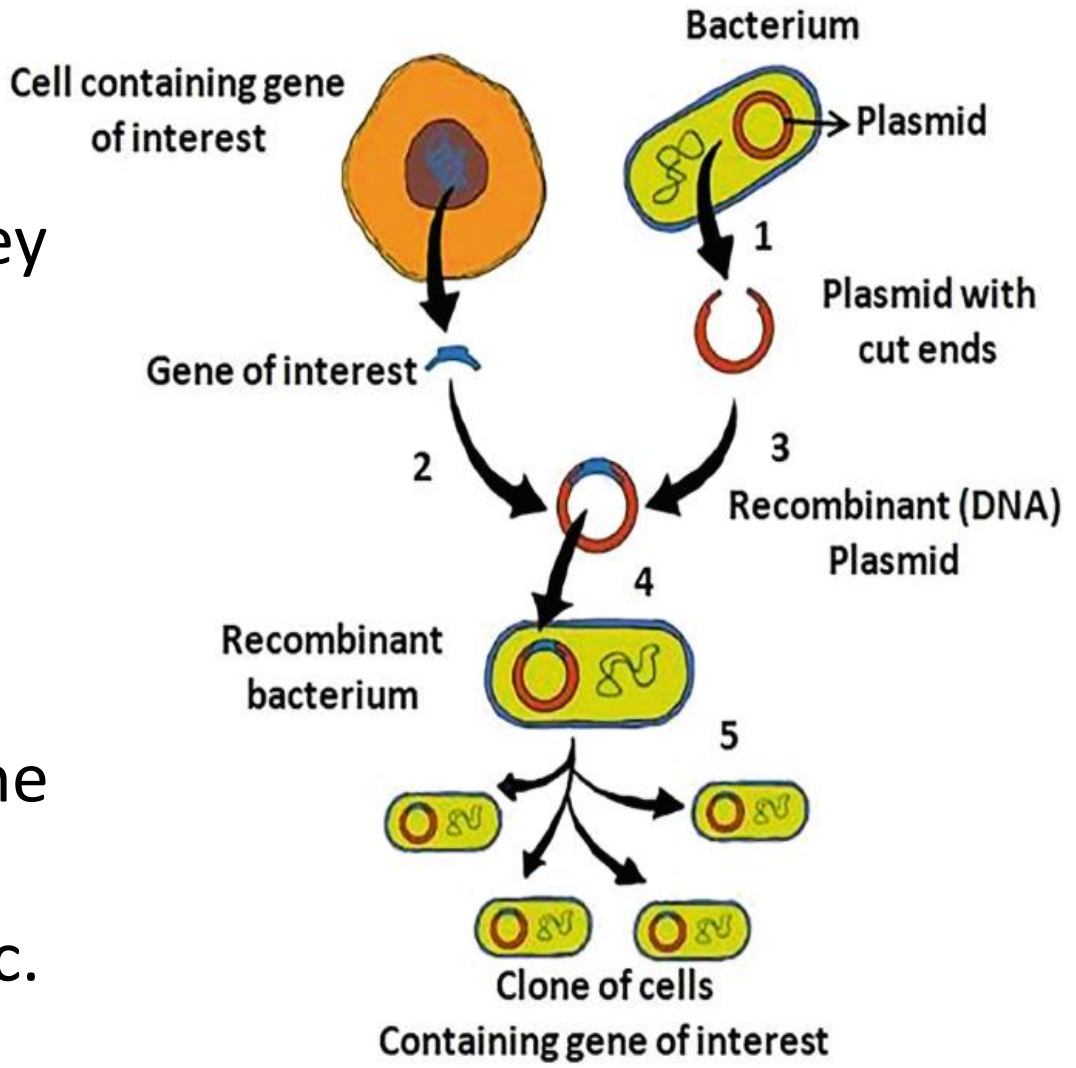


Strain	Key attribute	Optimized for:
BL21(DE3)	Expresses the T7 RNA Polymerase under the lacZ promoter (inducible by the lactose analog IPTG)	General expression of recombinant proteins
BL21(DE3) pLysE*	Lower basal expression levels of T7 RNA polymerase compared to BL21(DE3)	Expression of toxic recombinant proteins
DB3.1	Mutation in gyrA gene makes it resistant to toxin from ccdB gene	Propagation of plasmids expressing the ccdB gene (Gateway system)
DH5α	-	General cloning procedures
JM110	Lacks DNA methyltransferases	Growth of plasmids that must not be methylated
Origami2 (DE3)	Enhanced activity of enzymes that facilitate protein folding (reductases)	Expression of proteins poorly soluble
Rosetta2 (DE3)	Contains additional tRNAs for rare codons that are poorly expressed in E. Coli	Optimized expression of eukaryotic proteins by bypassing codon-bias problems
Stbl2	Lacks an enzyme involved in DNA recombination (recA)	Growth of plasmid containing with high potential to recombine (like lenti- and retroviral plasmids)
XL10 Gold	Exhibit the Hte (high transformation efficiency) phenotype	Transformation of large plasmids and preparation of DNA libraries

Gene Cloning

4. Transformation of recombinant vector into suitable host

- ✓ Some bacteria are natural transformers; they do the uptake the recombinant vector automatically.
- ✓ For example: *Bacillus*, *Haemophillus*, *Helicobacter pylori*, which are naturally competent bacterial cells.
- ✓ On the other hand some bacteria require the incorporation by artificial methods such as Ca^{++} ion treatment, electroporation, PEG etc.

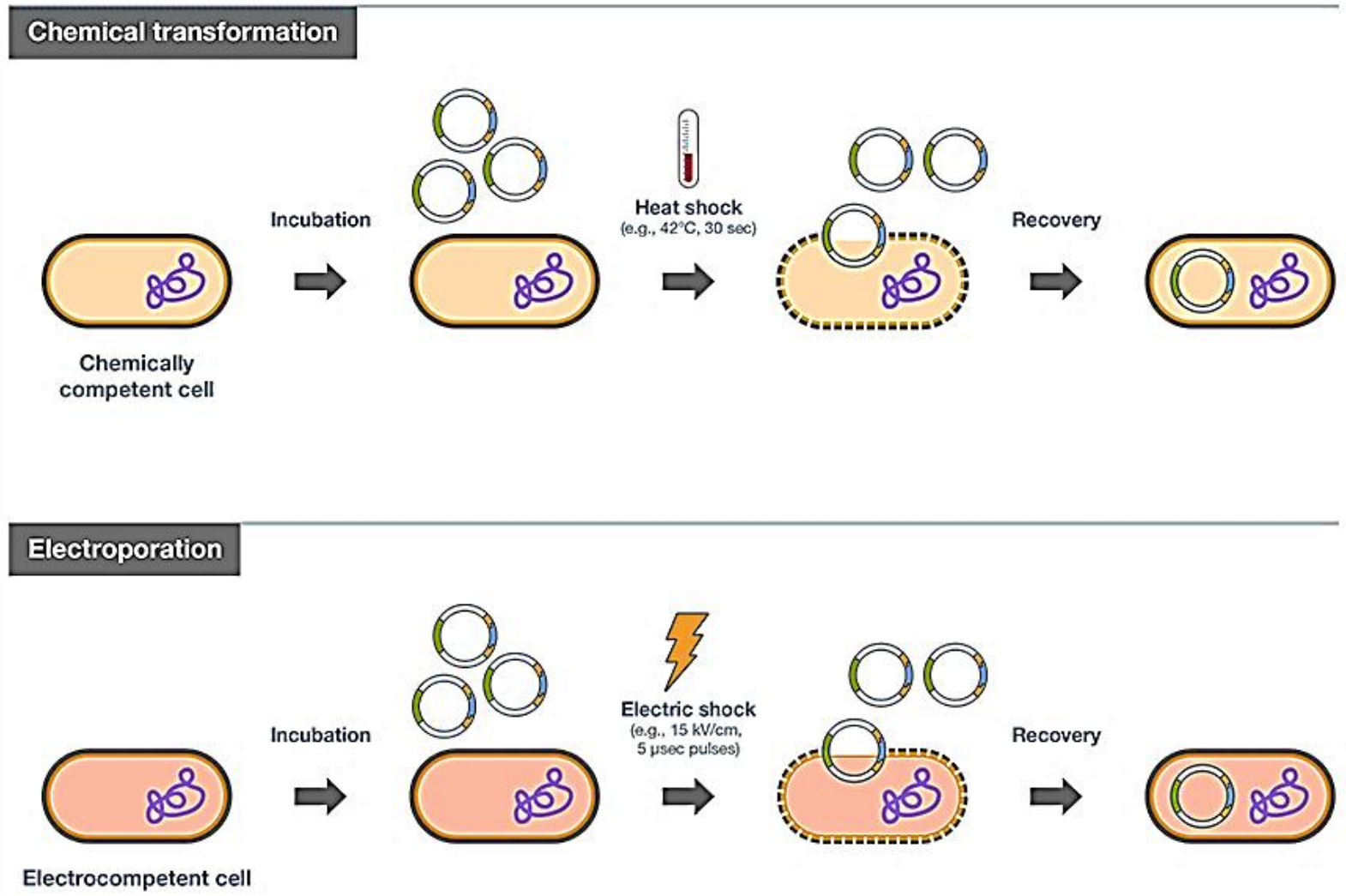


Gene Cloning

- *E. coli* can be made competent for DNA uptake in two main ways:

1. Chemical competence

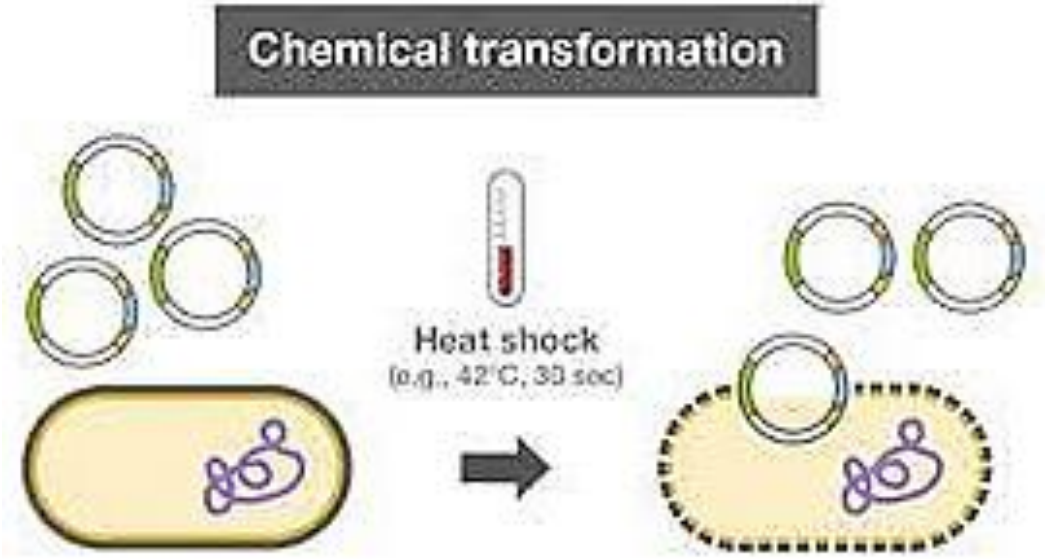
2. Electroporation



Gene Cloning

Chemical competence

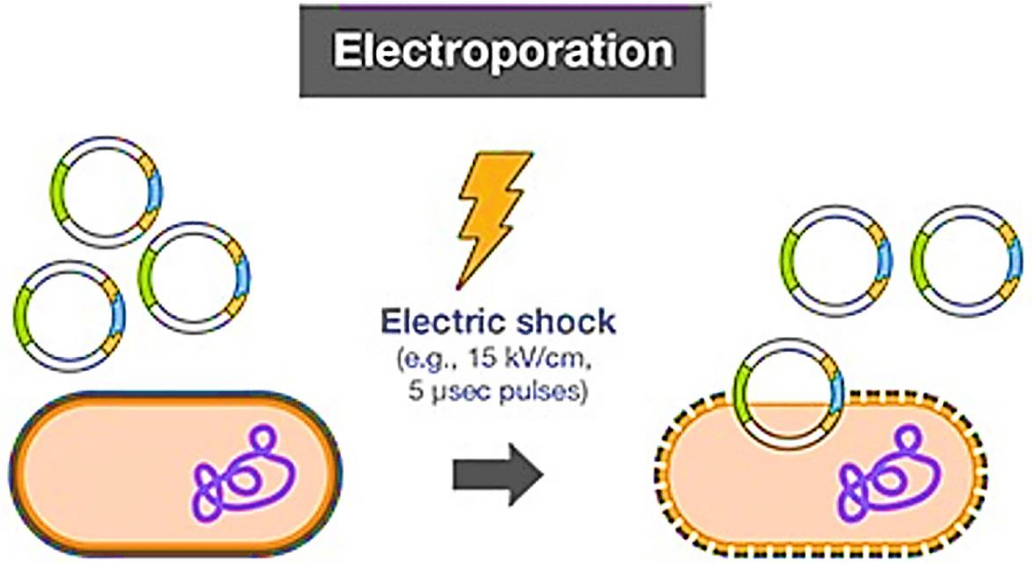
- This is achieved by pre-treating the cells with chemicals (often calcium chloride) under cold conditions, followed by a short pulse of heat shock, which together increases permeability of the cell membrane to the DNA.
- Plasmids up to 10 kb can be efficiently introduced using this method, and given its simplicity this is the most commonly used technique to introduce recombinant DNA into bacteria.

