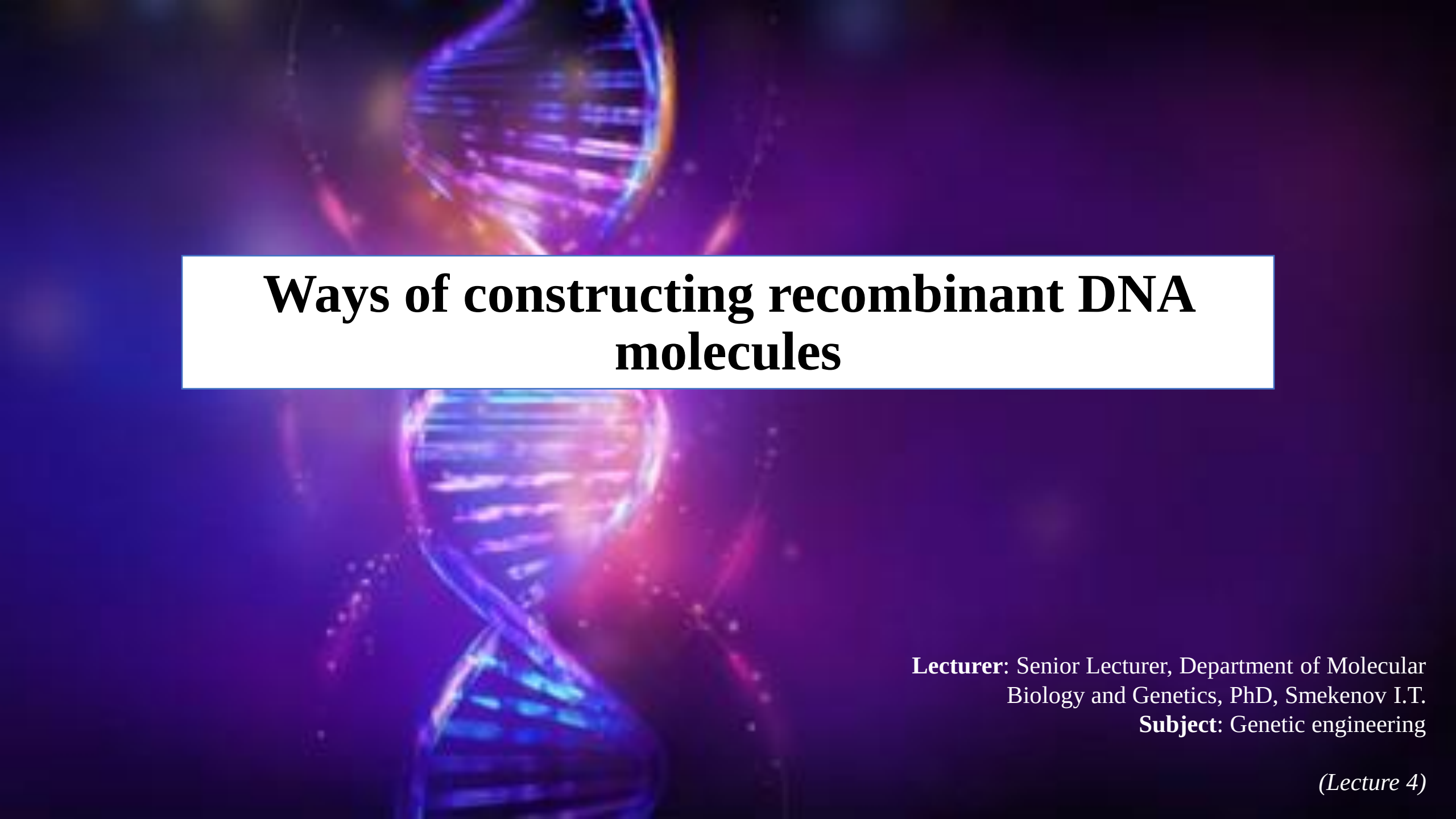


Ways of constructing recombinant DNA molecules

Lecturer: Senior Lecturer, Department of Molecular Biology and Genetics, PhD, Smekenov I.T.

Subject: Genetic engineering

(Lecture 4)

Lecture Goal:
To study and understand the main methods used for constructing recombinant DNA molecules, including their principles, advantages, and applications in genetic engineering.

Tasks:

1. To describe the restriction–ligation method of recombinant DNA construction.
2. To explain the principles of TA cloning and TOPO cloning.
3. To understand the concept of overlapping ends and their role in DNA fragment assembly.
4. To analyze the principles and workflow of PCR-based cloning.
5. To compare different cloning strategies in terms of efficiency, accuracy, and vector requirements.

Keywords: *Restriction–ligation method, sticky ends, blunt ends, restriction enzymes, DNA ligase, TA cloning, TOPO cloning, overlapping ends, homologous recombination, PCR cloning, vector, insert, recombinant DNA.*

Summary

1. Restriction–ligation method

The classical approach for creating recombinant DNA.

Involves cutting vector and insert DNA with the same **restriction enzymes** to generate compatible ends.

DNA ligase joins the fragments via phosphodiester bond formation.

Widely used but requires available restriction sites and multiple enzymatic steps.

2. TA cloning

Relies on the addition of a single **adenine (A)** overhang to PCR products by **Taq DNA polymerase**.

The vector has complementary **thymine (T)** overhangs, allowing direct ligation without restriction enzymes.

Fast and convenient for cloning PCR products, though orientation control is limited.

3. TOPO cloning

Utilizes **topoisomerase I** covalently attached to the vector ends, enabling rapid and efficient ligation of PCR products.

No ligase or restriction enzymes are needed.

Offers higher cloning efficiency and correct orientation, often used in commercial cloning kits.

4. Overlapping ends (homologous recombination cloning)

DNA fragments with **overlapping sequences** can be joined without restriction or ligation steps.

Achieved through recombination-based systems such as **Gibson Assembly**, **In-Fusion**, or **SLiCE**.

Produces seamless recombinant molecules with precise junctions.

5. PCR-based cloning

Involves direct amplification of the desired insert with primers containing sequences homologous to vector ends.

The amplified product recombines with the vector via homologous recombination or enzyme-mediated assembly.

Eliminates the need for restriction digestion and allows flexibility in insert design.

Key questions

1) What are the basic steps involved in the **restriction–ligation** method of recombinant DNA construction?

2) How do **TA cloning** and **TOPO cloning** differ in their mechanisms and efficiency?

3) What is the role of **overlapping ends** in DNA fragment assembly and seamless cloning?

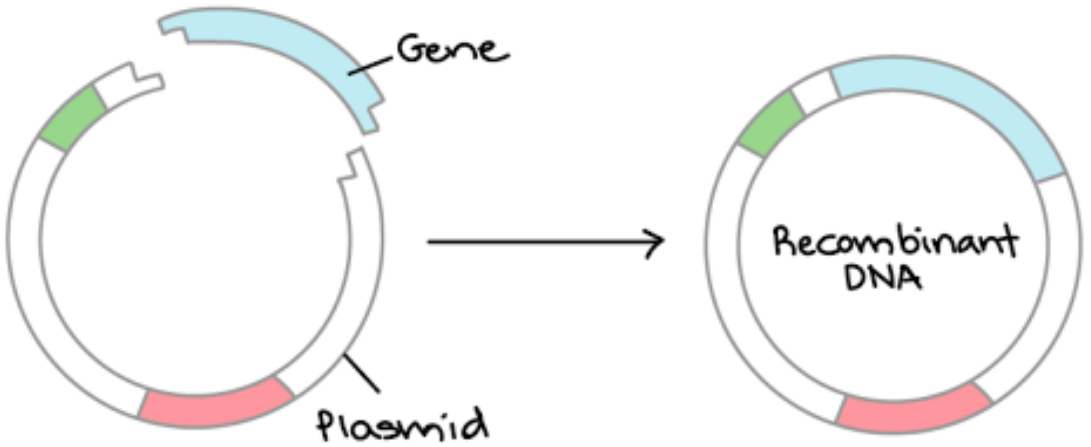
4) How is **PCR cloning** used to directly insert amplified DNA fragments into vectors?

