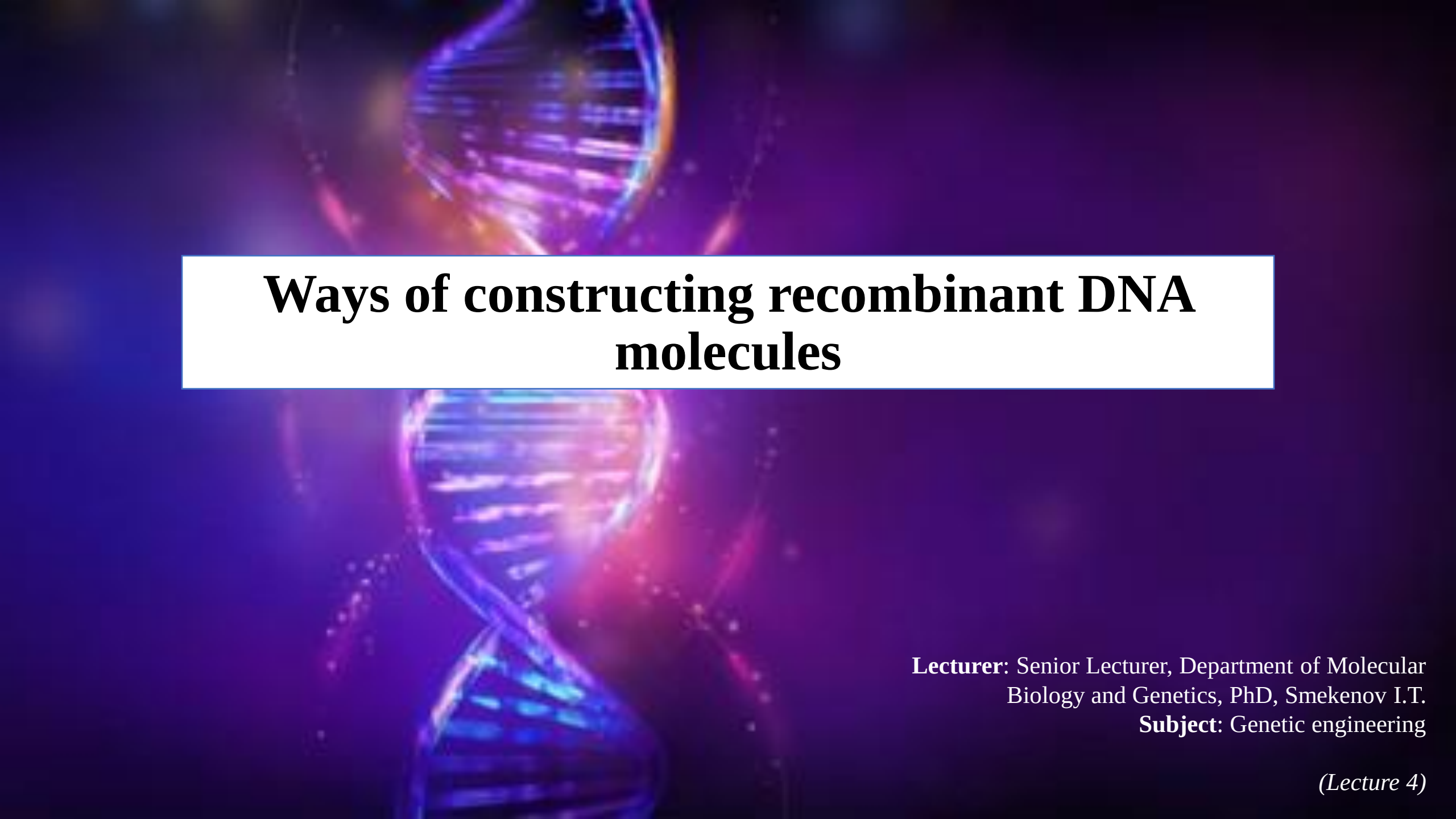


Ways of constructing recombinant DNA molecules

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

Subject: Genetic engineering

(Lecture 4)

Lecture Goal:
To explore the methods for constructing recombinant DNA molecules, focusing on selective and reporter genes used in genetic engineering.