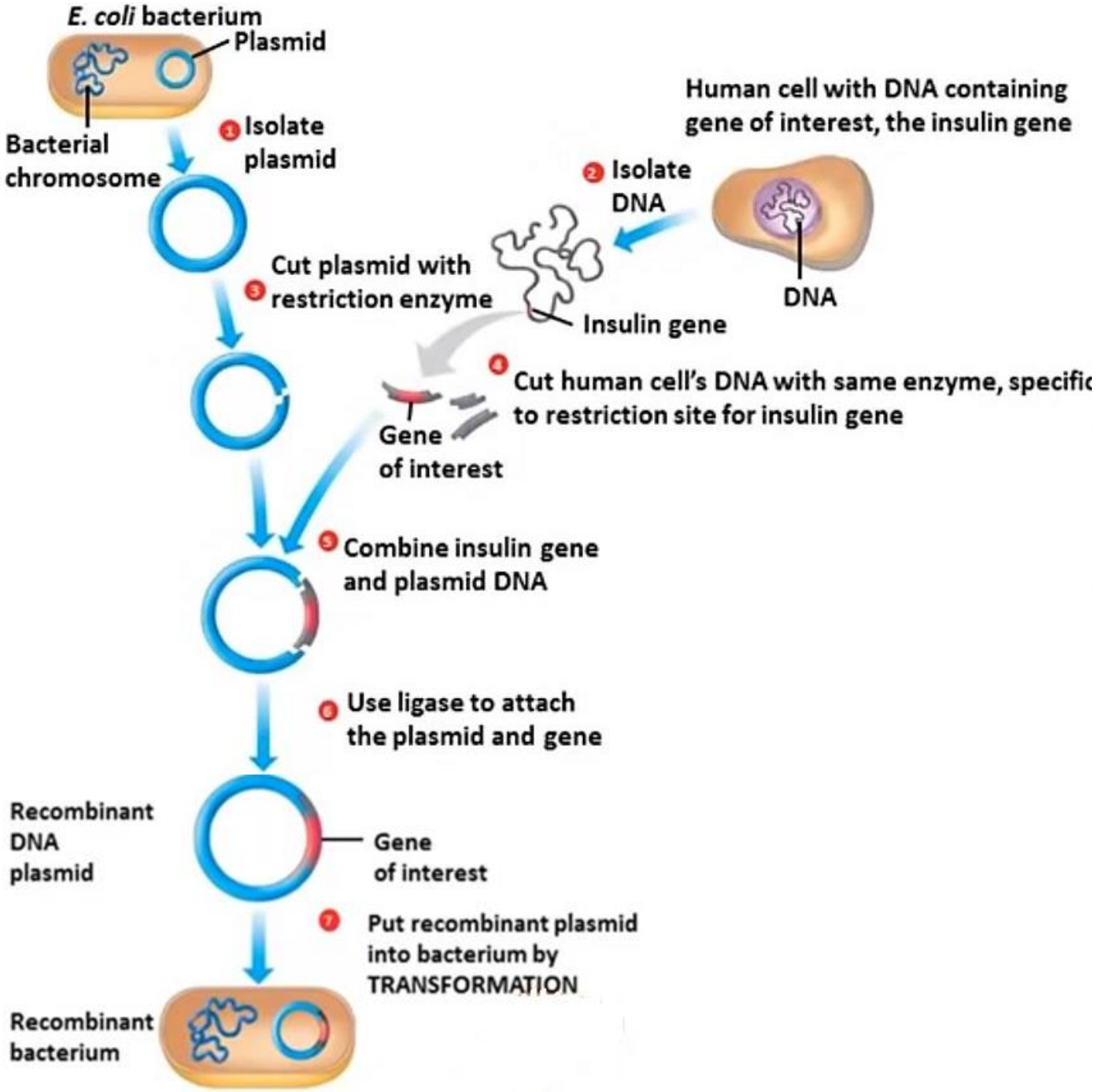


Gene Cloning

Electroporation

- The cells are subjected to a brief electric shock that generates small pores into the cell surface, thus allowing plasmid DNA to enter.
- This technology has a higher efficiency compared to chemical transformation and is used mostly to introduce very large plasmids.

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5 Combine insulin gene and plasmid DNA
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Recombinant DNA plasmid
Gene of interest
7 Put recombinant plasmid into bacterium by TRANSFORMATION
Recombinant bacterium

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Gene Cloning

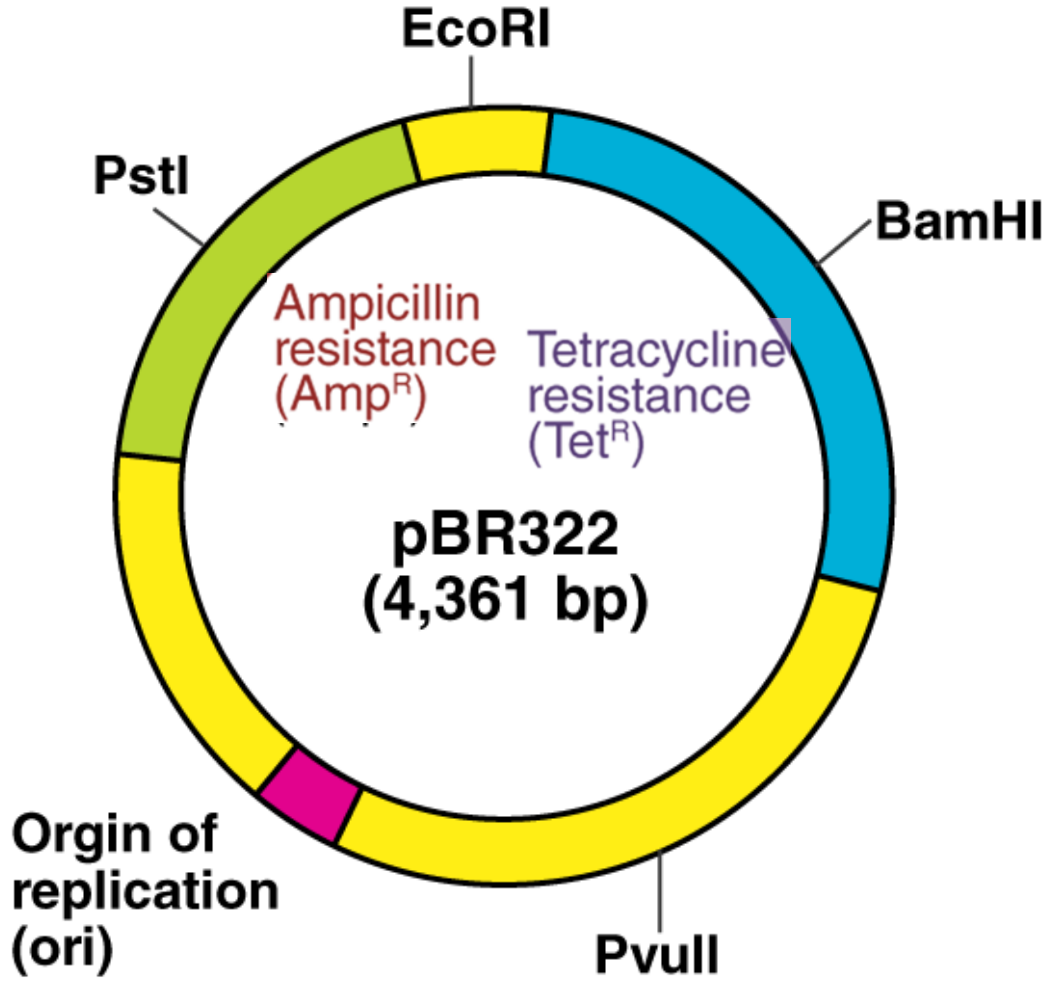
5. Isolation of Recombinant Cells

- The transformation generates both transformed and non-transformed host cells (a mixed population of cells in colony).
- The selectable marker gene of plasmid used as vector is used for isolation or selection of recombinant cell from non-recombinant cell.

Gene Cloning

5. Isolation of Recombinant Cells

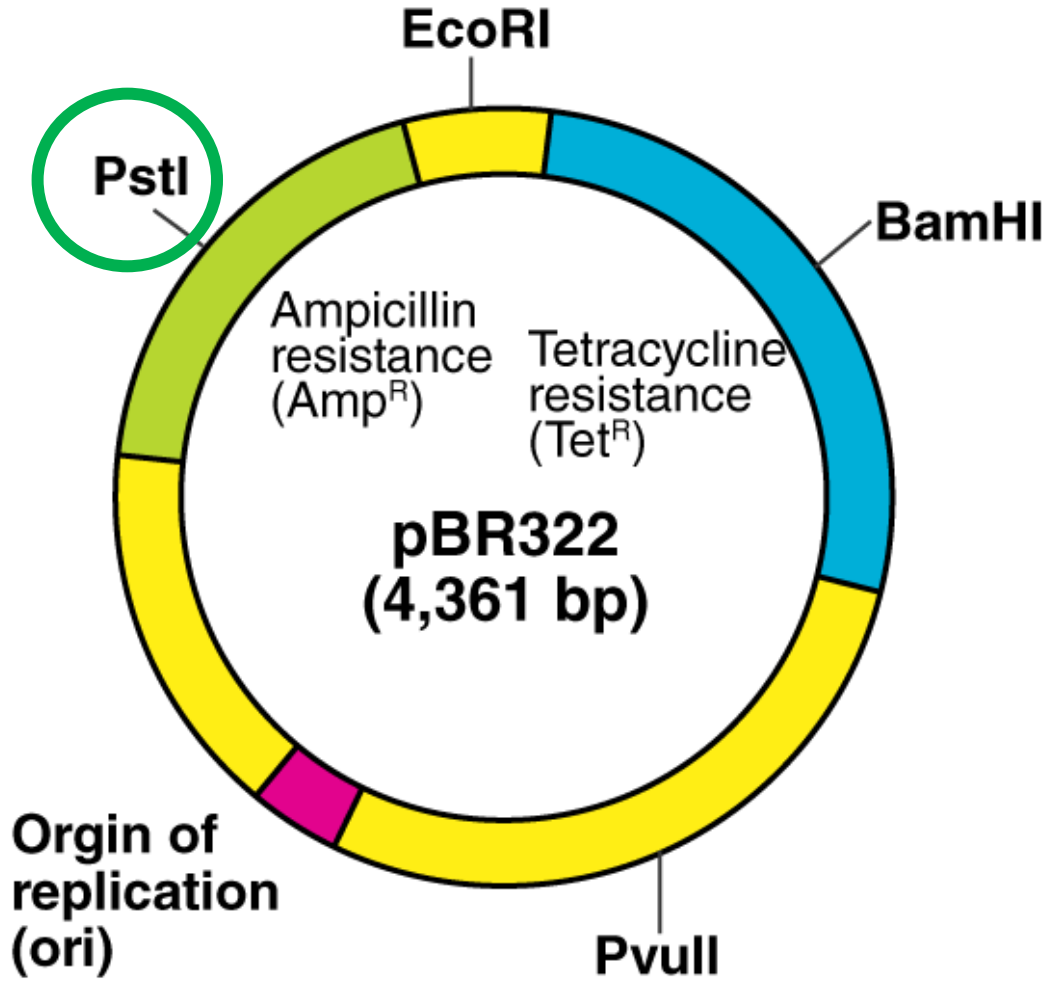
- For examples, pBR322 most common plasmid vector contains two selectable marker gene (Ampicillin resistant gene and Tetracycline resistant gene).



Gene Cloning

5. Isolation of Recombinant Cells

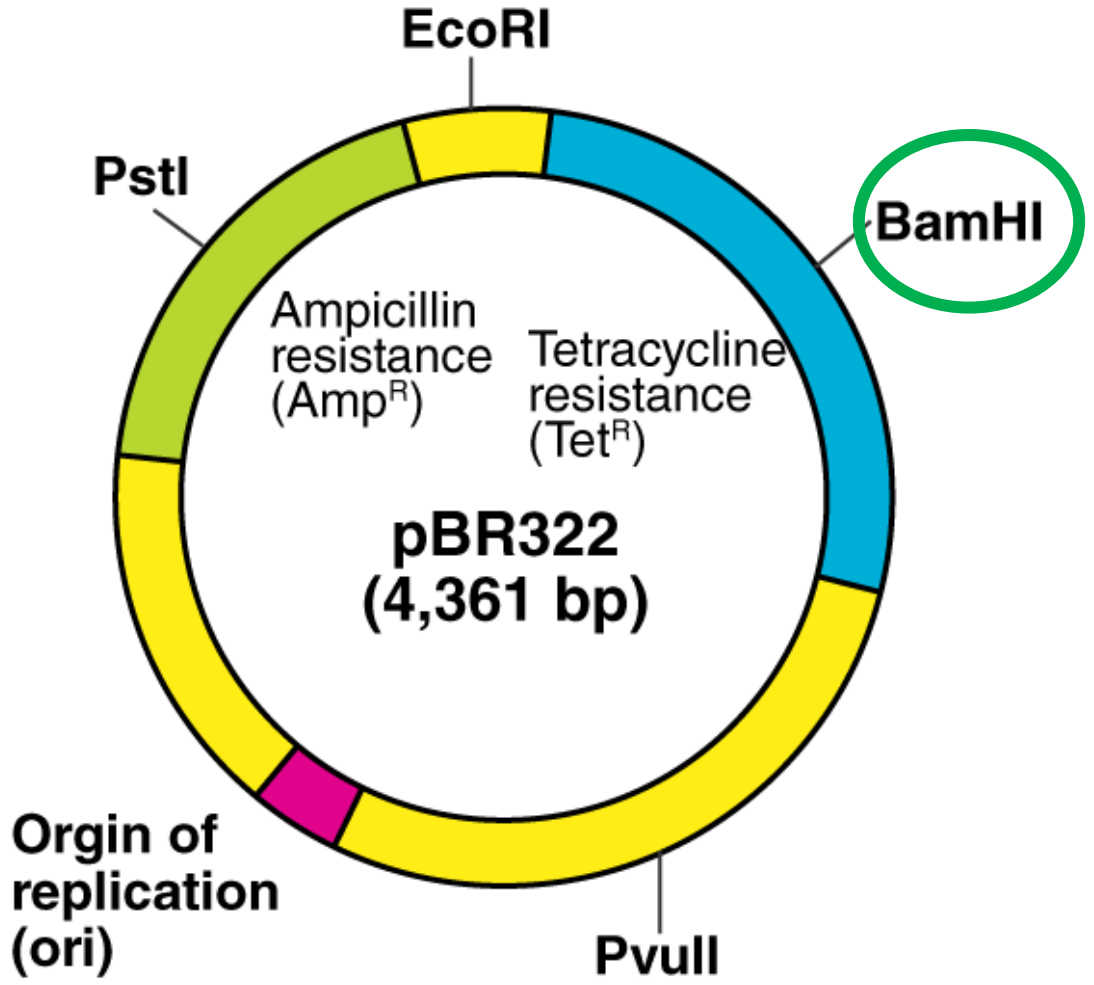
- When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the transformed cell become sensitive for the presence of Ampicillin.