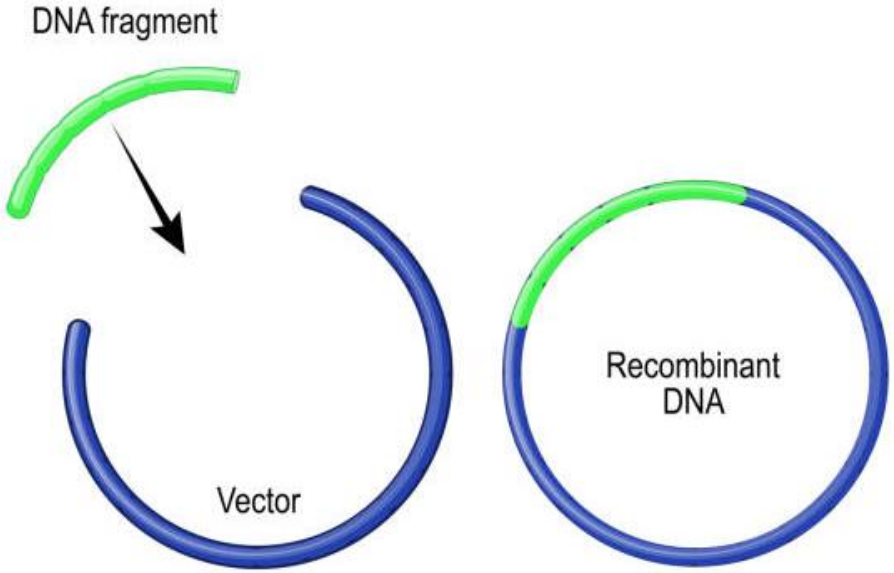
5) What factors influence the **efficiency and accuracy** of recombinant DNA construction?

6) Which methods are most suitable for high-throughput or error-free cloning?

Recombinant DNA Construction

- **Recombinant DNA construction** is a process in molecular biology that involves combining DNA from different sources to create a new sequence (hybrid) that can be used for various applications such as gene expression, cloning, or therapeutic purposes.

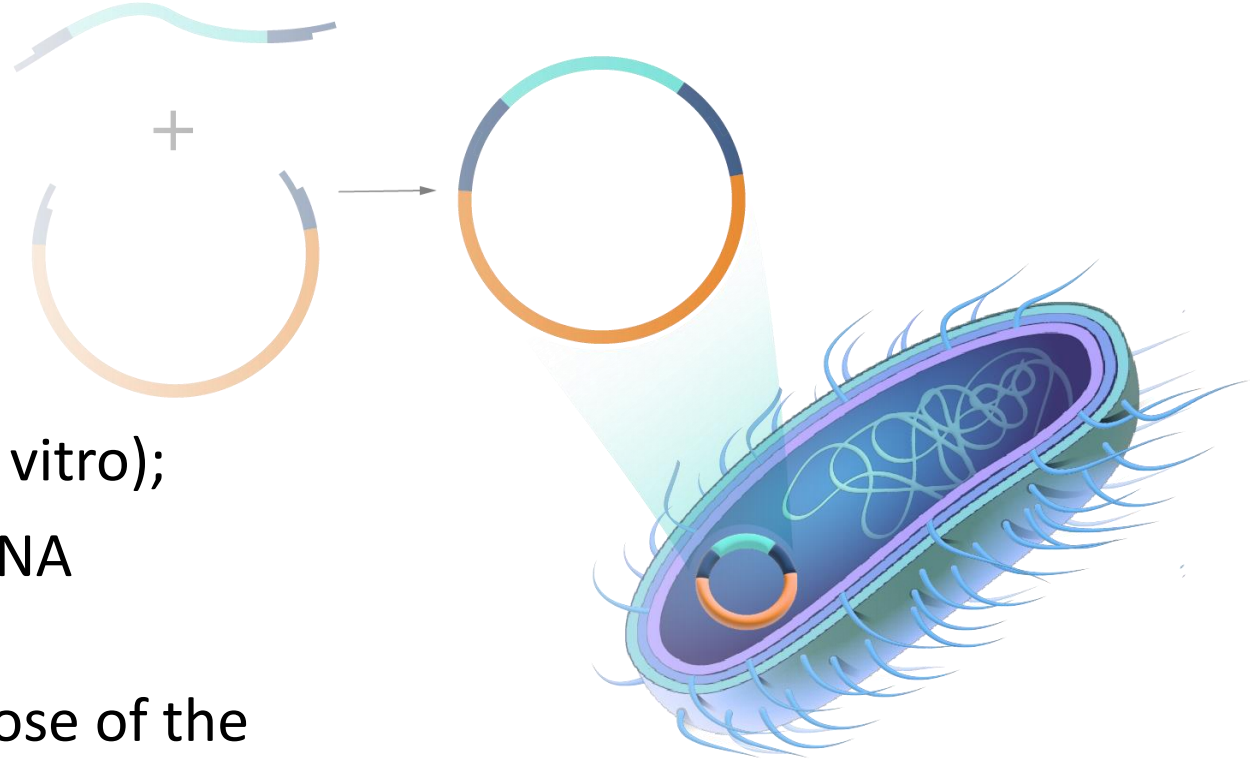




- ***Recombinant*** is understood as DNA formed by combining *in vitro* (in tube) two or more DNA fragments isolated from different biological sources.

Strategy for Recombinant DNA Construction

1. Choose a cloning method;
2. Find a suitable vector (plasmid);
3. Find gene sequence (insert);
4. Gene cloning using programs (analysis);
5. Creation of a recombinant construct (in vitro);
6. Analysis of the resulting recombinant DNA (sequencing);
7. Follow-up work depending on the purpose of the study;



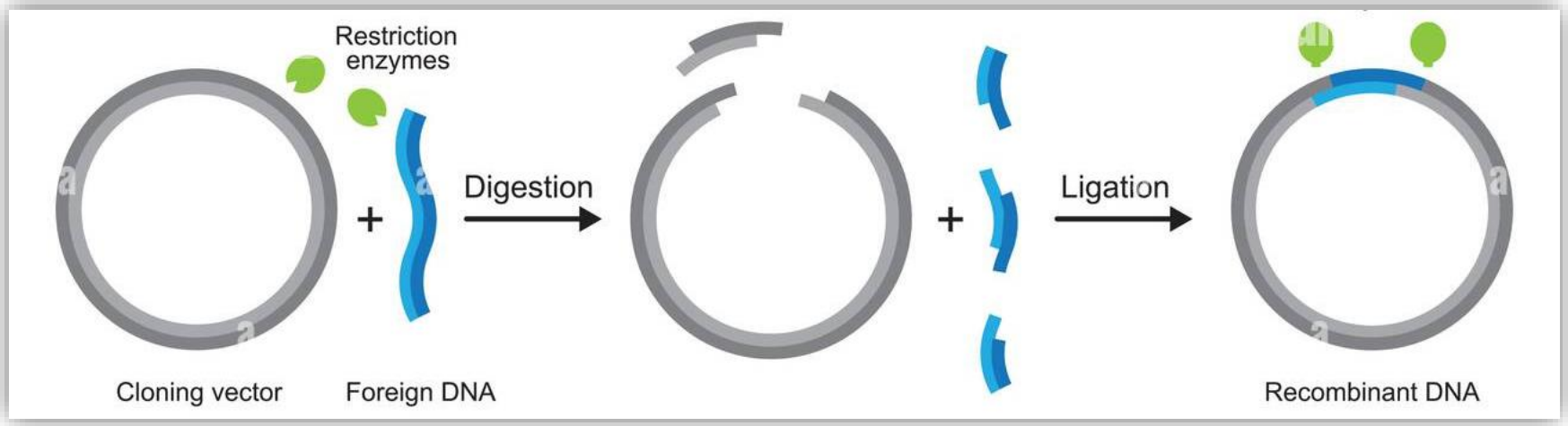
There are several methods for constructing recombinant DNA molecules

1. Restriction Enzyme-Based Cloning (Traditional Cloning)

This is the classical method that uses **restriction enzymes** to cut DNA at specific sites and **ligate** the desired fragment into a vector.

Advantages: Simple, inexpensive, and widely used.

Limitations: Dependent on the availability of restriction sites, may require extensive screening of colonies.