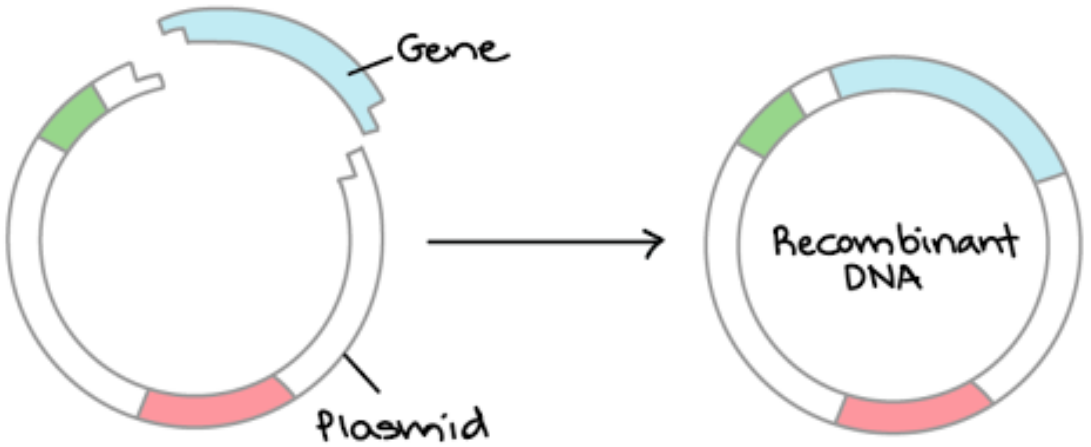
Tasks:

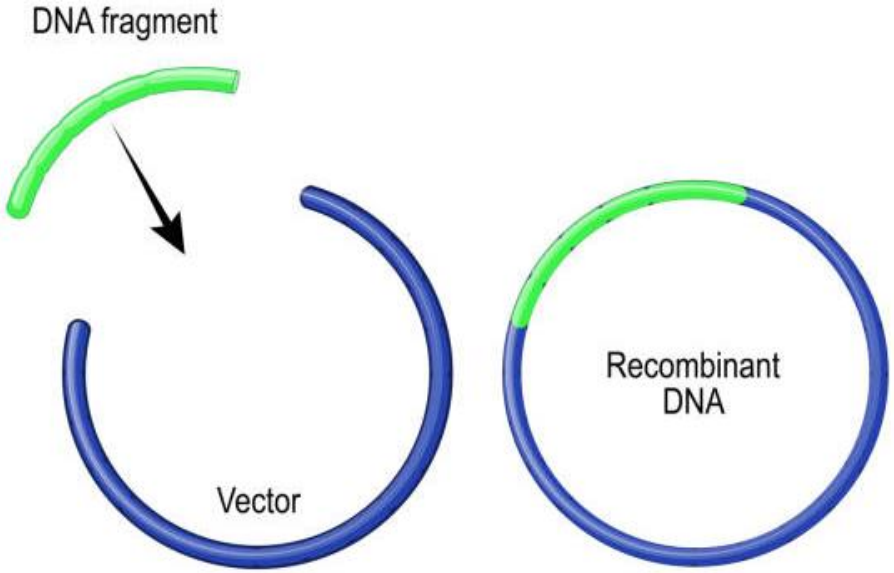
1. Discuss the main techniques for constructing recombinant DNA, emphasizing the role of selective genes (selection markers) in identifying successful recombinants.
2. Explain the function of reporter genes in monitoring gene expression, highlighting the most commonly used reporter genes such as β-glucuronidase (GUS), green fluorescent protein (GFP), luciferase (LUC), and chloramphenicol acetyltransferase (CAT).
3. Analyze why GUS and GFP are more frequently used than LUC and CAT in modern genetic experiments.