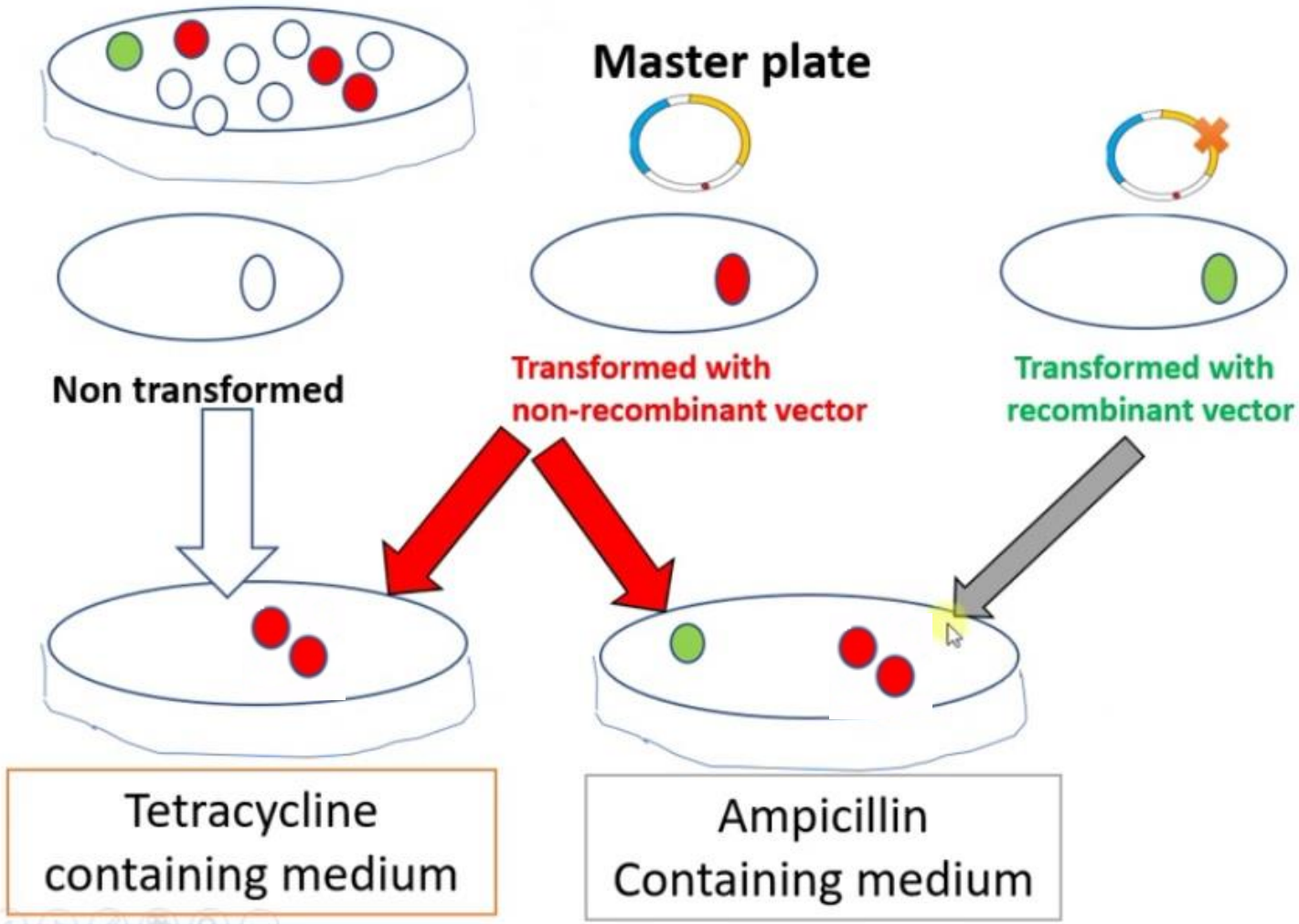
Gene Cloning

5. Isolation of Recombinant Cells

- When BamHI RE is used it knock out Tetracycline resistant gene from the plasmid, so that the transformed cell become sensitive for the presence of Tetracycline .



= majority non transformed without vector
= transformed with unaltered vector
= transformed with recombinant vector



After transformation , three types of colonies

1) Non-transformed: without our vector: Cannot grow in Amp or Tetr containing medium

2) Transformed:

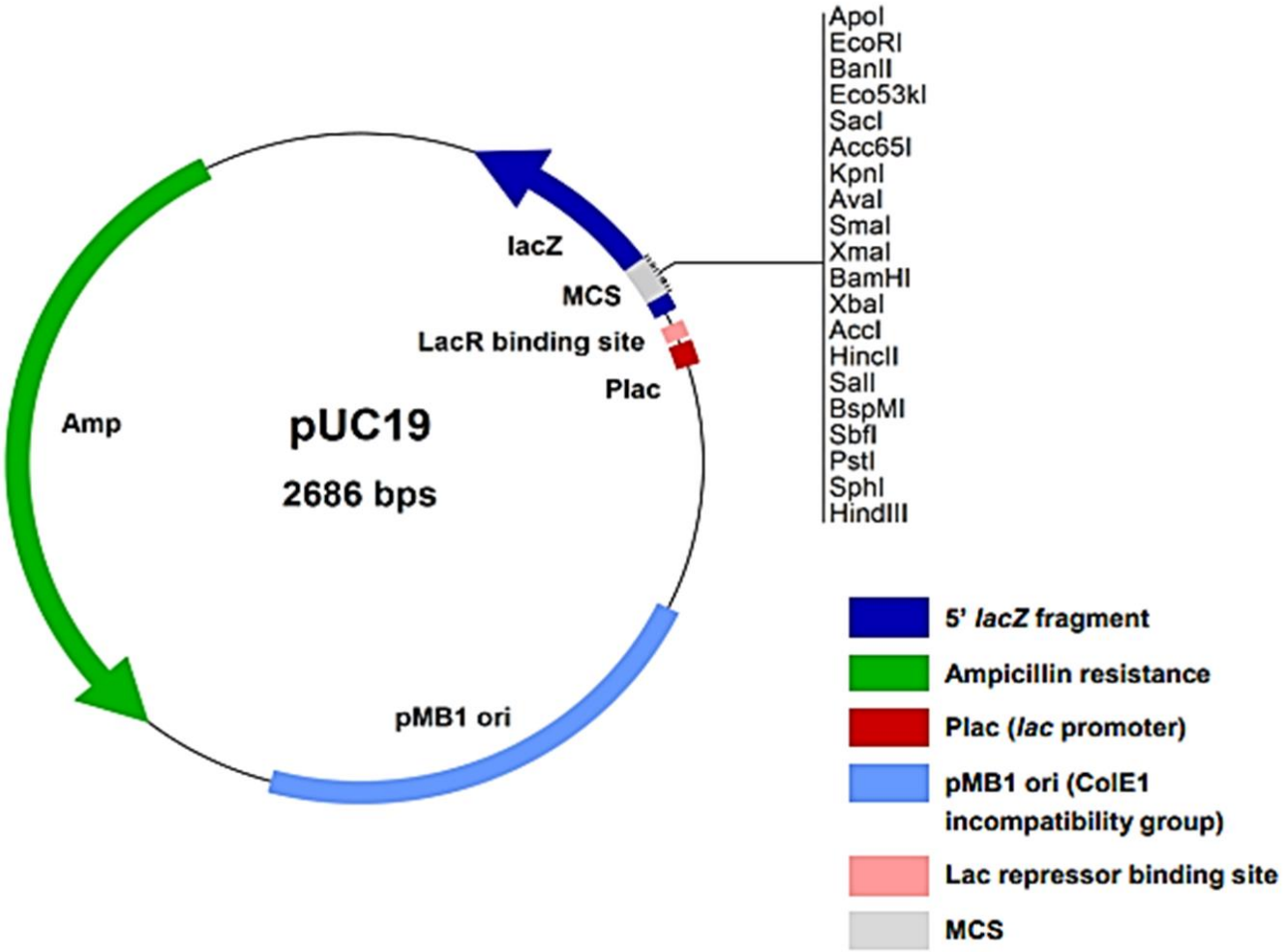
a) Transformed with non recombinant: Can grow in both

b)Transformed with recombinant vector : can grow only in Amp medium. we have inserted our gene of interest in tetracycline resistance region using Bam H1. Due to insertional inactivation Tet^r gene is no more functional.

Gene Cloning

5. Isolation of Recombinant Cells

- 2686 bp
- Selectable markers (**Resistant to ampicillin**)
- Multiple cloning site located within **lacZ**
- Origin of replication = pMB1
- Restriction sites

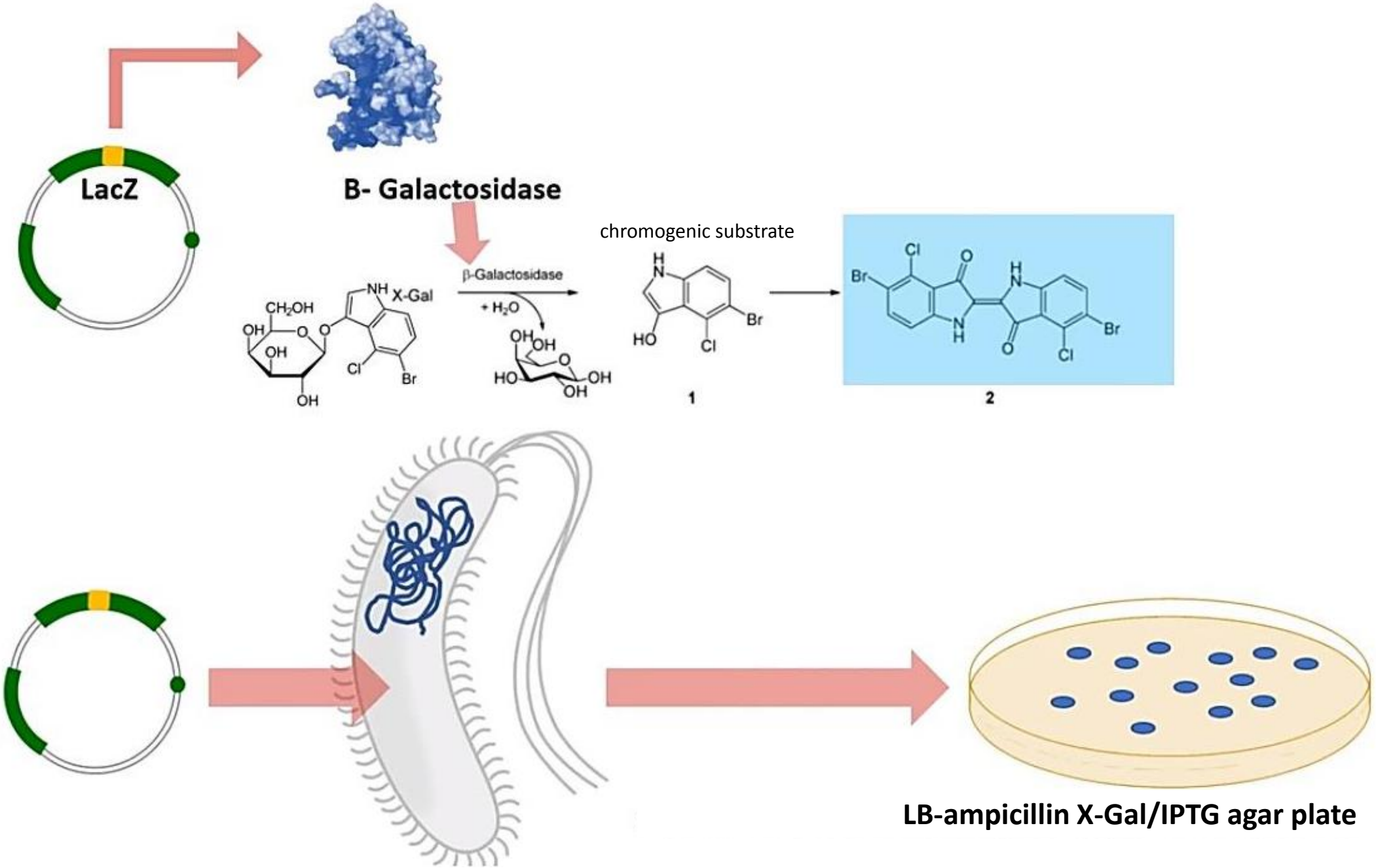


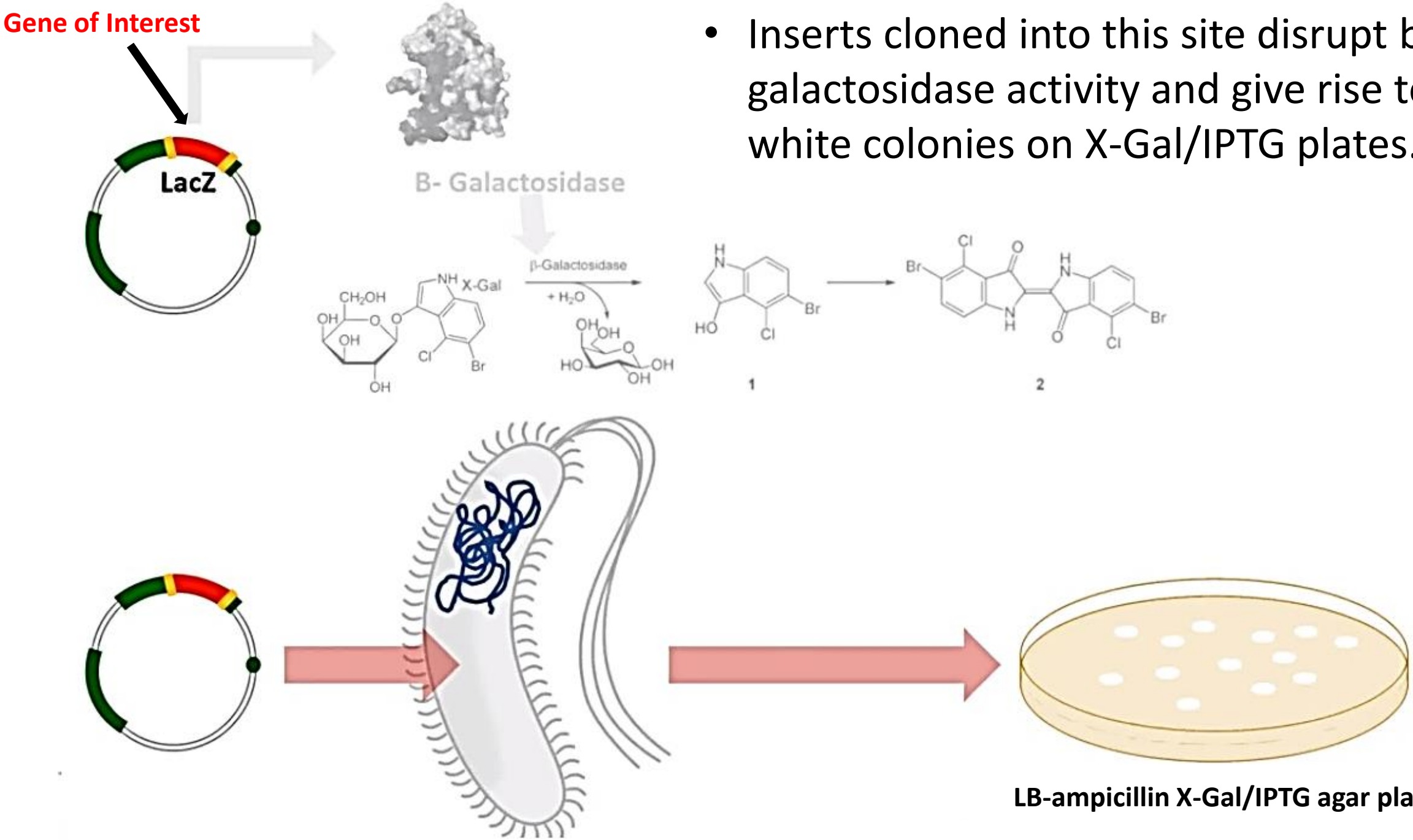
5. Isolation of Recombinant Cells

Blue-White Screening: Without the need for PCR or sequencing, desired (white) colonies can be selected simply by observing colony color.