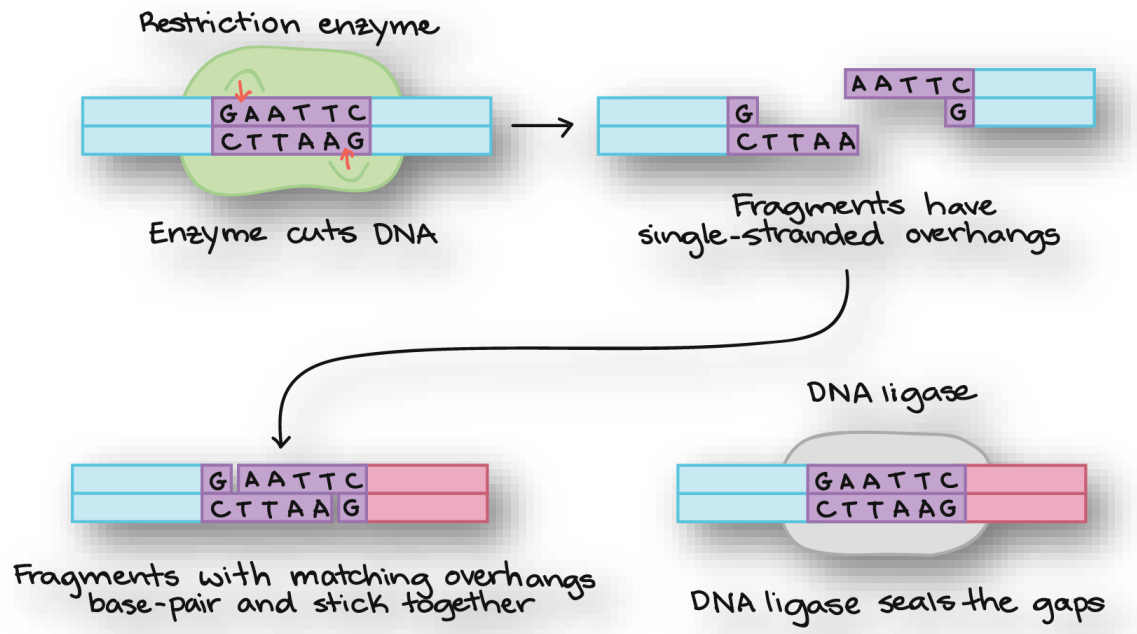


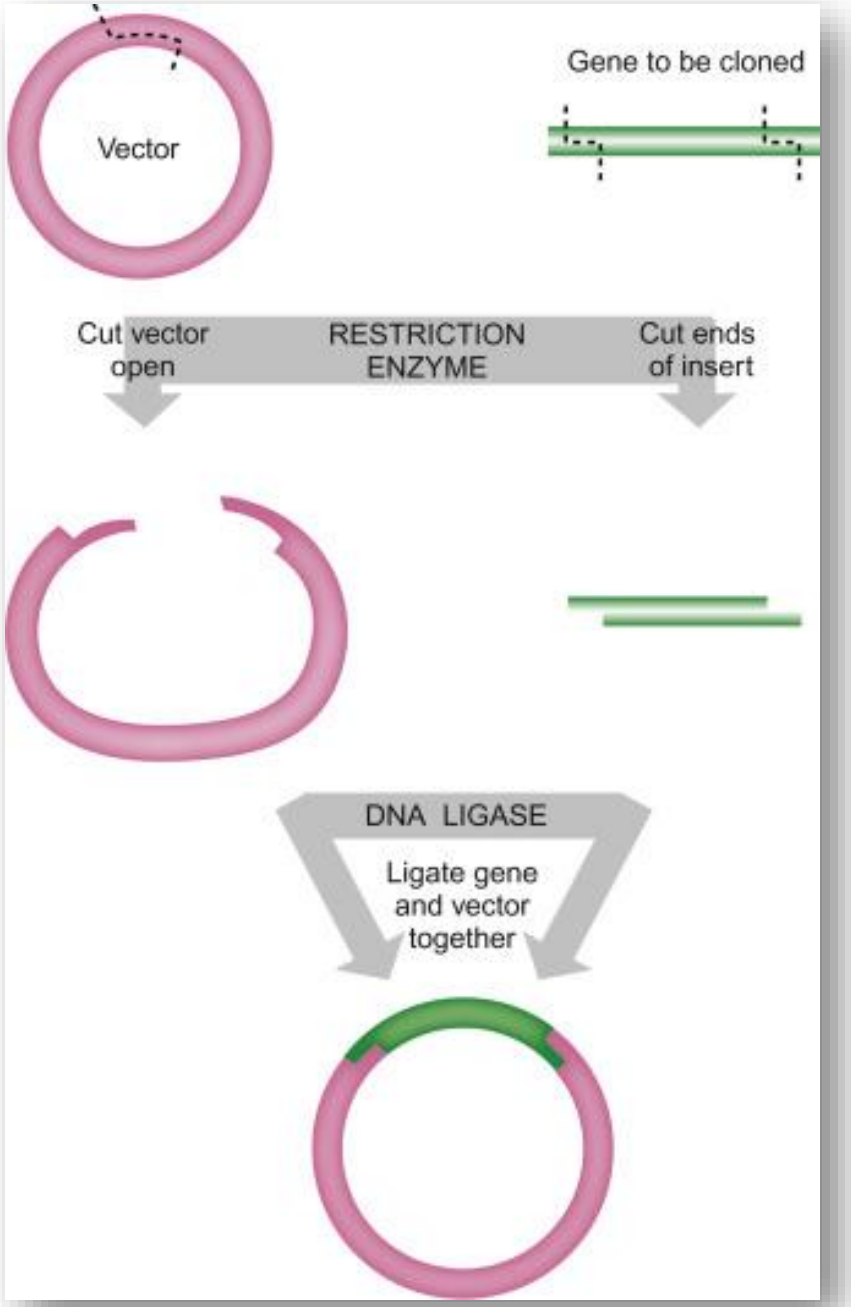
Steps:

1.Restriction enzymes cut both the target DNA and the vector at specific sequences, generating sticky or blunt ends.

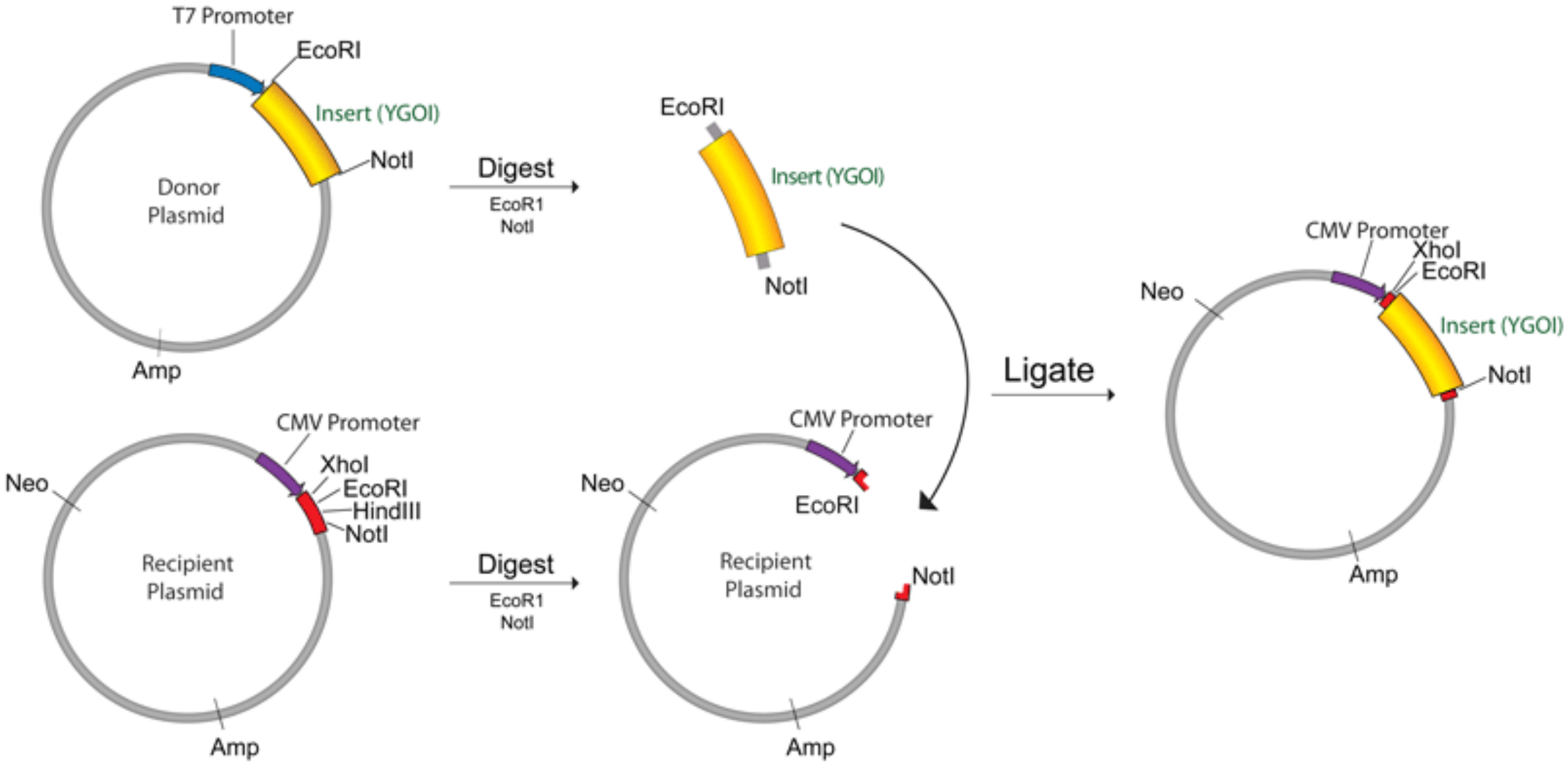
2.The cut DNA fragment is then inserted into the vector, and **DNA ligase** seals the nicks in the sugar-phosphate backbone.