Keywords: *Recombinant DNA, selective genes, selection markers, reporter genes, β-glucuronidase (GUS), green fluorescent protein (GFP), luciferase (LUC), chloramphenicol acetyltransferase (CAT), gene expression, genetic markers*

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.

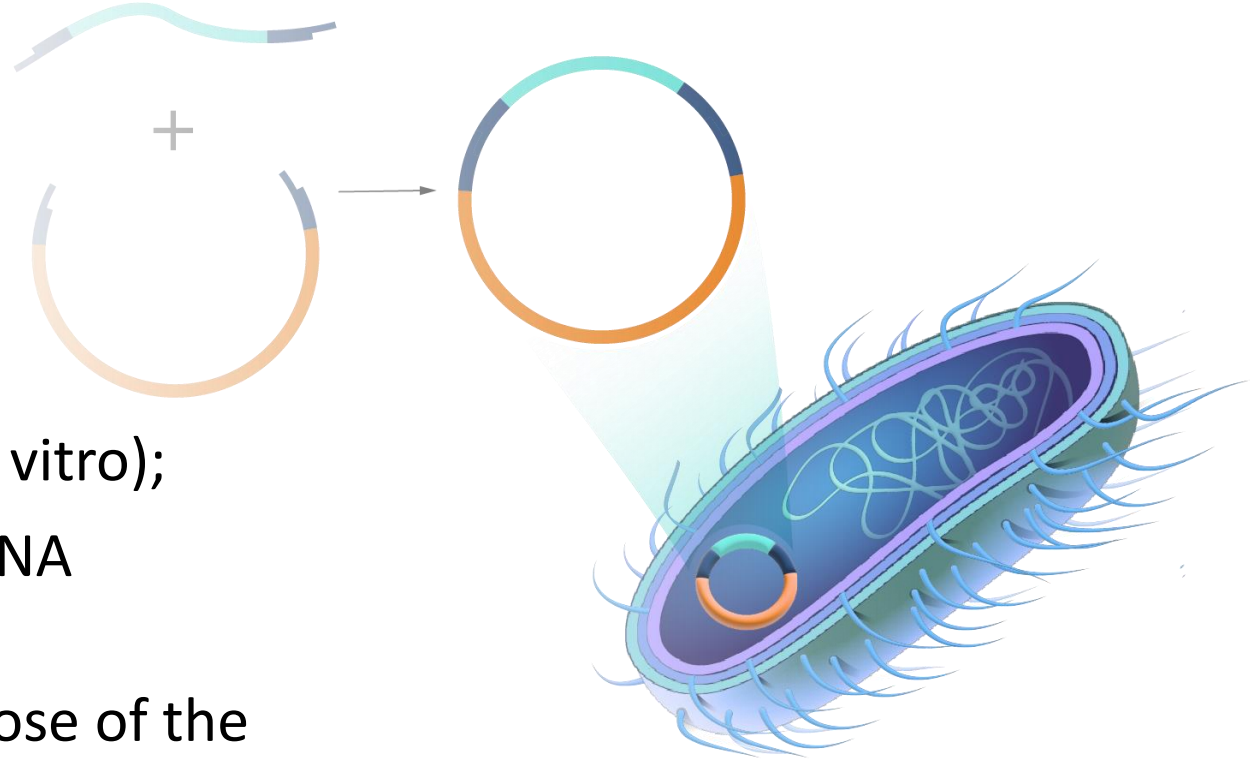




- ***Recombinant*** is understood as DNA formed by combining *in vitro* (in tube) two or more DNA fragments isolated from different biological sources.