LB-ampicillin X-Gal/IPTG agar plate

Component	Purpose
LB	Growth medium
Ampicillin	Selects for plasmid-containing cells
X-Gal	Colorimetric indicator for β-galactosidase activity
IPTG	Induces lacZ expression


LacZ
B- Galactosidase
chromogenic substrate
X-Gal
β-Galactosidase
+ H2O
1
2
LB-ampicillin X-Gal/IPTG agar plate



- Inserts cloned into this site disrupt beta - galactosidase activity and give rise to white colonies on X-Gal/IPTG plates.

Gene Cloning

Gene cloning involves following 7 essential steps:

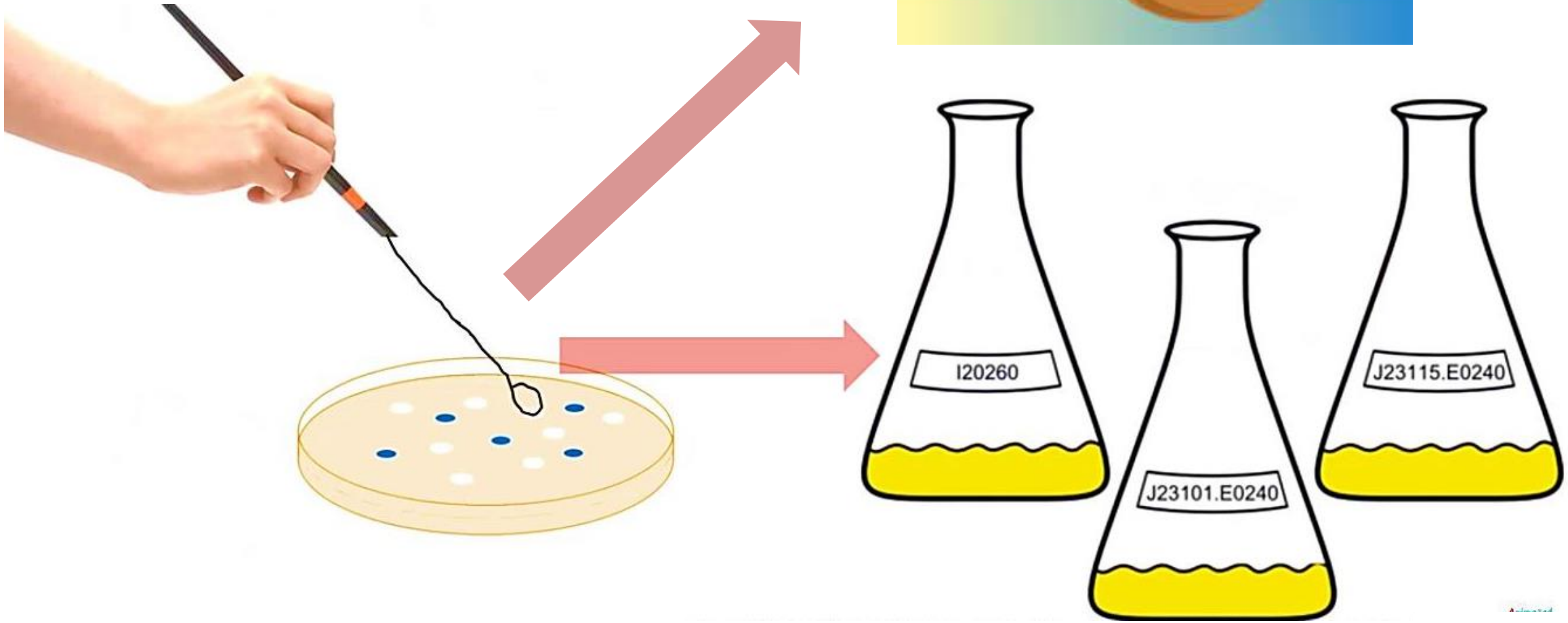
1. Isolation of specific DNA fragment containing gene of interest
2. Selection of suitable cloning vector
3. Formation of Recombinant DNA (Ligation)
4. Transformation of recombinant vector into suitable host
5. Isolation of Recombinant Cells
6. **Multiplication of Selected Host Cells**
7. Isolation and Purification of the Product

Gene Cloning

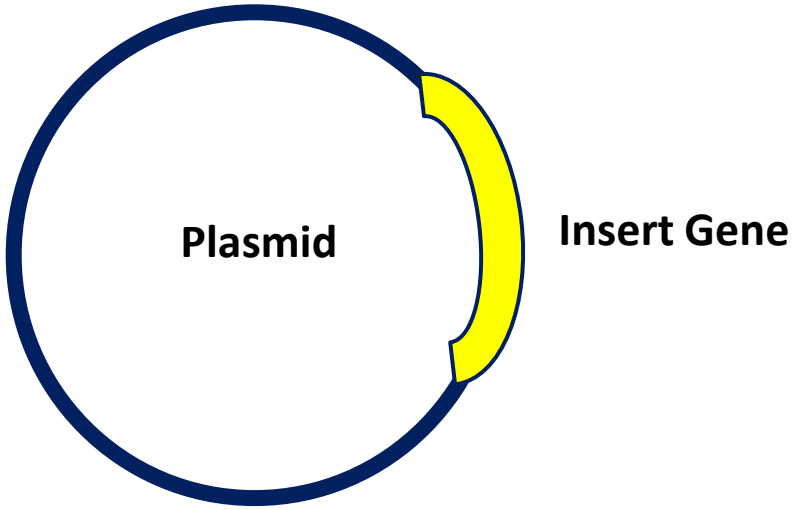
6. Multiplication of Selected Host Cells

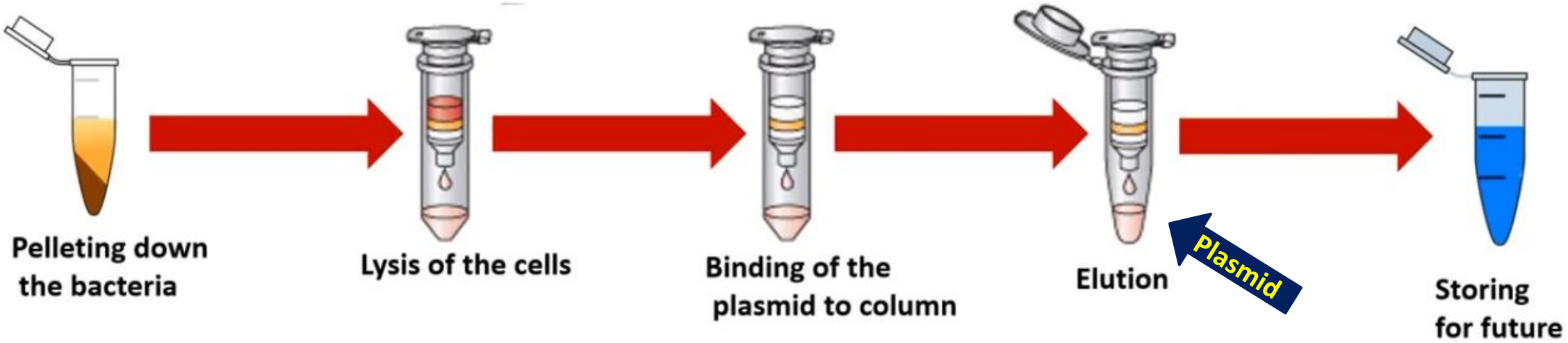
- After selecting and separating the transformed host cells by the screening methods; it becomes necessary to provide them optimum parameters to grow and multiply for generation of transformed colonies.
- In order to achieve this transformed host cells are introduced into fresh culture media.
- The host cells divide and re-divide along with the replication of the recombinant DNA carried by them and so generating multiple copies of desired gene.


Polymerase Chain Reaction
PCR


I20260
J23101.E0240
J23115.E0240

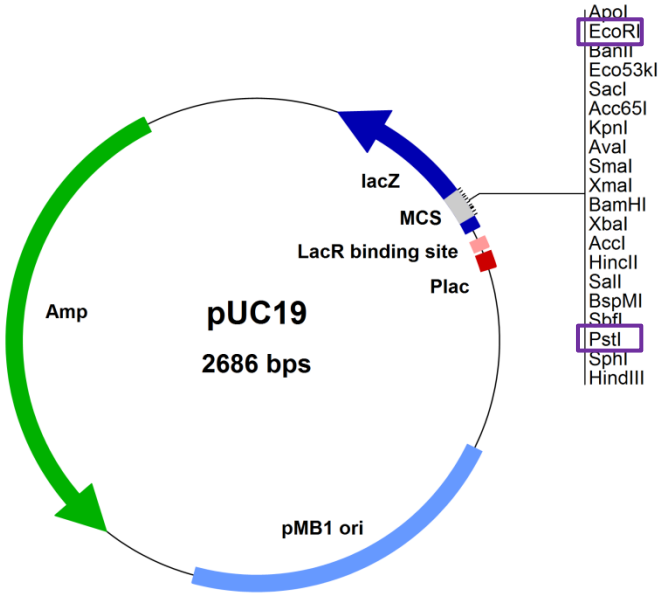
- **Plasmid isolation**

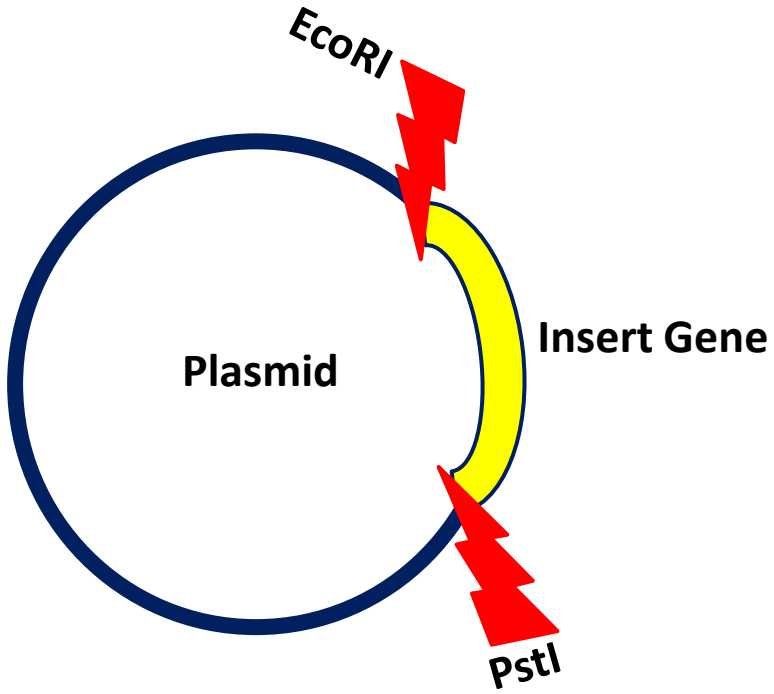


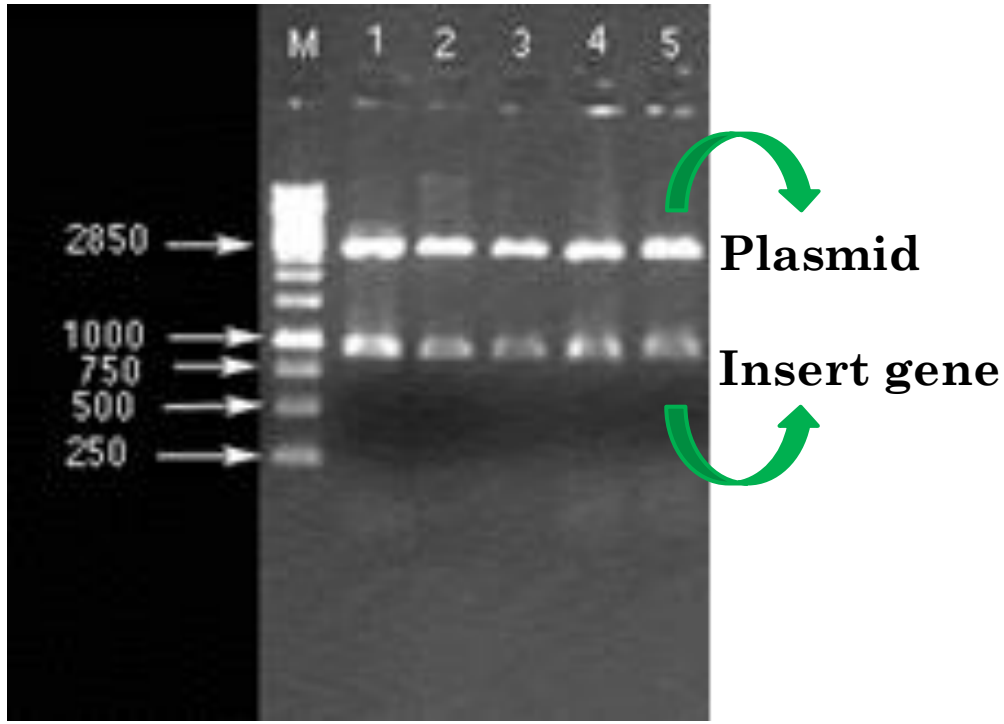


- **Restriction Enzyme Digestion**

Restriction enzyme digestion is a fundamental technique in molecular biology used to cut DNA molecules at specific recognition sequences. These enzymes, also known as restriction endonucleases, are naturally produced by bacteria as a defense mechanism against viral DNA.