3.Transform the recombinant vector into a host organism (e.g., **E. coli**) for replication or protein expression.





Restriction ligase method



2. TA Cloning

Overview: Utilizes the ability of **Taq DNA polymerase** to add a single **adenine (A)** overhang to the 3' ends of PCR products.

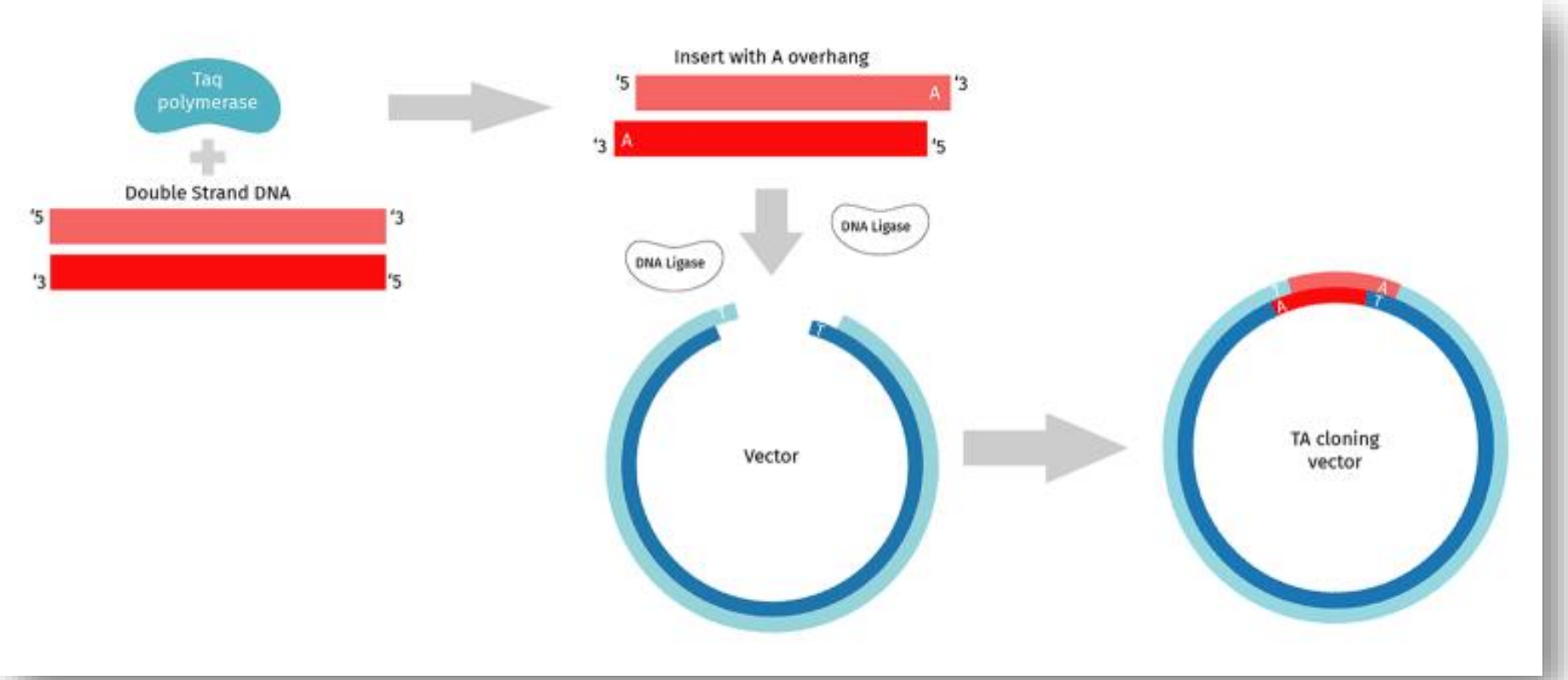
Advantages: Simple and fast; no need for restriction enzymes.

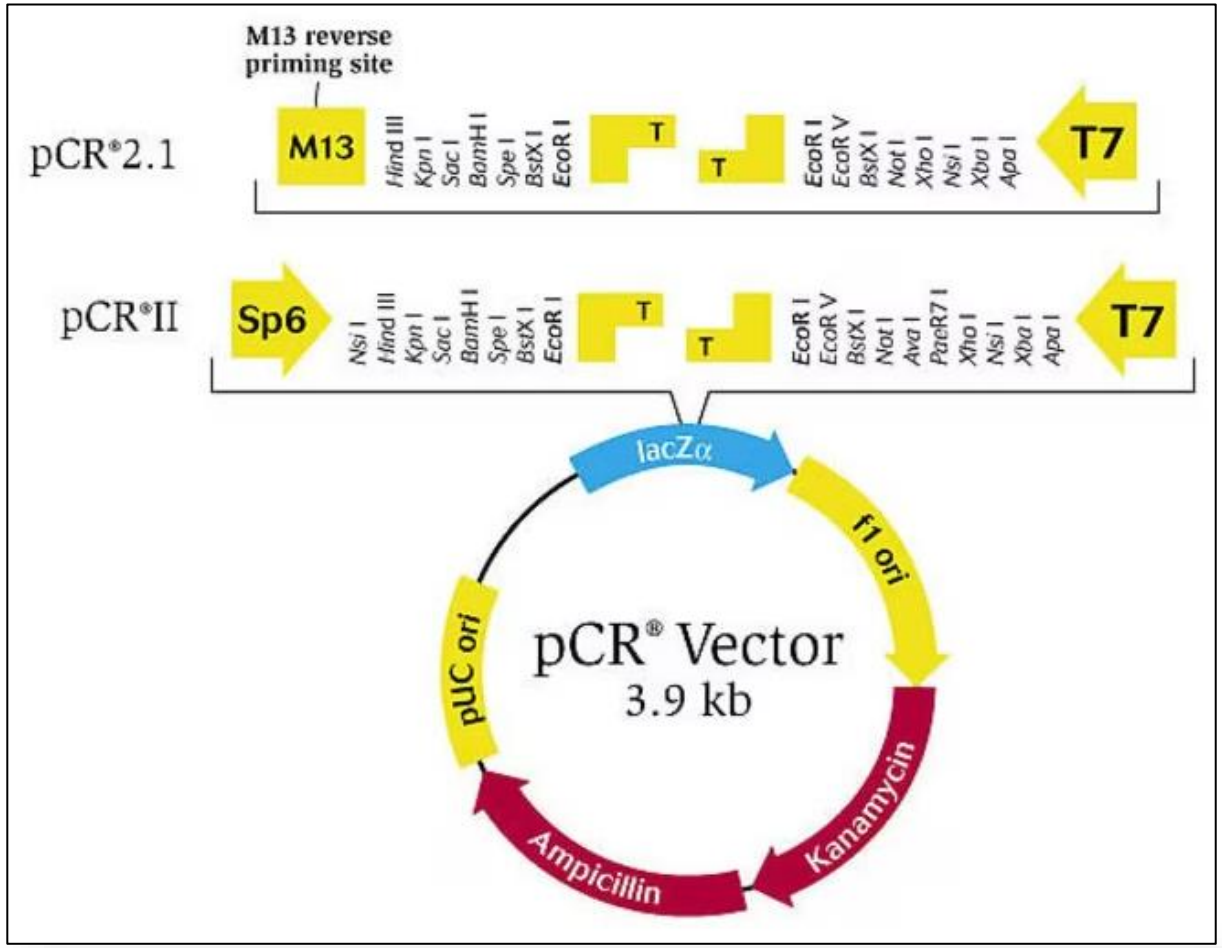
Limitations: Suitable mainly for PCR-amplified products.

Steps:

1.PCR-amplified DNA fragments (with A overhangs) are ligated into a vector with complementary **thymine (T)** overhangs.

2.The recombinant DNA is then transformed into competent cells.





Comments for pCR®2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

TA Cloning™ Kit, with pCR™2.1 Vector, without competent cells

https://www.thermofisher.com/order/catalog/product/K202020

3. TOPO Cloning

Overview: TOPO TA is an adaptation of standard TA cloning that uses the enzyme Topoisomerase I.

This technique uses the enzyme **topoisomerase** I to catalyze the ligation of a PCR product into a specially designed vector.