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;



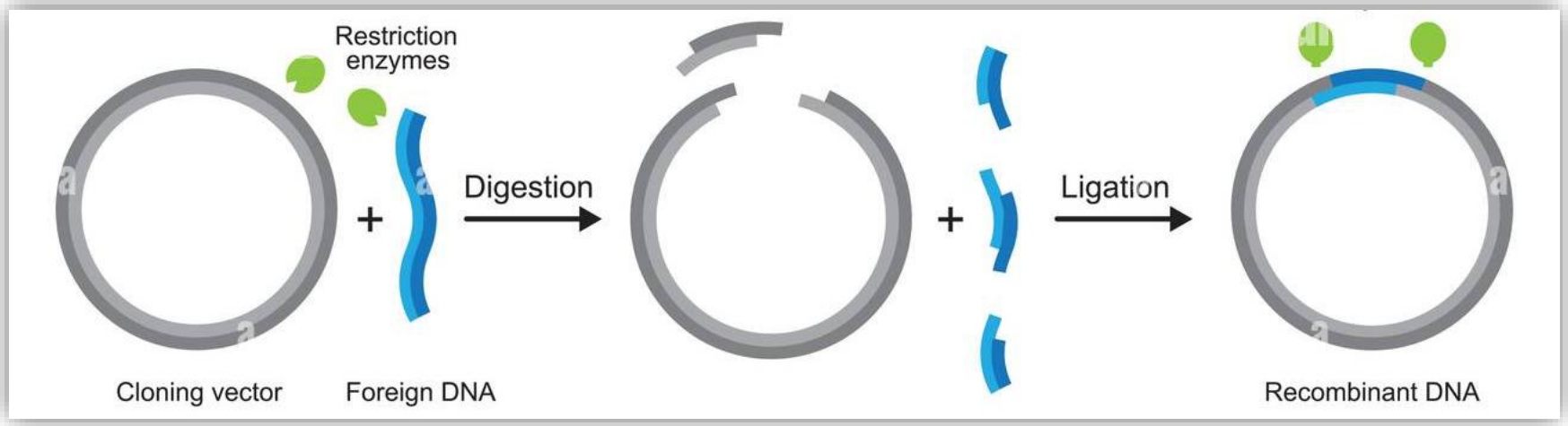
There are several methods for constructing recombinant DNA molecules

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.