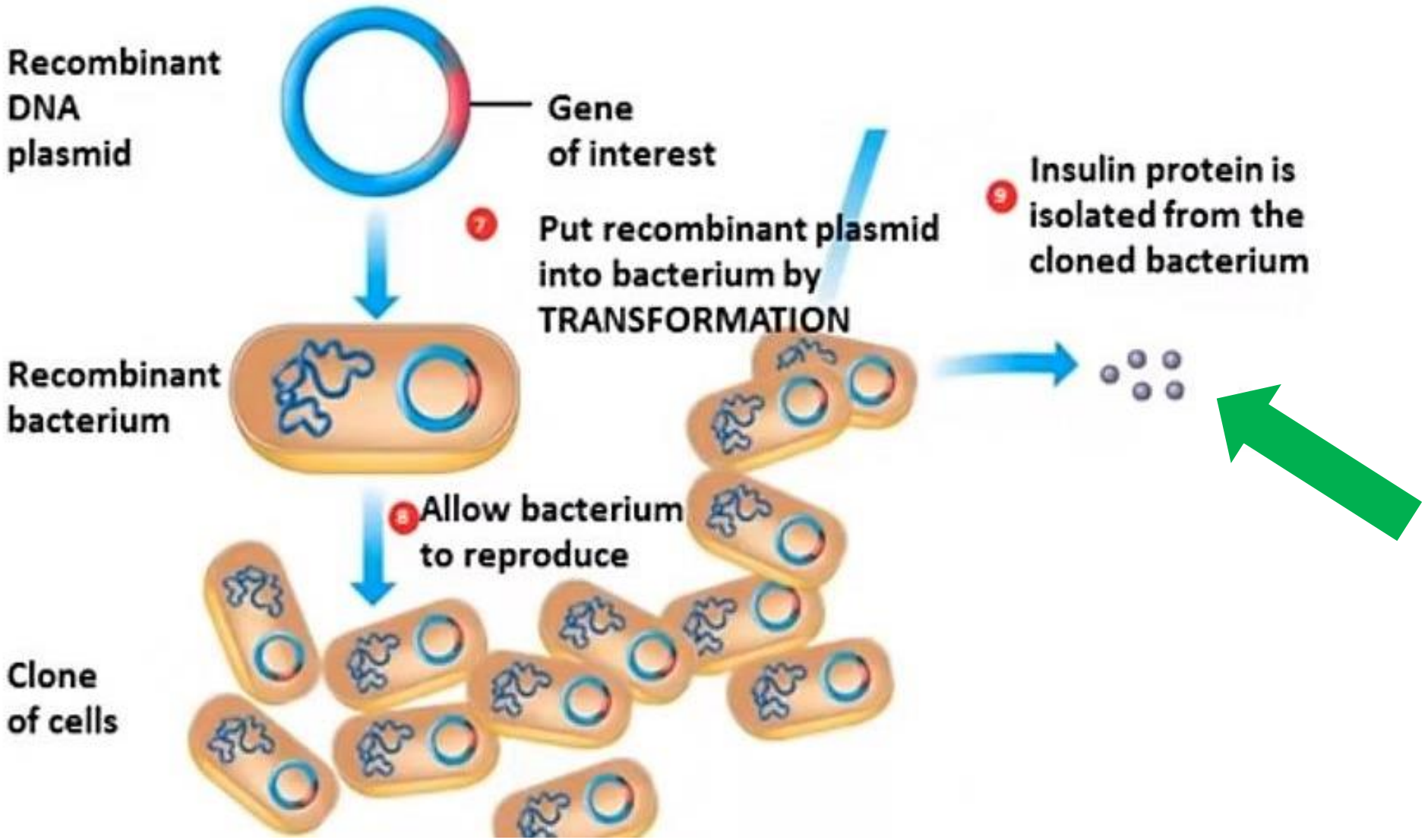
Agarose Gel Electrophoresis

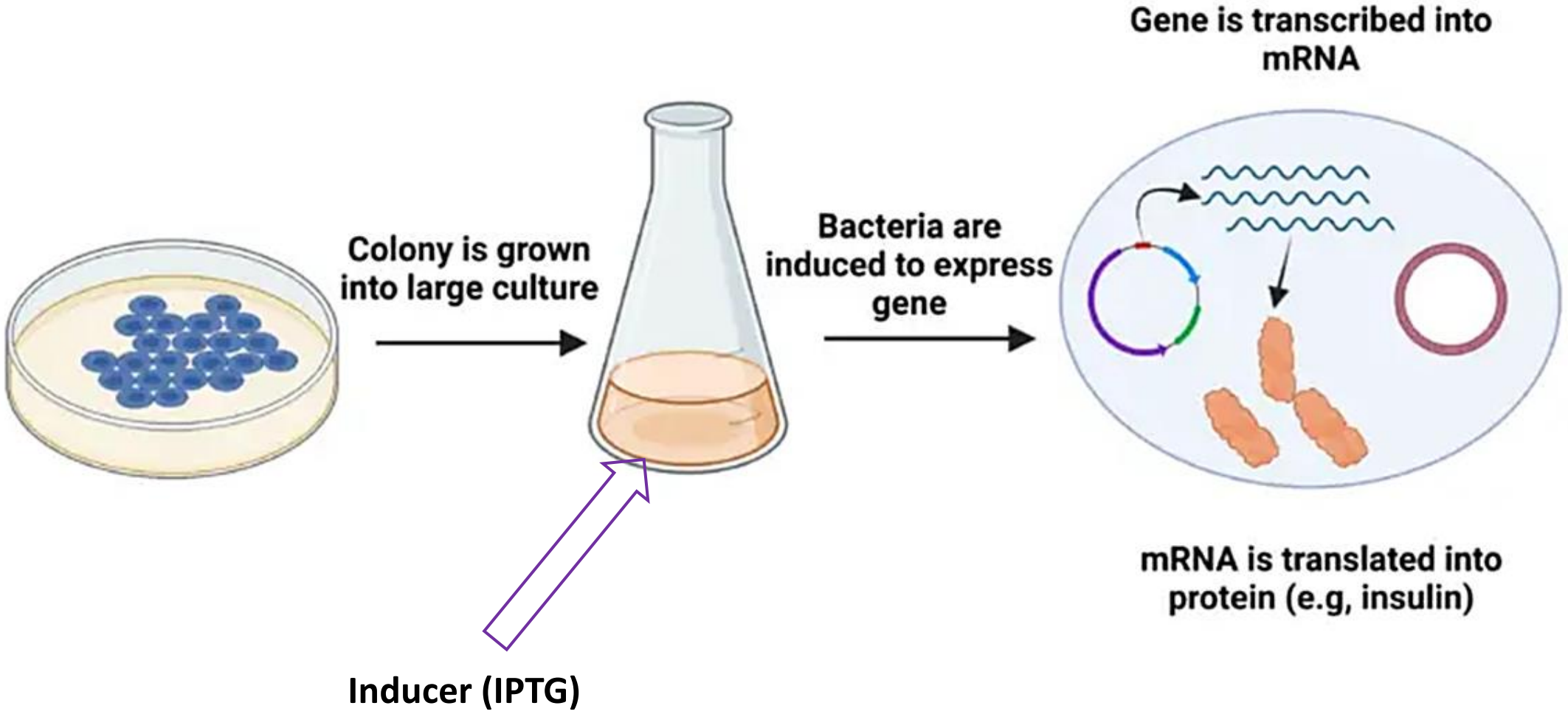
Gene Cloning

6. Multiplication of Selected Host Cells

- If the aim is obtaining numerous copies of gene of interest, then simply replication of the host cell is allowed.

- For obtaining the product of interest like protein, hormones, secondary metabolites, favourable conditions must be provided so that the gene of interest inserted in the vector expresses the product of interest.


Recombinant DNA plasmid
Gene of interest
7 Put recombinant plasmid into bacterium by TRANSFORMATION
9 Insulin protein is isolated from the cloned bacterium
Recombinant bacterium
8 Allow bacterium to reproduce
Clone of cells


Gene is transcribed into mRNA
Colony is grown into large culture
Bacteria are induced to express gene
mRNA is translated into protein (e.g, insulin)
Inducer (IPTG)

Gene Cloning

Gene cloning involves following 7 essential steps:

1. Isolation of specific DNA fragment containing gene of interest
2. Selection of suitable cloning vector
3. Formation of Recombinant DNA (Ligation)
4. Transformation of recombinant vector into suitable host
5. Isolation of Recombinant Cells
6. Multiplication of Selected Host Cells
7. **Isolation and Purification of the Product**

Gene Cloning

7. Isolation and Purification of the Product

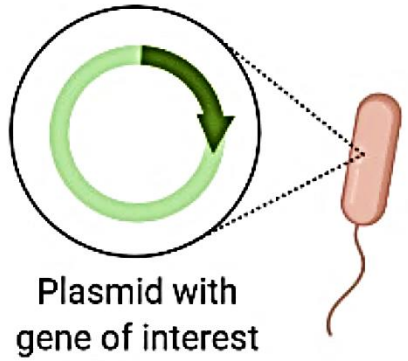
- The last step involves segregation of the multiplied gene of interest attached with the vector or to isolate the product of interest encoded by the gene of interest .
- This is carried by purification processes of the isolated gene copy/protein.

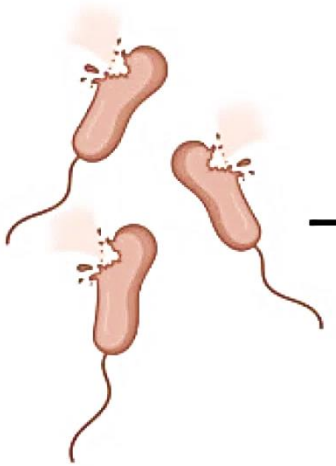
1 Transformation

2 Selection

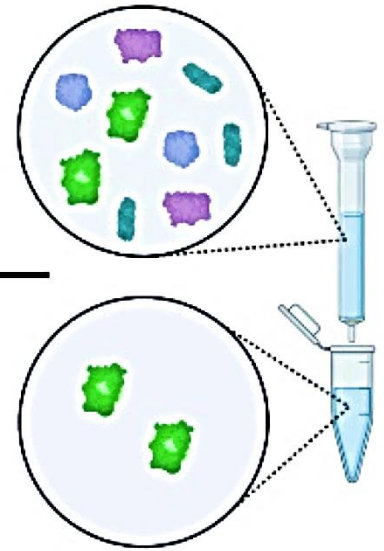
3 Cell growth and protein production

4 Cell lysis



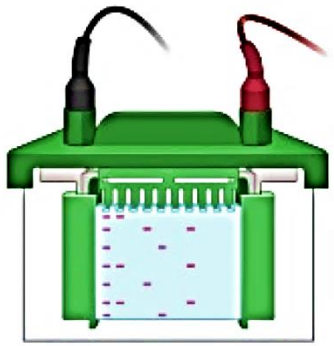


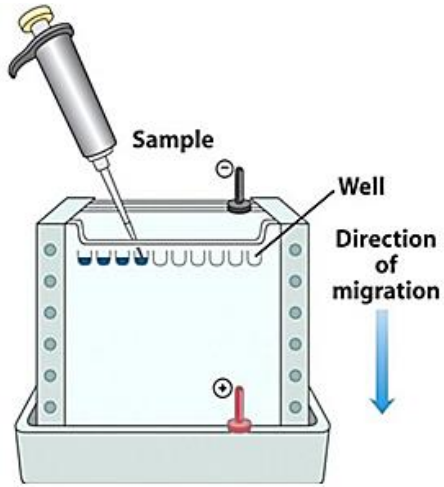
5 Affinity chromatography

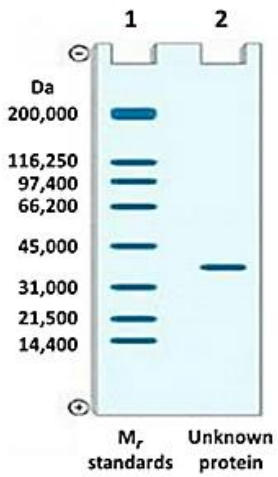


6 Dialysis

7 SDS-PAGE







- **Affinity Chromatography (Specific Ligand–Protein Interaction)**

Highly specific purification of a target protein from complex mixtures.
Tagged target protein (His-tag) ⇄ Immobilized ligand on resin (Ni-NTA)

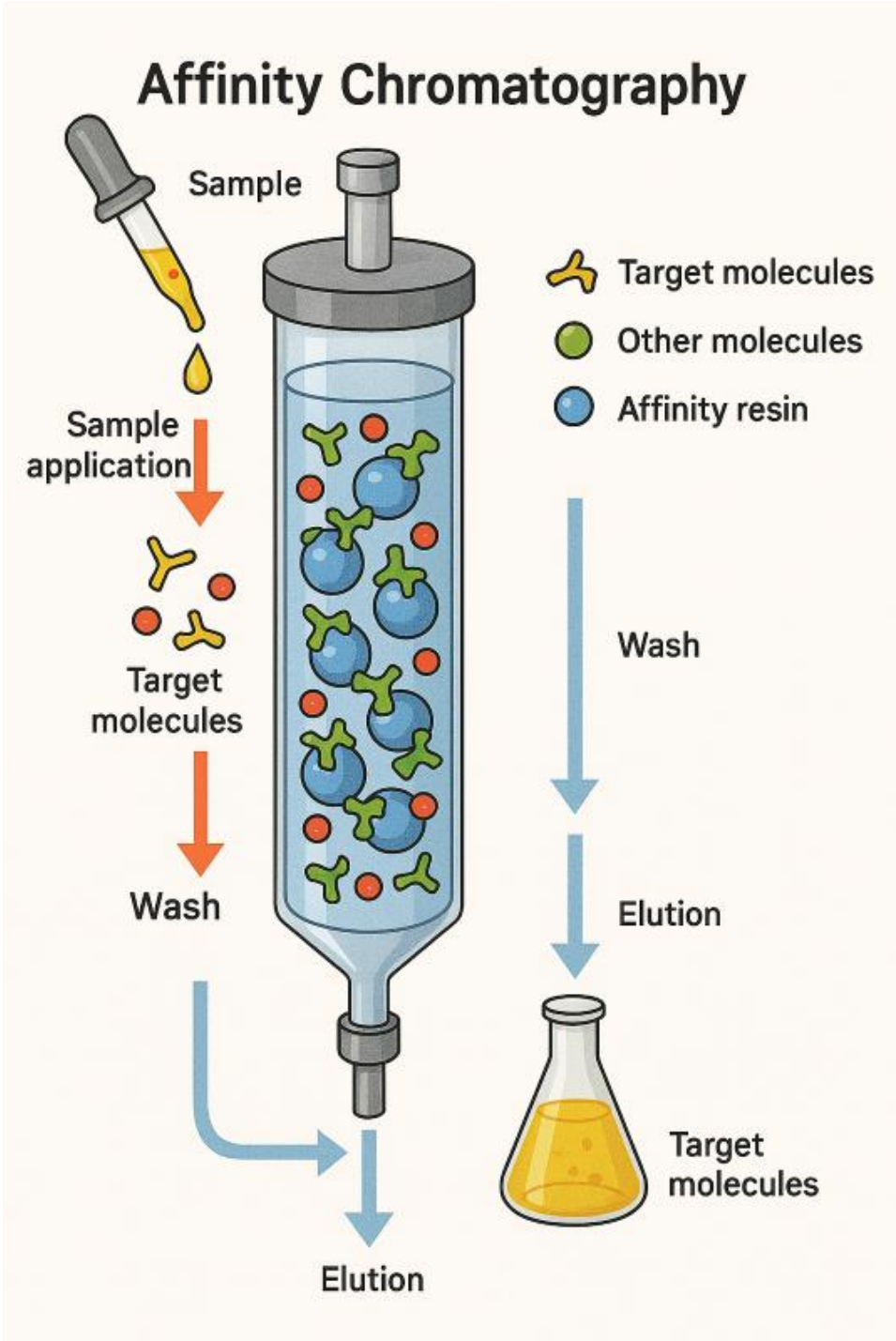
Step 1: Sample Preparation
- Cell lysis of expression system (e.g., E. coli, mammalian cells)
- Clarification by centrifugation and/or filtration