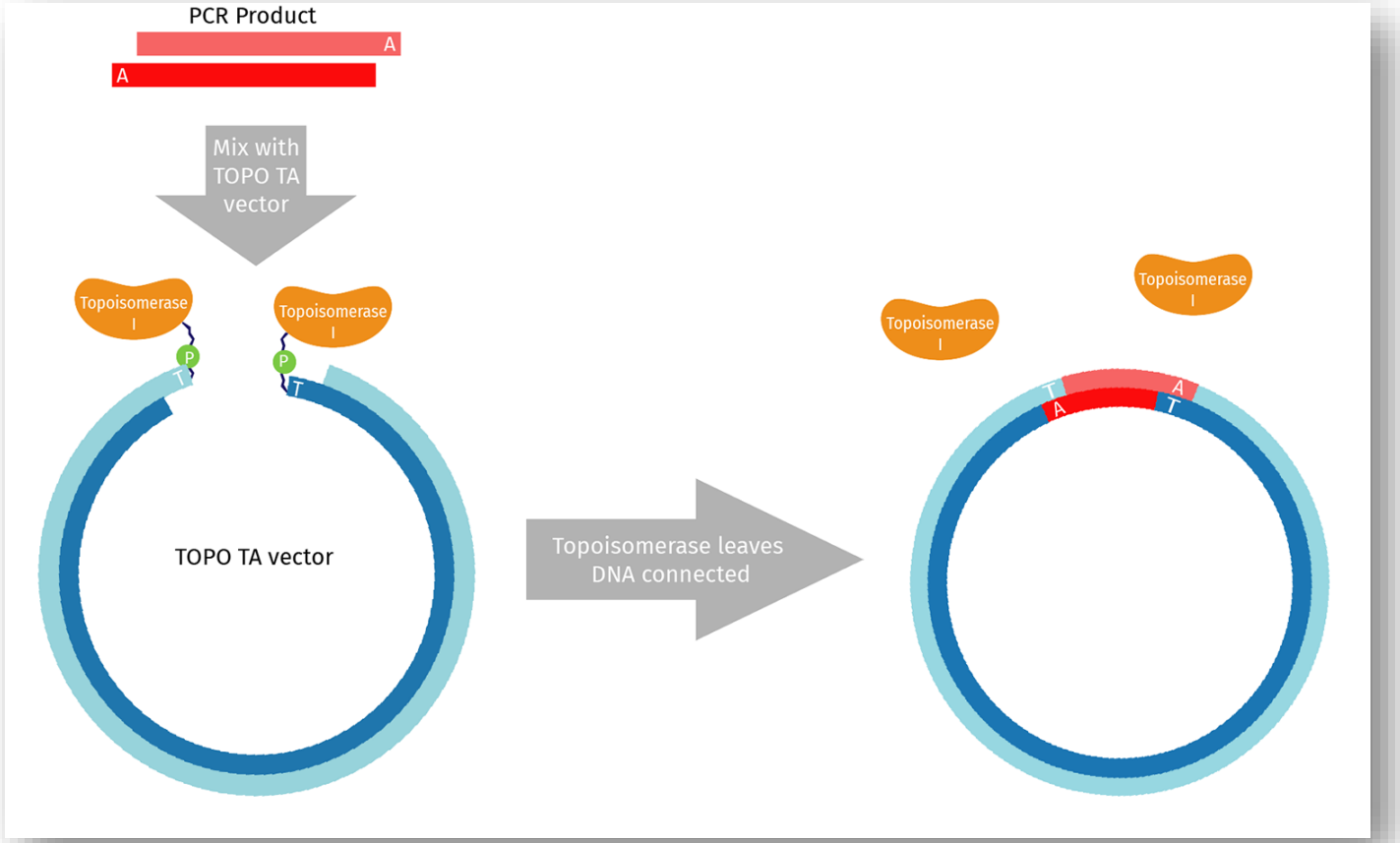
Advantages: Highly efficient and quick, no need for restriction enzymes or ligase.

Limitations: Relatively expensive; limited to topoisomerase-specific vectors.

- ✓ **When purchased** for use in the lab, topoisomerase is pre-assembled on both ends of a linearized TOPO vector. The topoisomerase/vector complex is poised to complete the re-ligation step once a suitable DNA substrate is provided.
- ✓ **As a result**, TOPO cloning is generally very efficient with no background. A TOPO reaction can be transformed after a 5-minute incubation.

Steps:

- ➢ The vector has ***topoisomerase*** covalently attached to it, which allows the rapid ligation of the PCR product (usually with A overhangs).



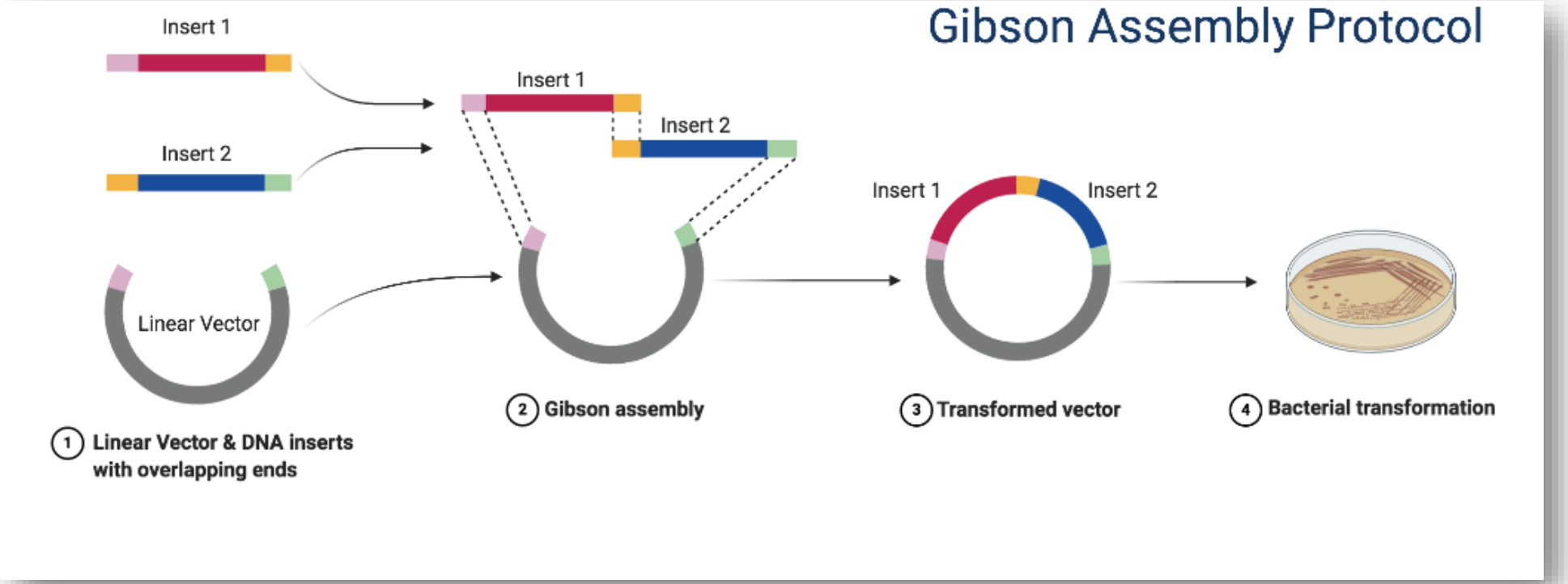
4. Gibson Assembly

Overview: Gibson Assembly® is a recombination-based molecular cloning method for the in vitro assembly of DNA fragments. Developed by Daniel G. Gibson and his colleagues in 2009, this methodology enables easy assembly of multiple DNA fragments into a circular plasmid in a single-tube isothermal reaction. The result is a scarless DNA molecule of up to 15 kb in size.

A method that allows the assembly of multiple DNA fragments in a single reaction using **overlapping sequences** and a mix of enzymes (exonuclease, polymerase, ligase).

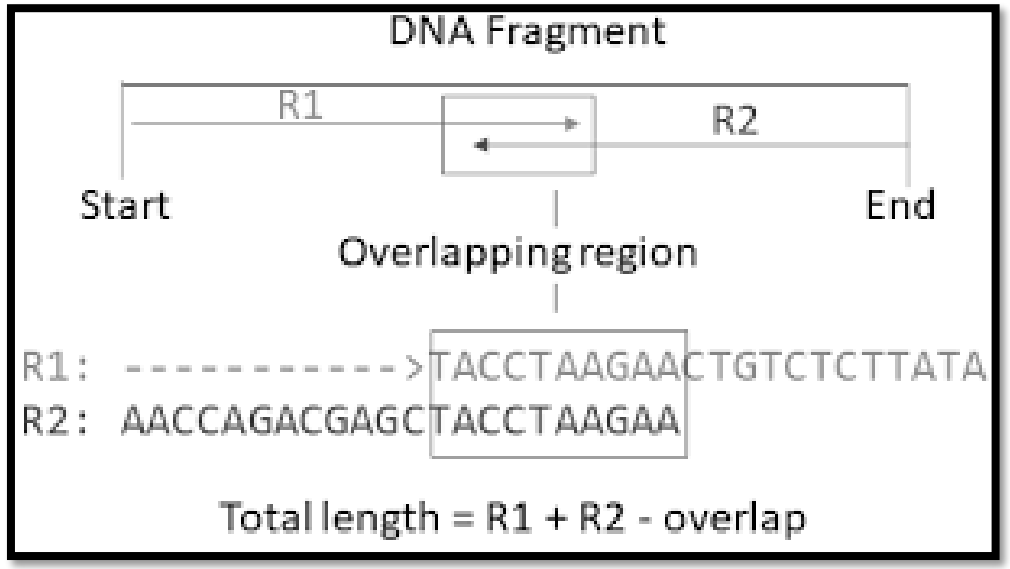
Advantages: Can assemble multiple DNA fragments in one reaction, no need for restriction sites.