Step 2: Sample Loading
- Load clarified lysate onto affinity column
- Target protein binds specifically to immobilized ligand

Step 3: Washing
- Remove non-specifically bound contaminants
- Example: 20–40 mM imidazole for His-tagged proteins

Step 4: Elution
- Release target protein using competitive or disruptive conditions
- Example: 250–500 mM imidazole, low pH, or free ligand

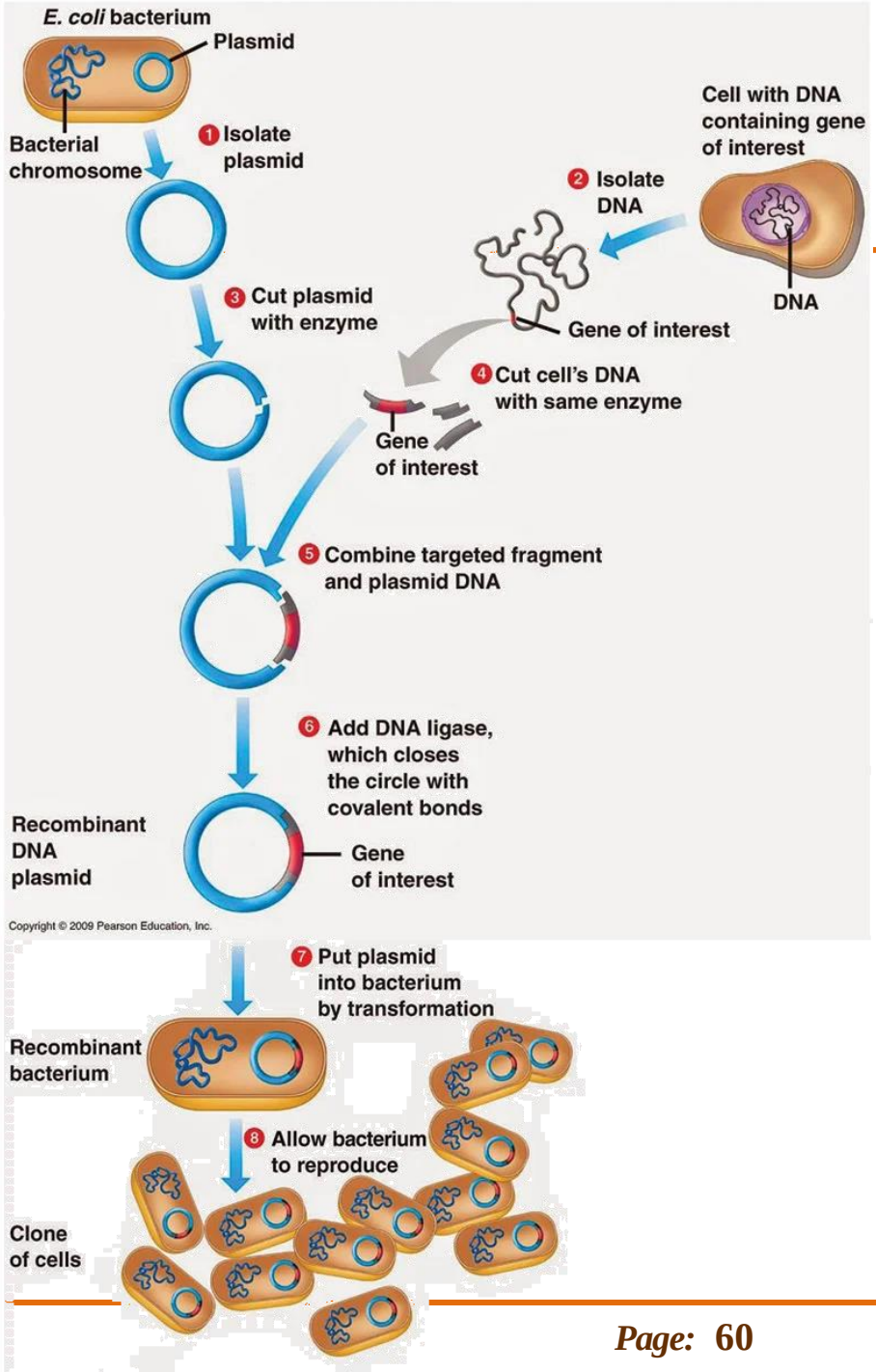
Step 5: Buffer Exchange & Storage
- Desalting or dialysis into storage buffer (e.g., PBS, Tris-HCl)
- Assess purity and concentration (SDS-PAGE, Bradford assay)



Cloning Strategies

Cloning Strategies

- Cloning strategies refer to the various molecular techniques used to insert a DNA fragment of interest into a vector for replication, expression, or functional analysis.
- The choice of strategy depends on the experimental goal, insert size, vector type, and required efficiency.

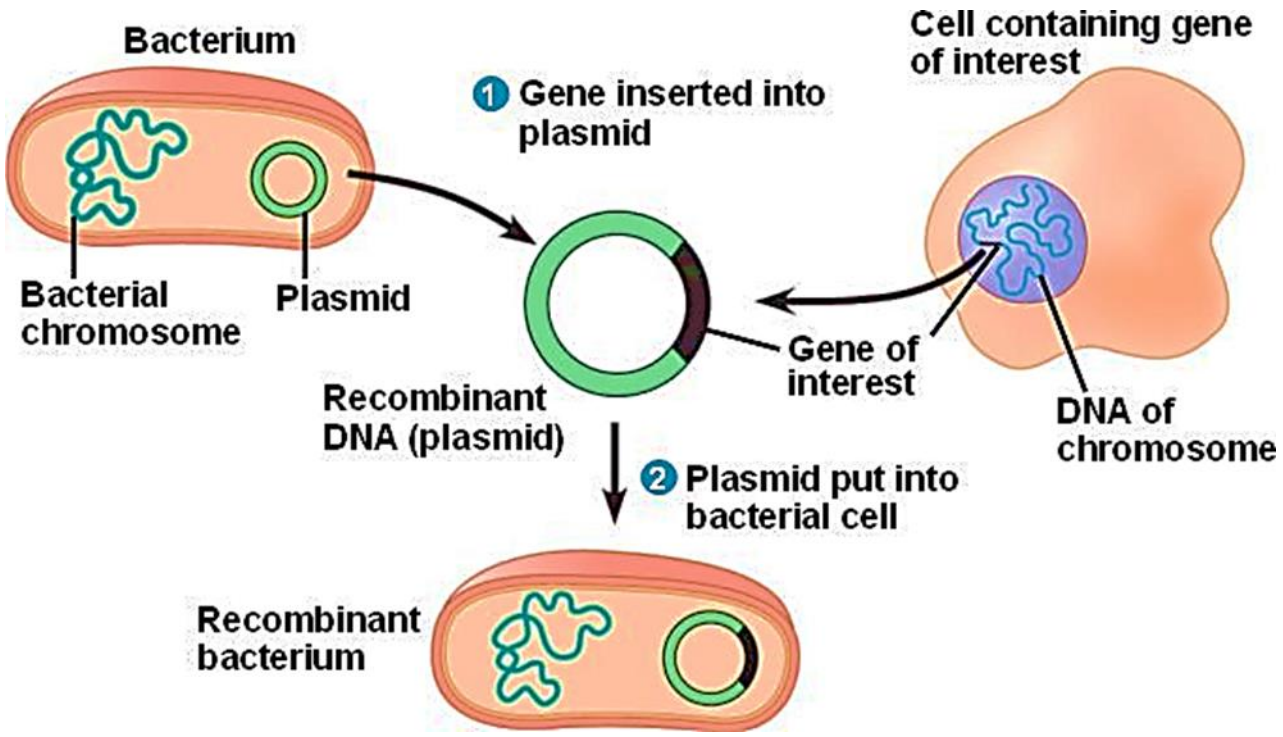


Gene Cloning

Gene cloning can be achieved by following two different methods:

1. Cell based DNA cloning

2. Cell-free DNA cloning (PCR)



Comparison: Cell-based Cloning vs. Cell-free (PCR) Cloning

Feature	Cell-based Cloning	Cell-free (PCR) Cloning
Requires Cells	Yes	No
Time Required	Long (days)	Short (hours)
Accuracy / Fidelity	High	Moderate (depends on PCR fidelity)
Insert Size Capacity	Large (up to 100+ kb)	Small to medium (up to ~10 kb)
Application in Protein Engineering	Protein expression & production	Rapid mutagenesis & sequence modifications

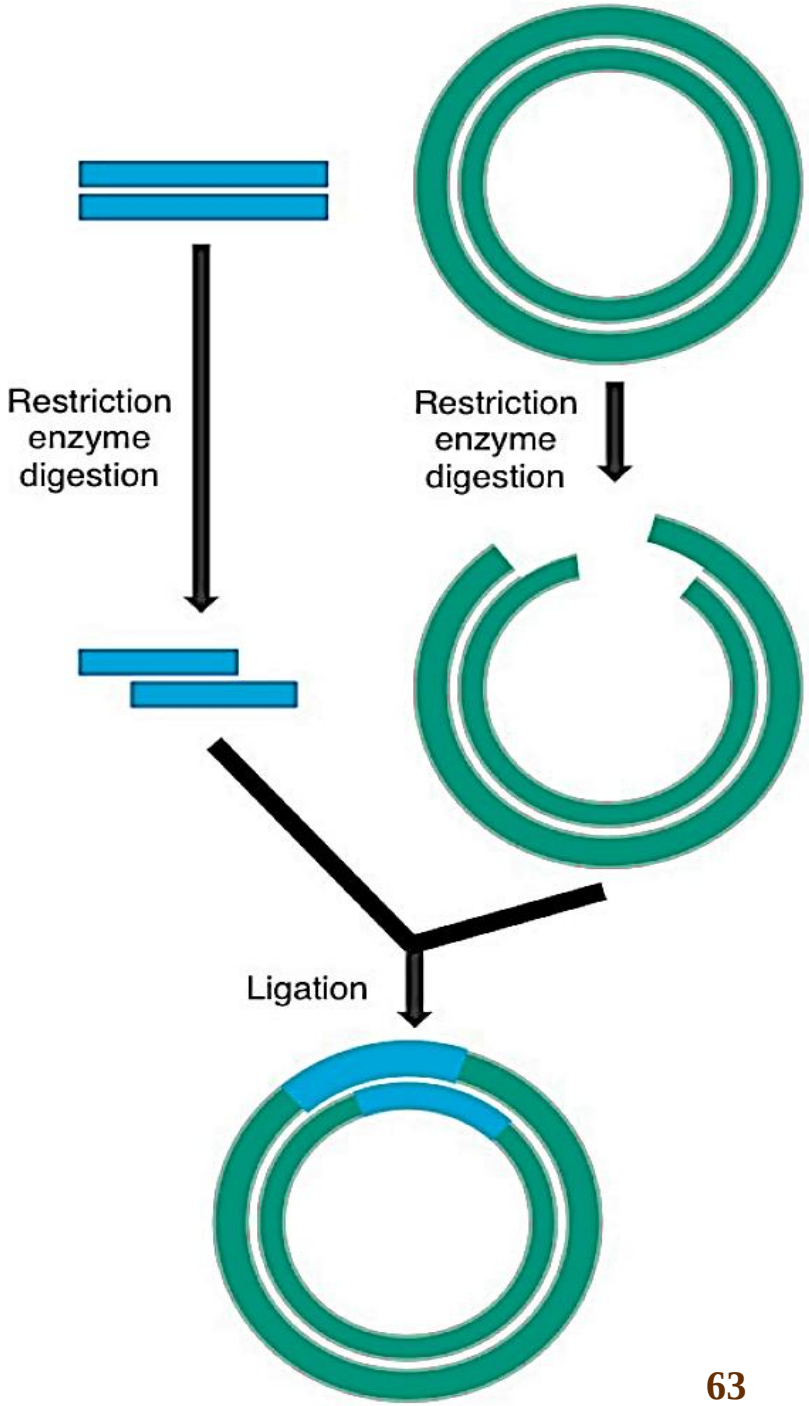
 Notes:

- Cell-based cloning relies on bacterial or eukaryotic host cells for replication and selection.
- Cell-free cloning (e.g., Gibson Assembly, TA/TOPO cloning, or PCR-based methods) is ideal for quick edits, site-directed mutagenesis, or assembling small constructs without transformation.

Restriction Enzyme-Based Cloning (Traditional Cloning)

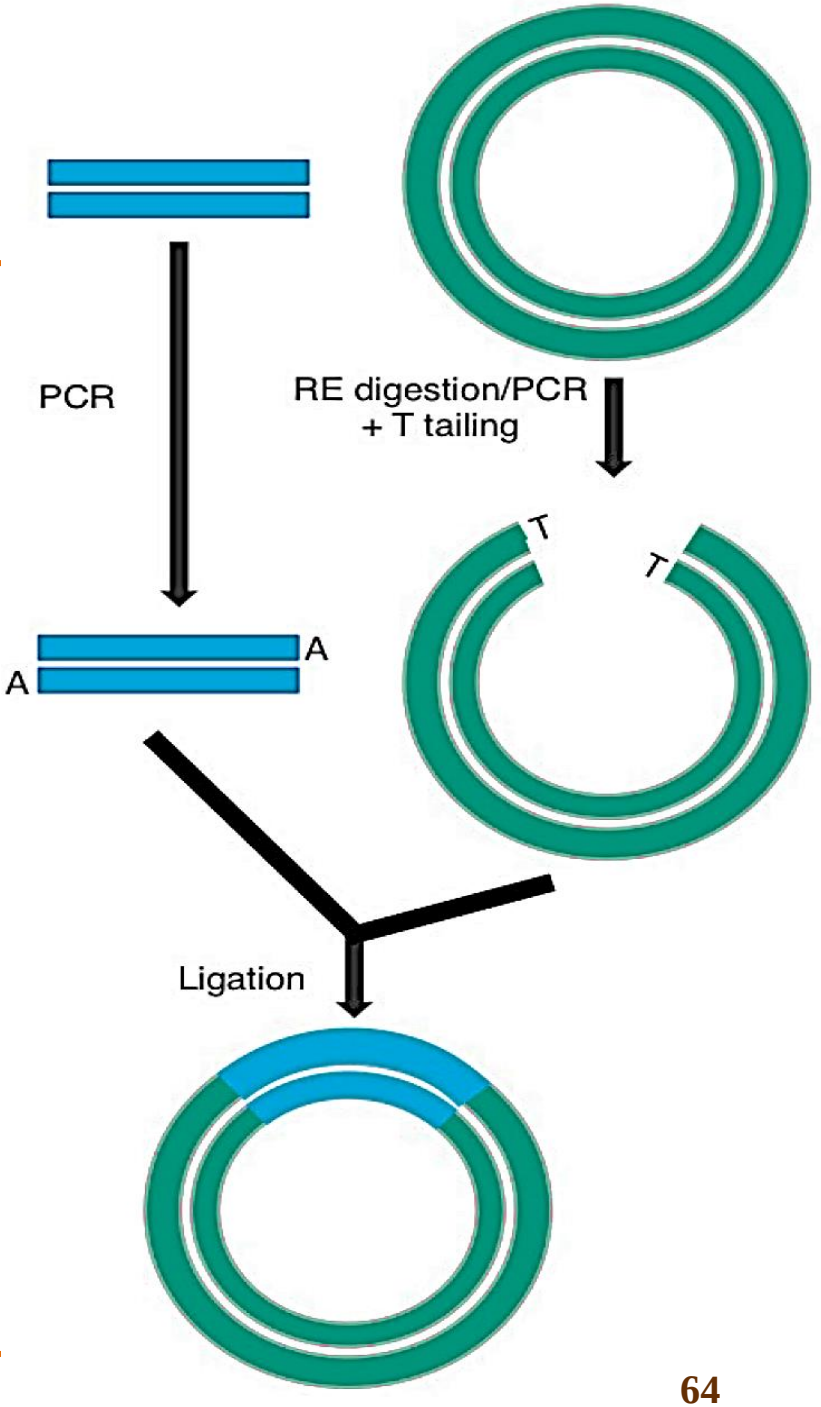
- Uses restriction enzymes to cut both the insert and vector at specific sites, creating compatible ends (sticky or blunt).
- DNA ligase joins the fragments.