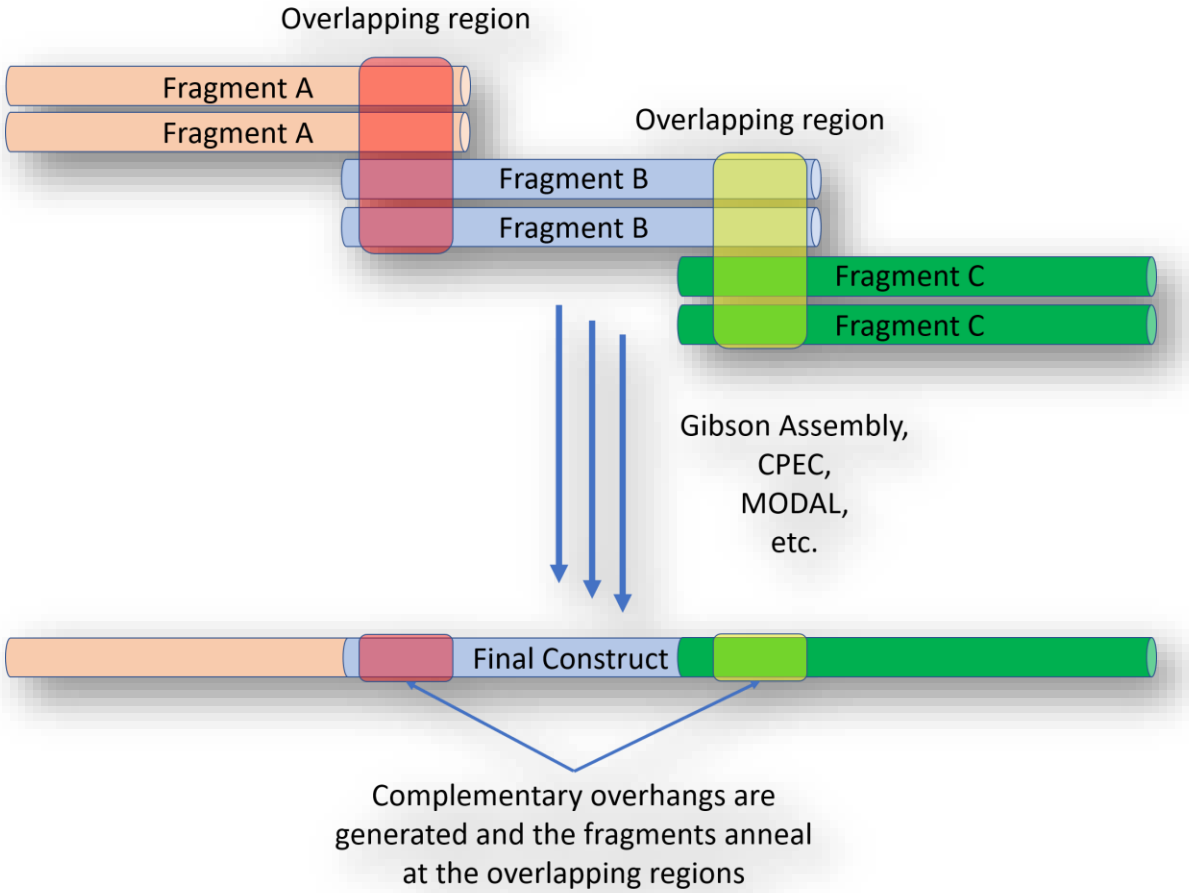
Limitations: Requires precise design of overlapping sequences.



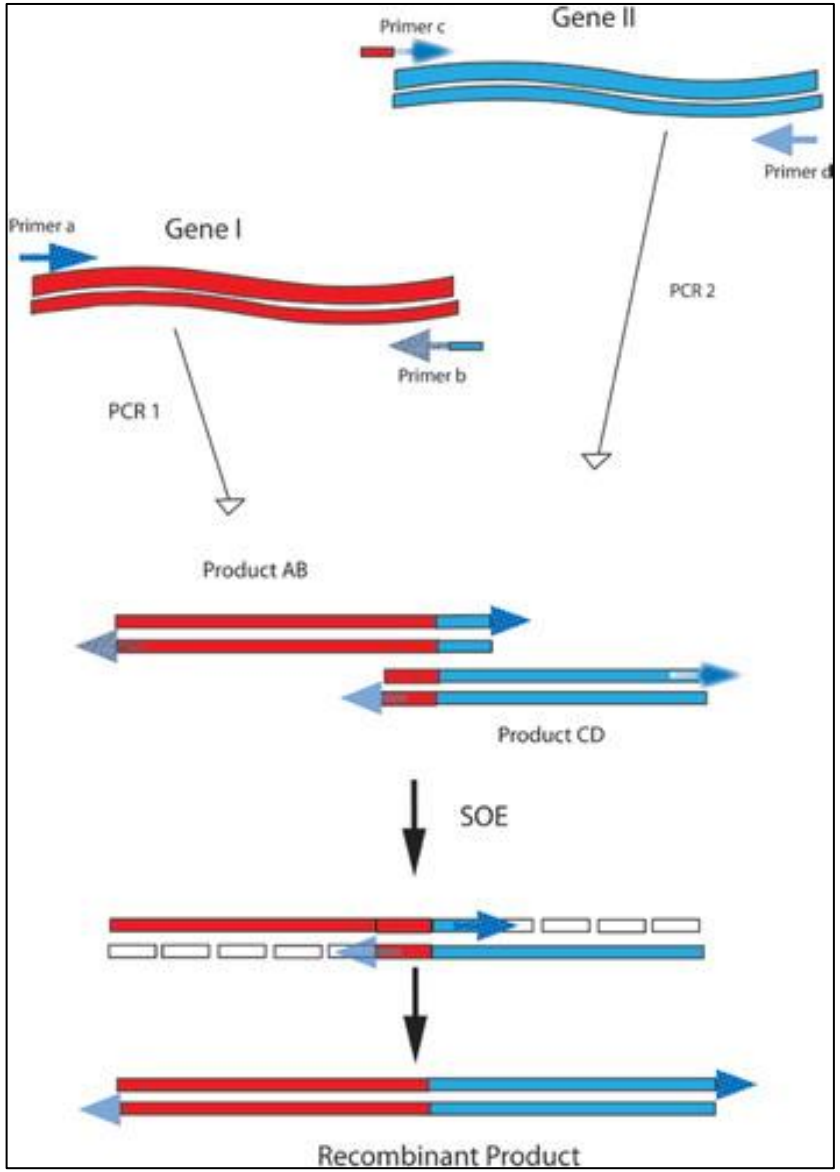
Steps:

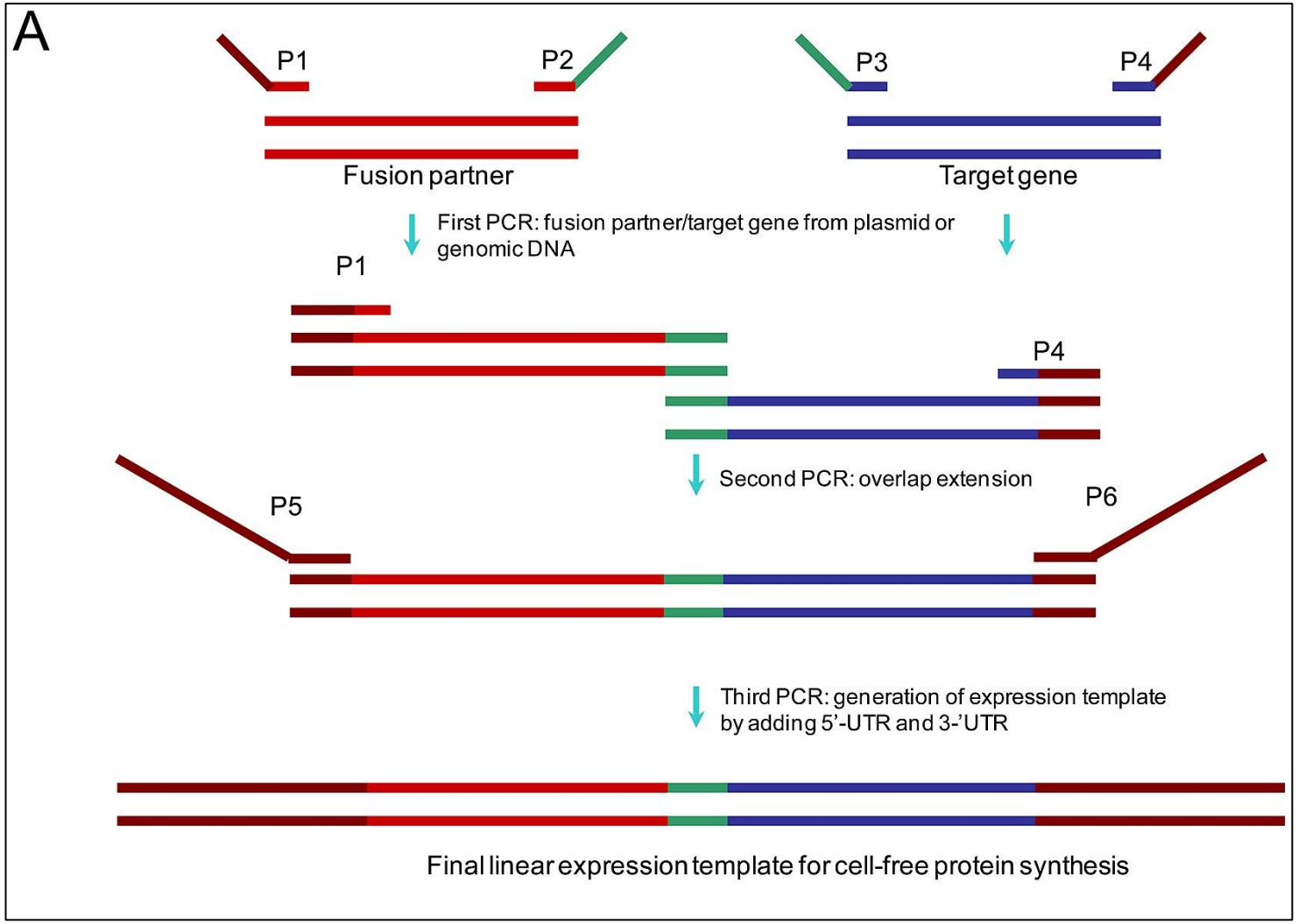
1. Design your plasmid and order primers;
2. Generate DNA segments by PCR. DNA fragments with overlapping regions (20-40 bp) are joined together. Exonuclease chews back the 5' ends to expose complementary sequences, polymerase fills in the gaps, and ligase seals the nicks.