- Pros: Simple, widely understood.
- Cons: Limited by restriction site availability; may leave unwanted "scar" sequences.



TA and Blunt-End Cloning

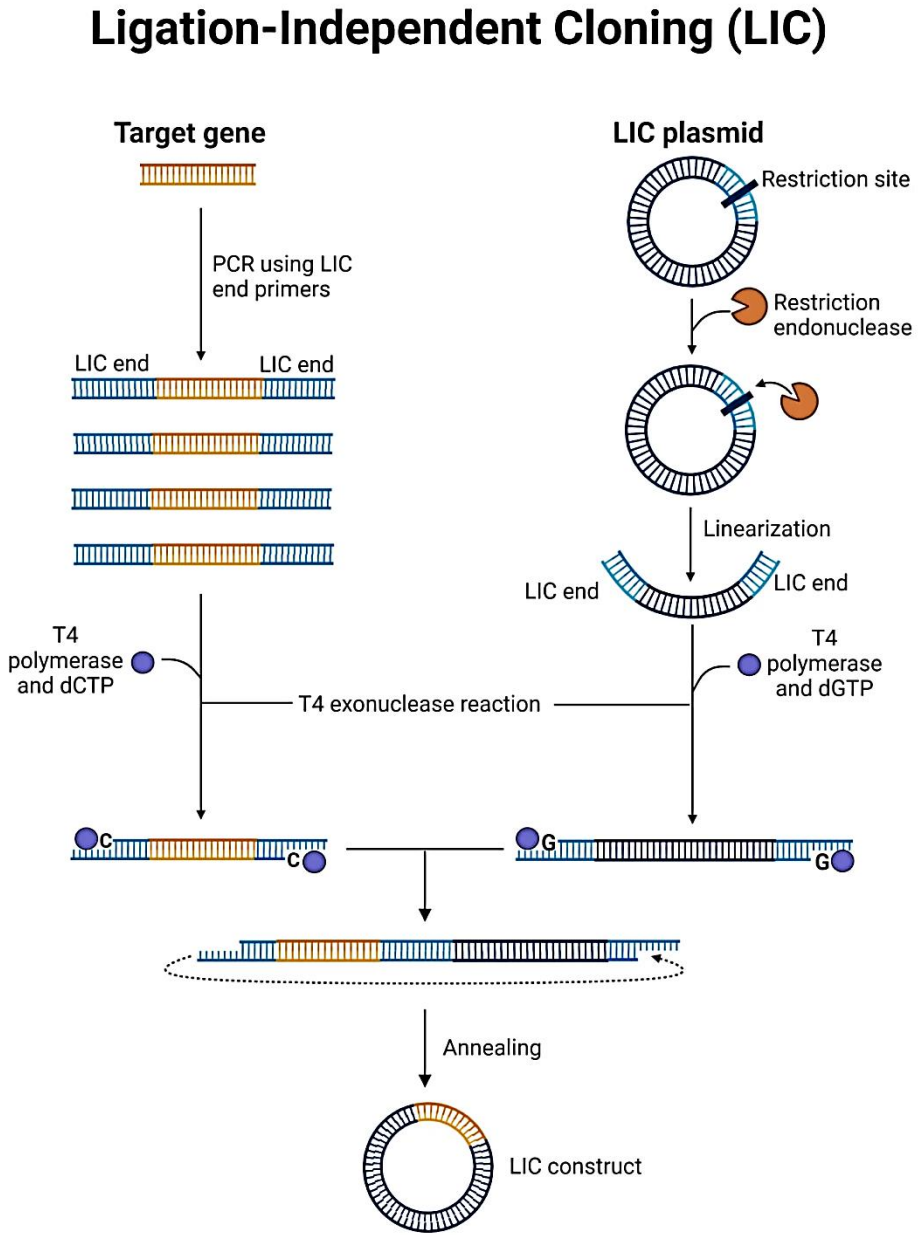
- Cell-free DNA cloning / PCR-based cloning involves the direct ligation of a PCR-generated DNA fragment without using restriction enzymes to cut the insert.
- One of the most commonly used PCR cloning method takes advantage of an adenine (A) residue that is added by the Taq polymerase at the 3’ ends of the DNA fragments during the amplification process.
- These “A-tailed” products can be directly ligated with “T-tailed” vectors.



Ligation independent cloning

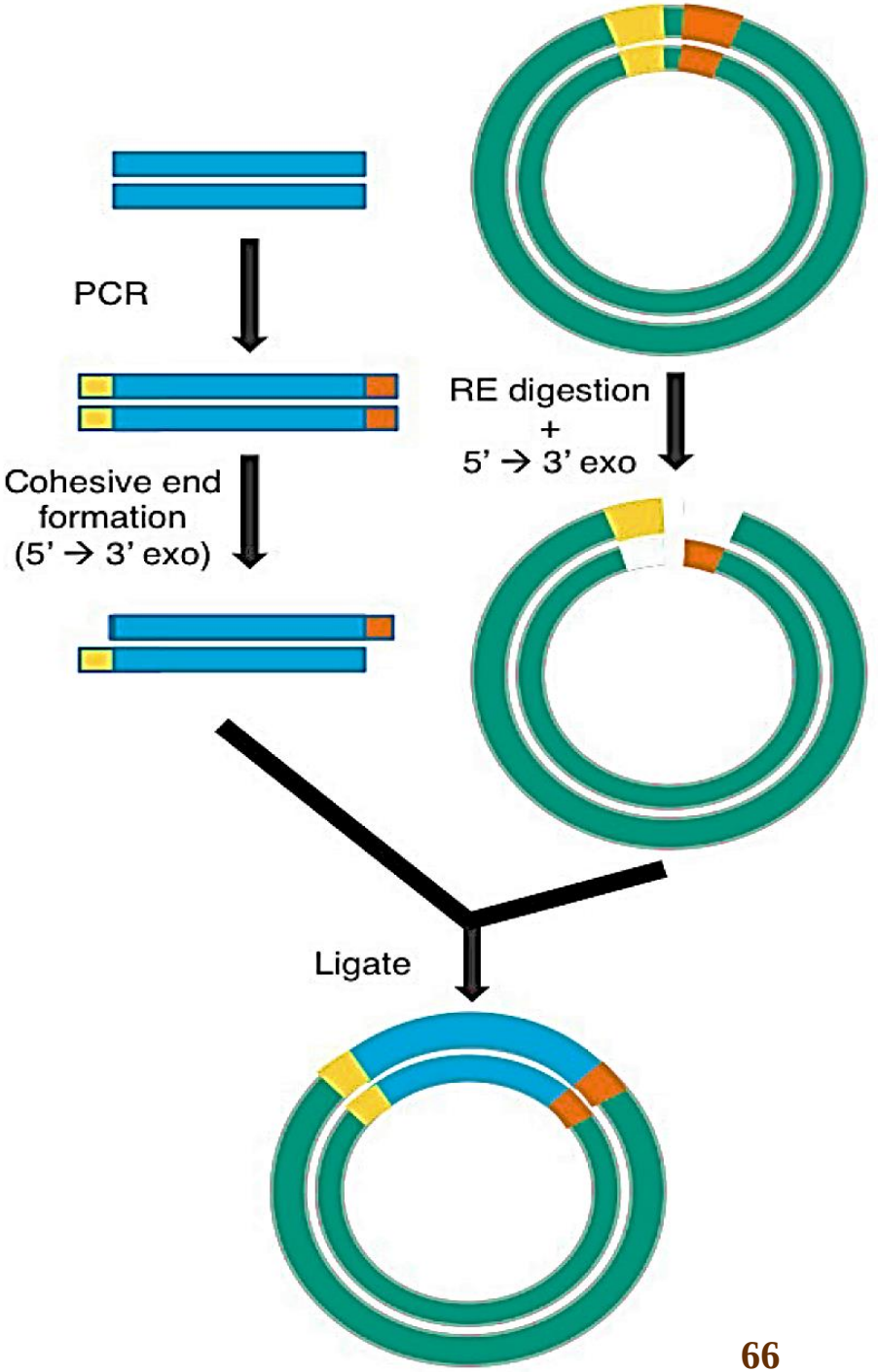
- Ligation independent cloning (LIC) is usually carried out by adding short sequences of DNA to the fragment to be cloned that are homologous to the destination vector.

- Complementary cohesive ends between the vector and insert are then formed by using enzymes with 3’ to 5’ exonuclease activity, and the resulting two molecules are then mixed together and annealed.



Seamless cloning

- Seamless cloning (SC) is a method that relies on matching short sequences at the ends of a DNA fragment with corresponding short sequences on a vector. It is similar to the LIC method.
- In SC, an enzyme with 5′ to 3′ exonuclease activity is used to create 3′ overhangs on the DNA fragment.
- The most well known of these methods is the Gibson Assembly Method, in which up to ten fragments can be easily combined.



Gibson Assembly®

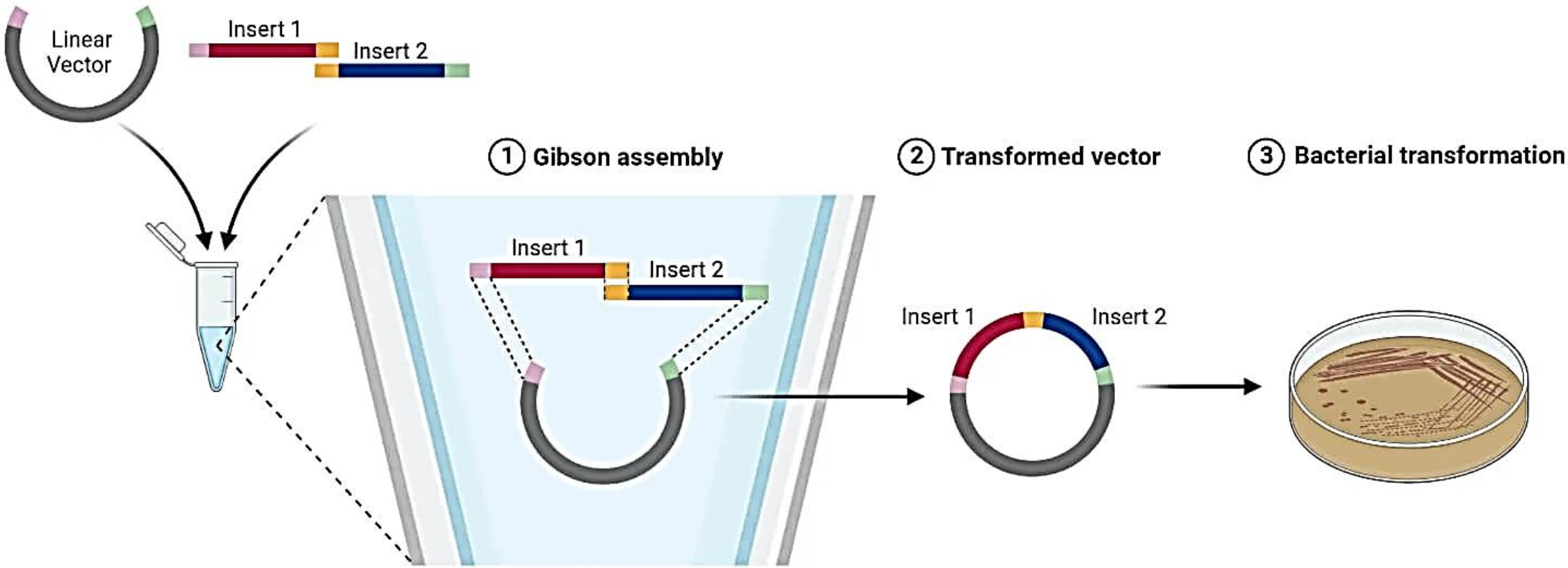
This method enables the seamless assembly of DNA fragments without the need for restriction enzymes and without creating a "scar" sequence.

The reaction relies on a master mix containing three key enzymes:

- A **5′ exonuclease** that chews back the 5′ ends of DNA fragments to generate complementary single-stranded overhangs (typically 20–40 bp),
- A **DNA polymerase** that fills in any gaps after the overlapping ends anneal, and
- A **DNA ligase** that covalently seals the nicks, producing a fully functional, circular plasmid.

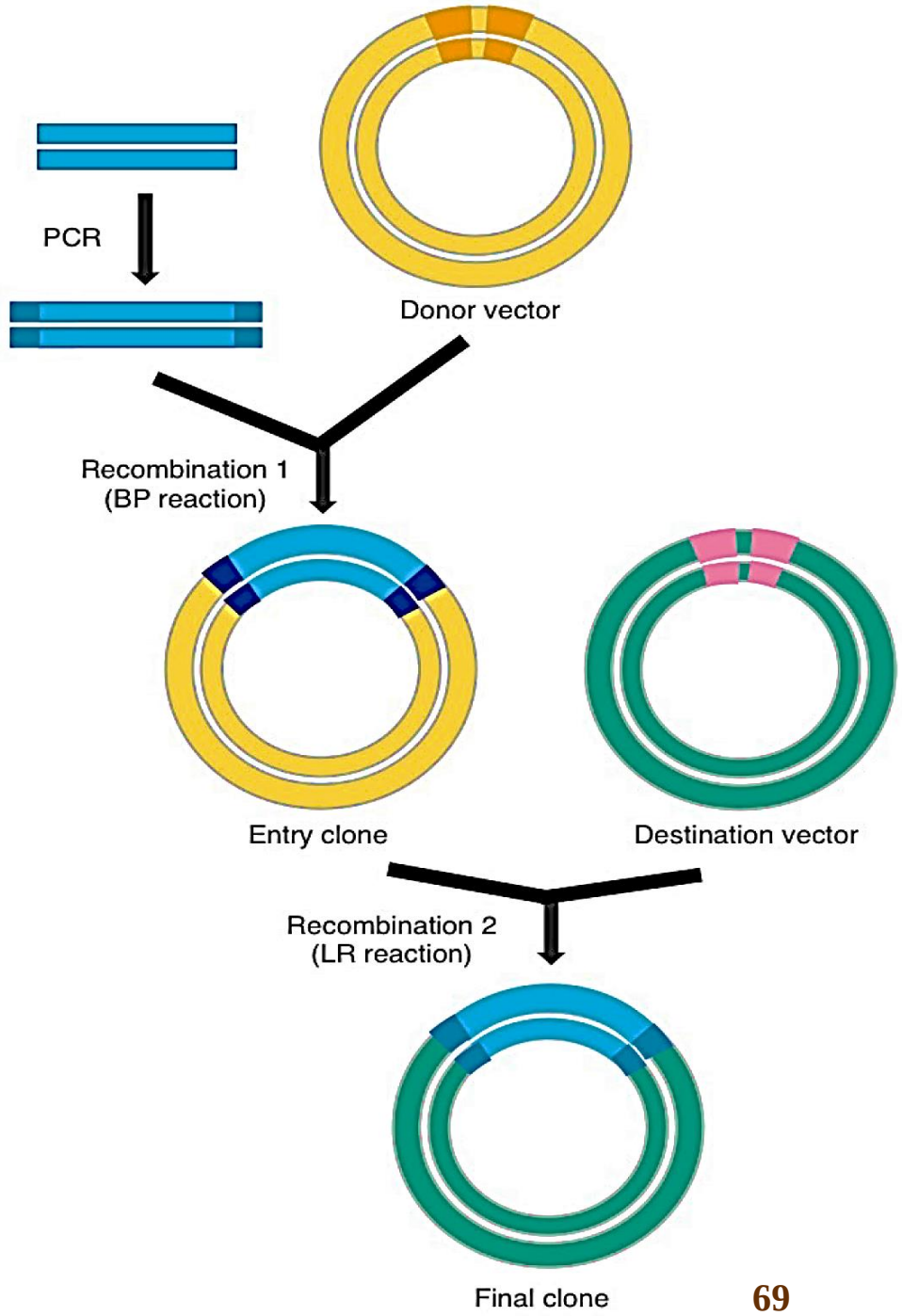
Gibson Assembly® has become a cornerstone of modern molecular cloning due to its speed, flexibility, and reliability in constructing complex genetic circuits and recombinant vectors.

Gibson Assembly Protocol



Gateway cloning

- Gateway® Cloning is a highly efficient, site-specific recombination-based cloning system .
- Gateway® cloning is based on the **bacteriophage λ recombination system**, which uses specific DNA sequences called **att sites** and proprietary enzyme mixes (**BP Clonase®** and **LR Clonase®**).
- The interplay between the lambda phage and a bacterium is hinged on regions of DNA known as the attP site within the phage and the attB site in the bacterium's genome.



Gateway cloning

- Two Main Steps

1. BP Reaction (Entry Clone Creation)

Your gene (flanked by attB sites) + Donor Vector (with attP sites)

→ BP Clonase® mediates recombination

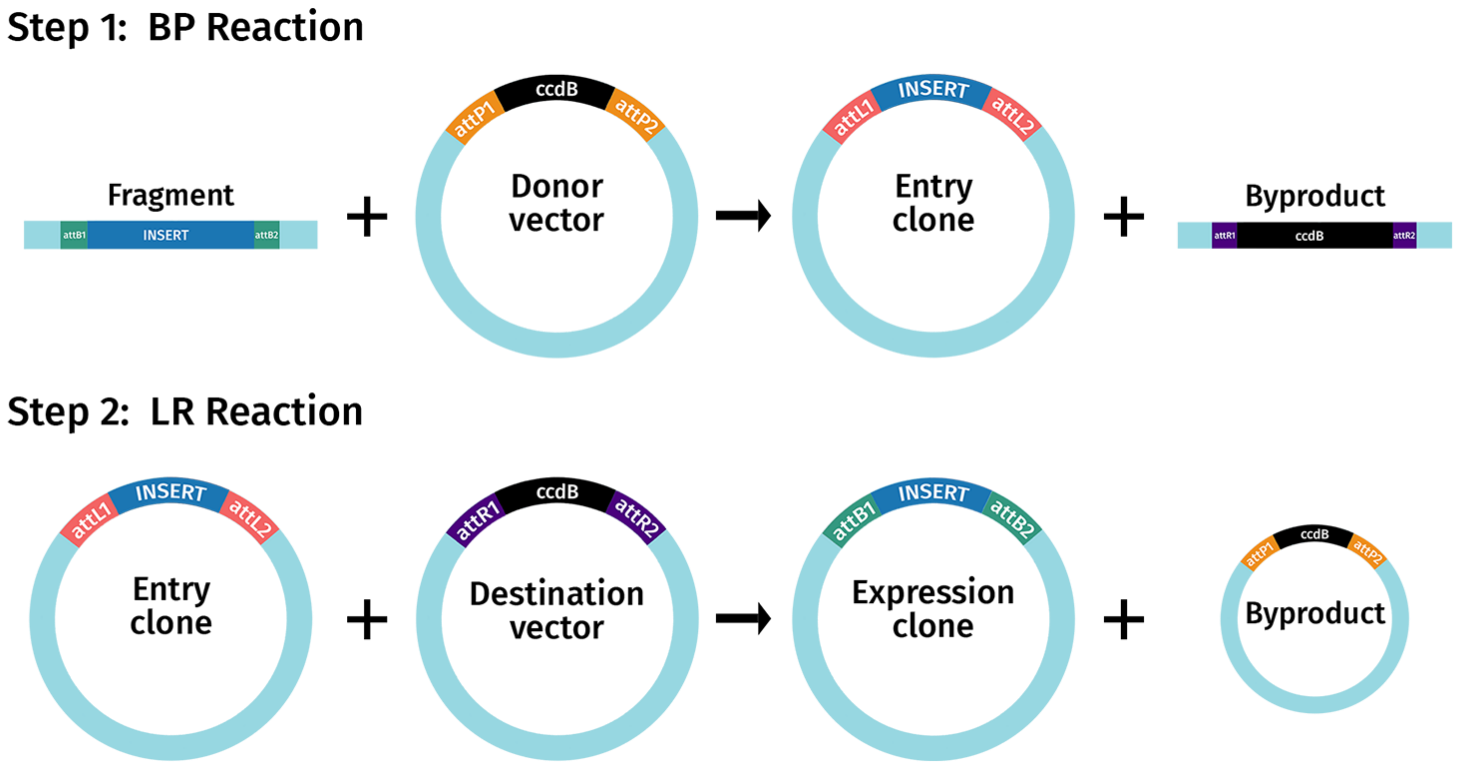
→ Produces an Entry Clone (with attL sites) + a byproduct (ccdB gene in a small plasmid).

2. LR Reaction (Expression Clone Creation)

Entry Clone (attL) + Destination Vector (attR, containing promoter, tag, selection marker, etc.)

→ LR Clonase® mediates recombination

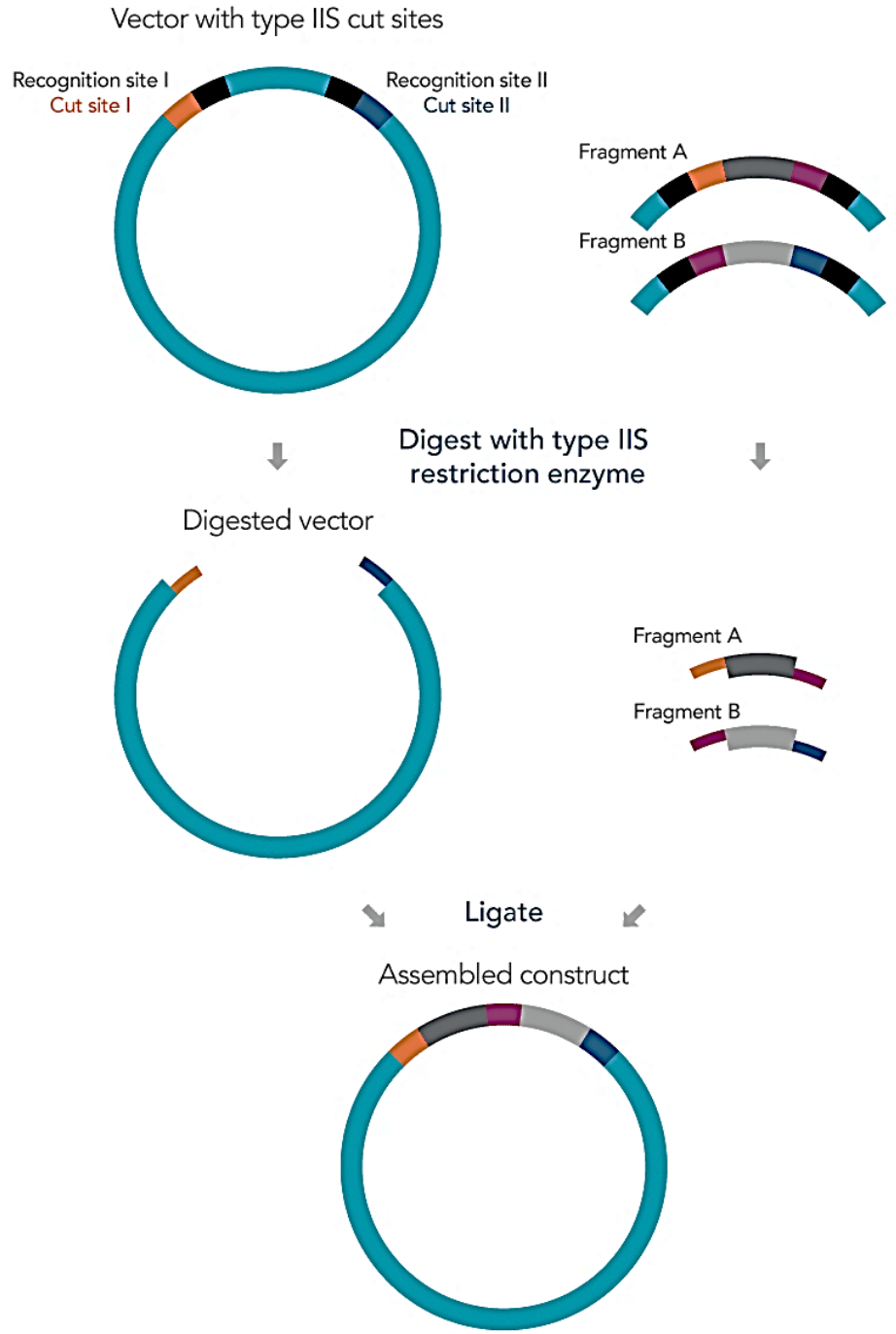
→ Produces the final Expression Clone (attB-flanked gene in expression vector) + Donor plasmid (with ccdB).



Golden Gate Cloning

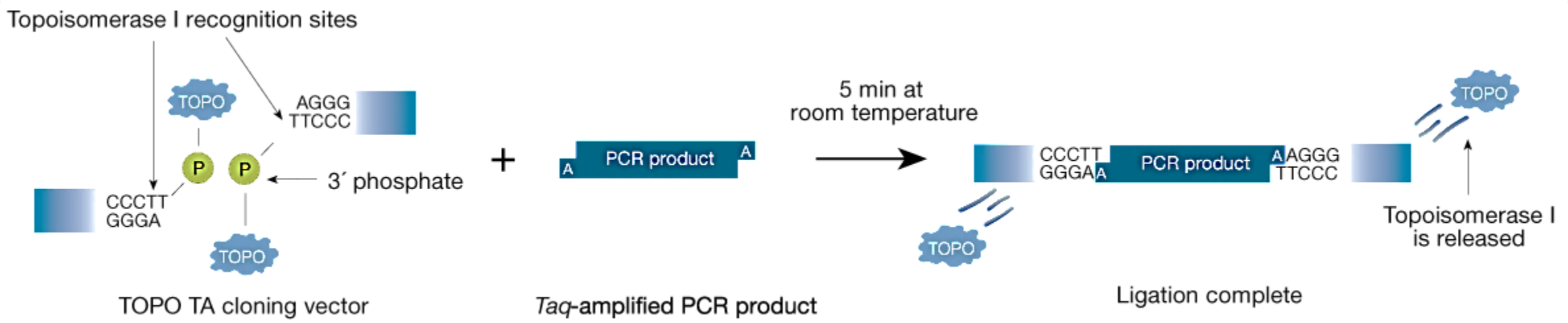
Golden Gate Cloning is a highly efficient, one-pot DNA assembly technique that uses Type IIS restriction enzymes to assemble multiple DNA fragments in a single reaction — with perfect accuracy, no scars, and defined order and orientation.

- Uses Type IIS restriction enzymes (e.g., BsaI, BbsI) that cut outside their recognition sites.
- Allows seamless, scarless, and modular assembly of multiple DNA parts in a single reaction.
- Widely used in standardized DNA assembly.



TOPO® Cloning

- Topoisomerase-based cloning (TOPO cloning) is a molecular biology technique of Cell-free DNA cloning / PCR-based cloning in which DNA fragments are cloned into specific vectors without the requirement for DNA ligases. Taq polymerase has a non template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3'-end of the PCR products.
- Relies on topoisomerase I activity to ligate PCR products directly into vectors.



Cloning method	Cost	Sequence dependency	Throughput	Assembly of multiple fragments	Directional cloning	Need for dedicated vectors	Examples of commercially available products
Traditional cloning (restriction enzyme-based)	Low	Yes (restriction enzyme sites)	Low to mid (can be increased by using ligation adapters)	Difficult for more than 2 fragments	Possible	No	-
PCR cloning	Medium (vectors)	No	High	Challenging (requires special modifications)	Difficult	Yes (for certain applications)	TOPO® TA
Ligation independent cloning	Medium (reagents)	Limited (vector)	Low	Yes	Yes	No	In-Fusion®
Seamless cloning	High (reagents)	No	Low	Yes	Yes	No	Gibson assembly GeneArt®
Recombinatorial cloning	High (reagents and vectors)	No	High	Challenging (requires special modifications)	Yes	Yes	Gateway® Echo Cloning™ Creator™

THANK YOU