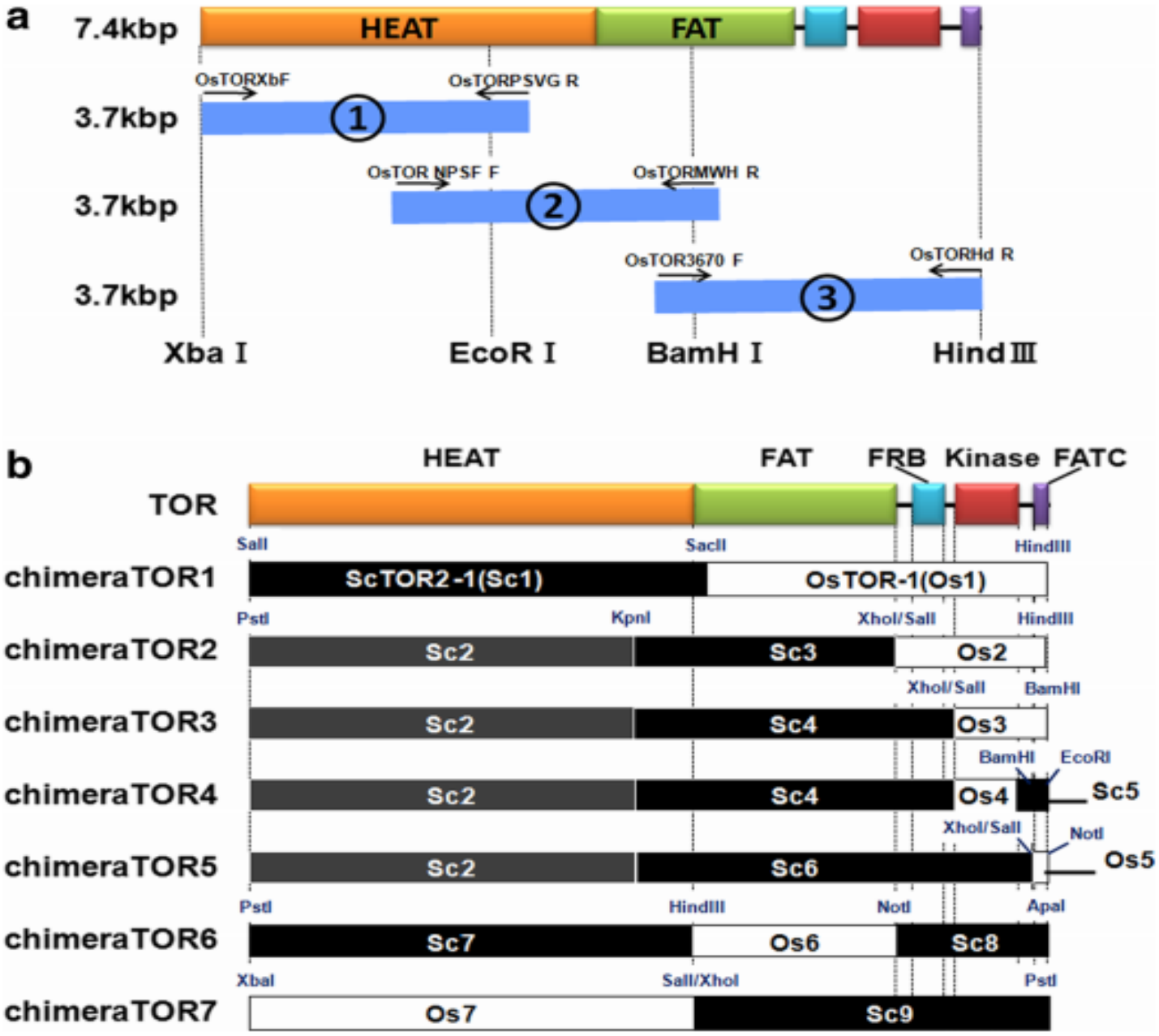


Overlapping extinction PCR





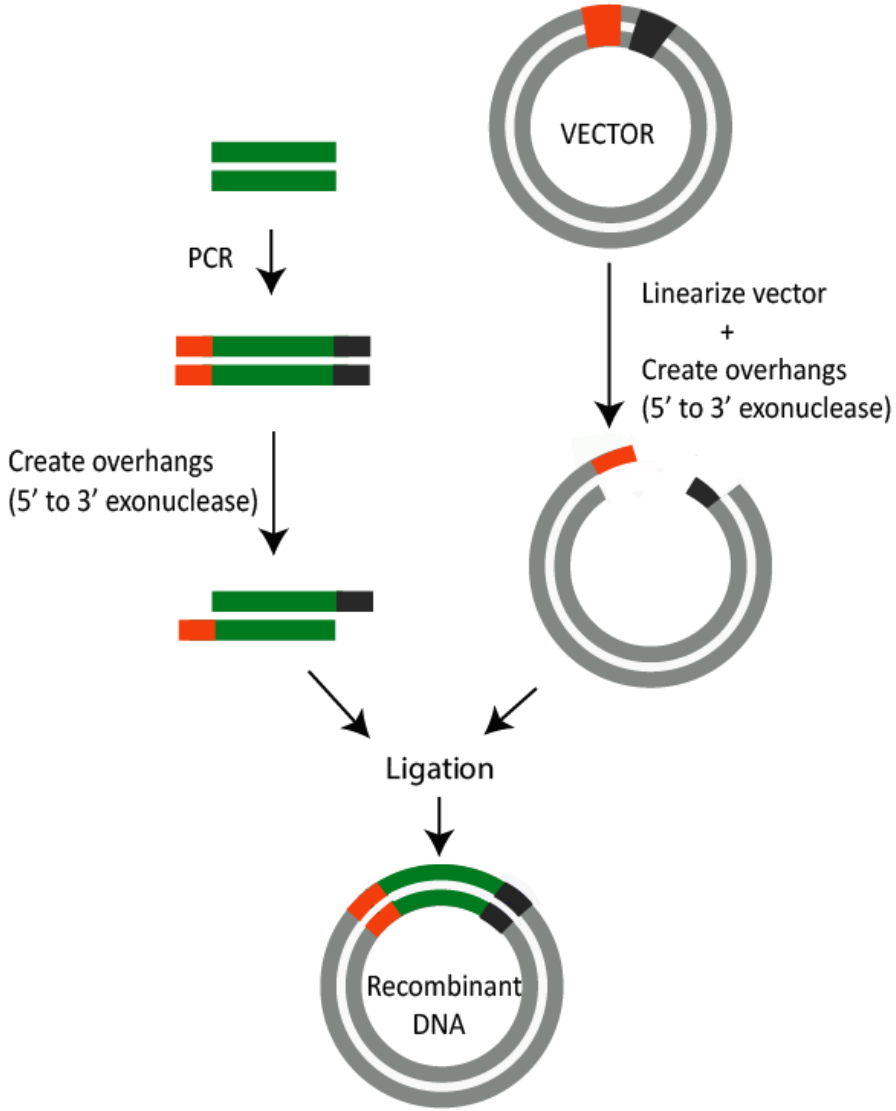
Creation of chimeric genes



4. PCR Cloning Method

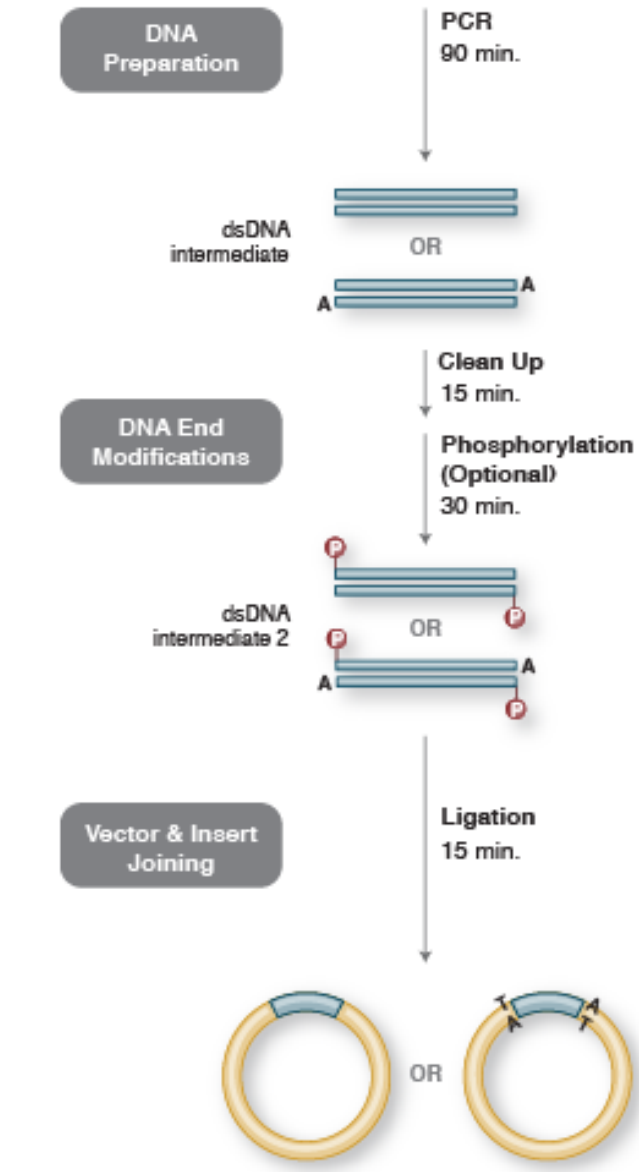
Overview: PCR cloning differs from traditional cloning in that the DNA fragment of interest, and even the vector, can be amplified by the Polymerase Chain Reaction (PCR) and ligated together, without the use of restriction enzymes.

PCR cloning is a rapid method for cloning genes, and is often used for projects that require higher throughput than traditional cloning methods can accommodate. It allows for the cloning of DNA fragments that are not available in large amounts.



Advantages:
- High efficiency, with dedicated vectors
- Amenable to high throughput

Disadvantages:
- Limited vector choices
- Higher cost
- Lack of sequence control at junction
- Multi-fragment cloning is not straight forward
- Directional cloning is difficult


DNA Preparation
PCR
90 min.
dsDNA intermediate
OR
A
A
Clean Up
15 min.
DNA End Modifications
Phosphorylation (Optional)
30 min.
dsDNA intermediate 2
OR
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P
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Vector & Insert Joining
Ligation
15 min.
OR
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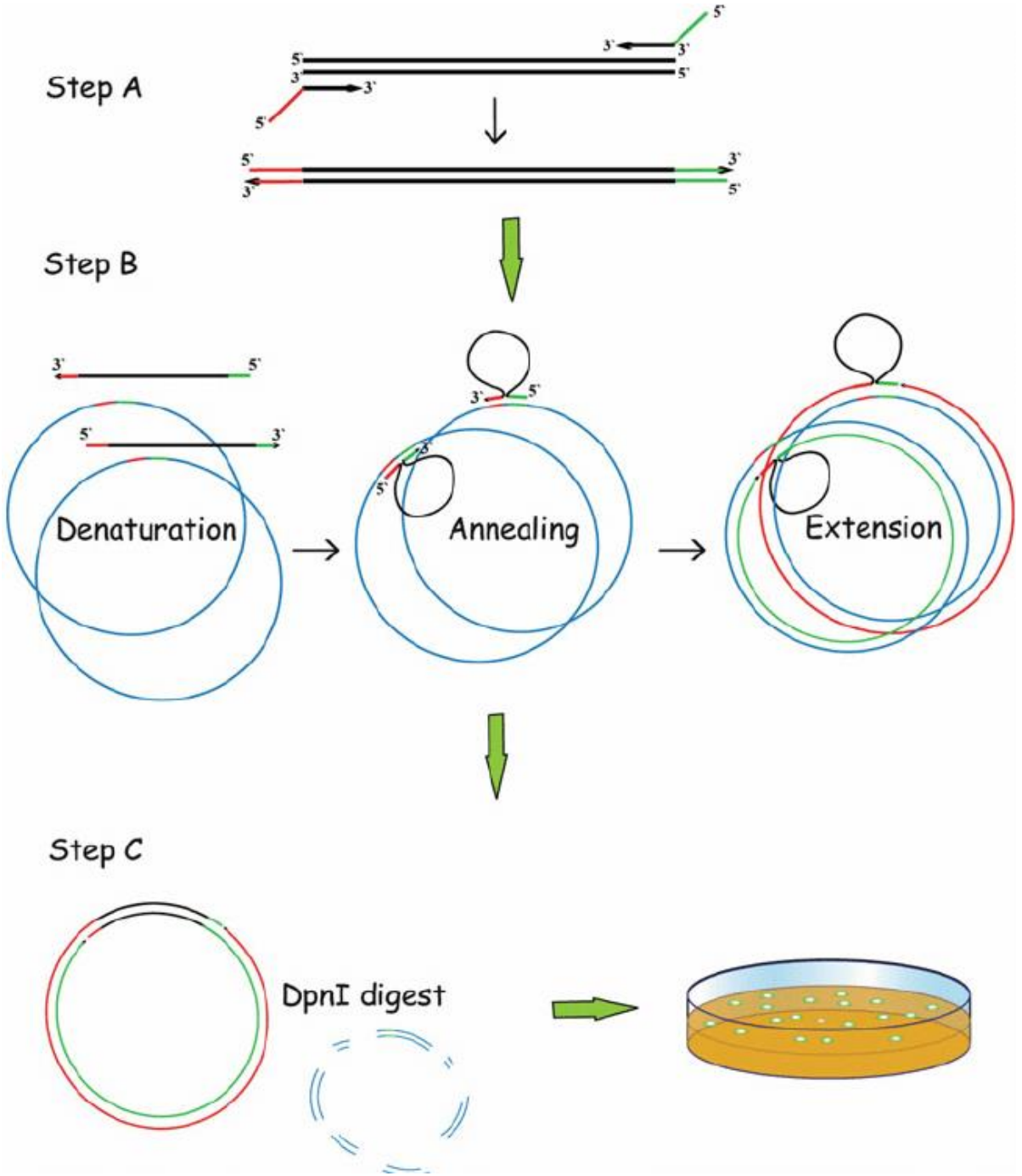
Estimated total time
2 hr. – 2 hr., 30 min.


Step A
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Step B
3'
5'
5'
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Denaturation
3'
5'
5'
3'
Annealing
Extension
Step C
DpnI digest

References

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