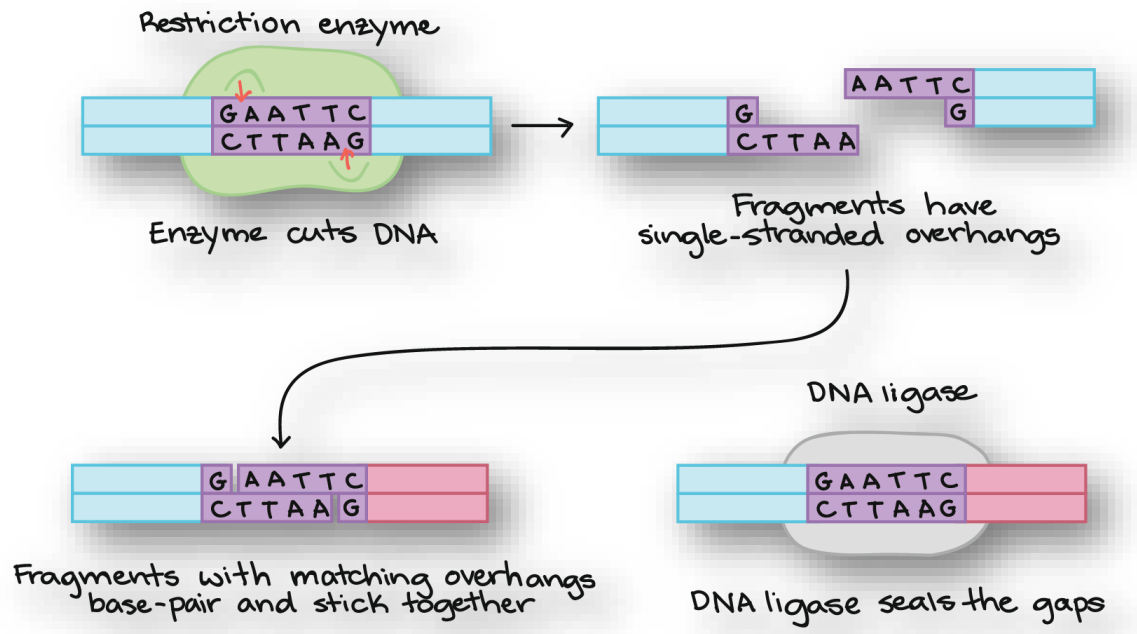


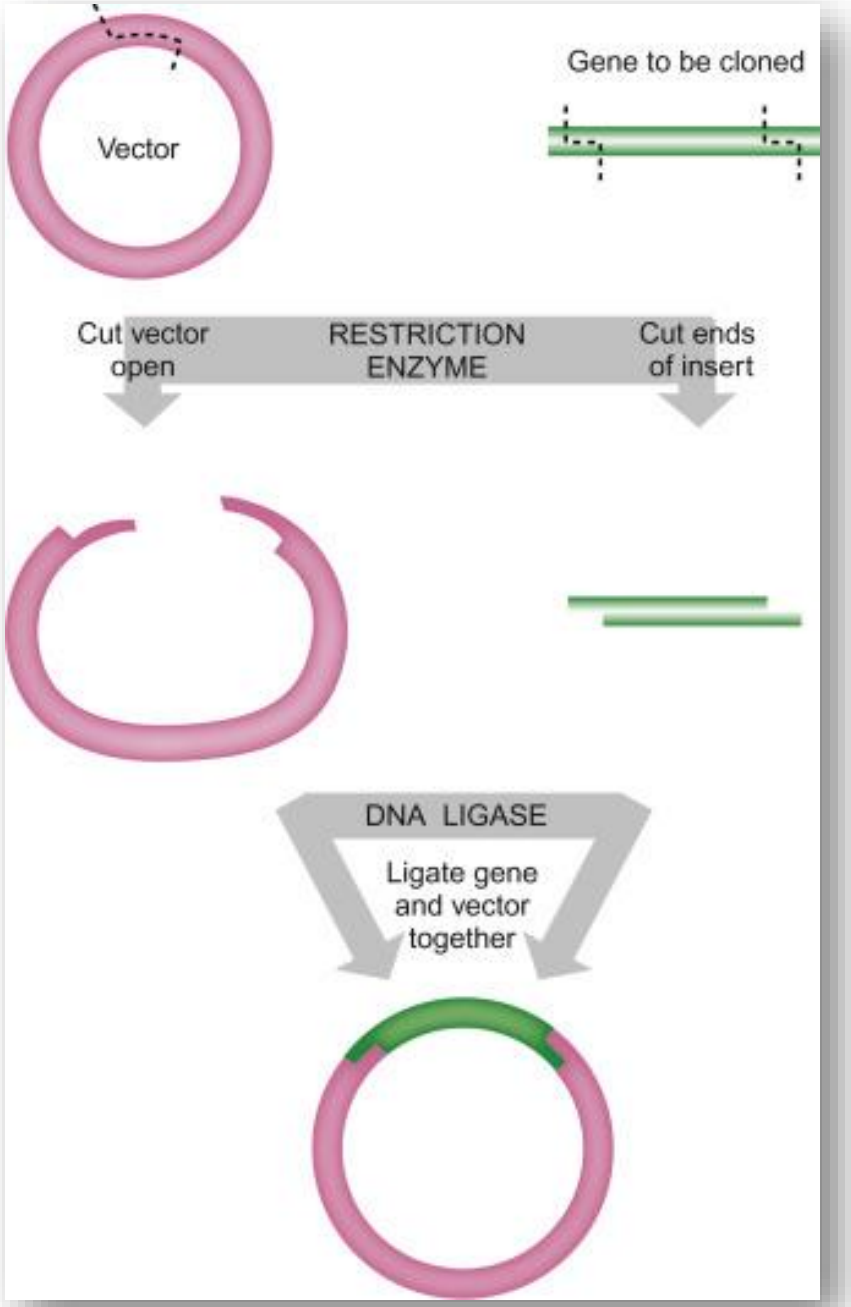
Steps:

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

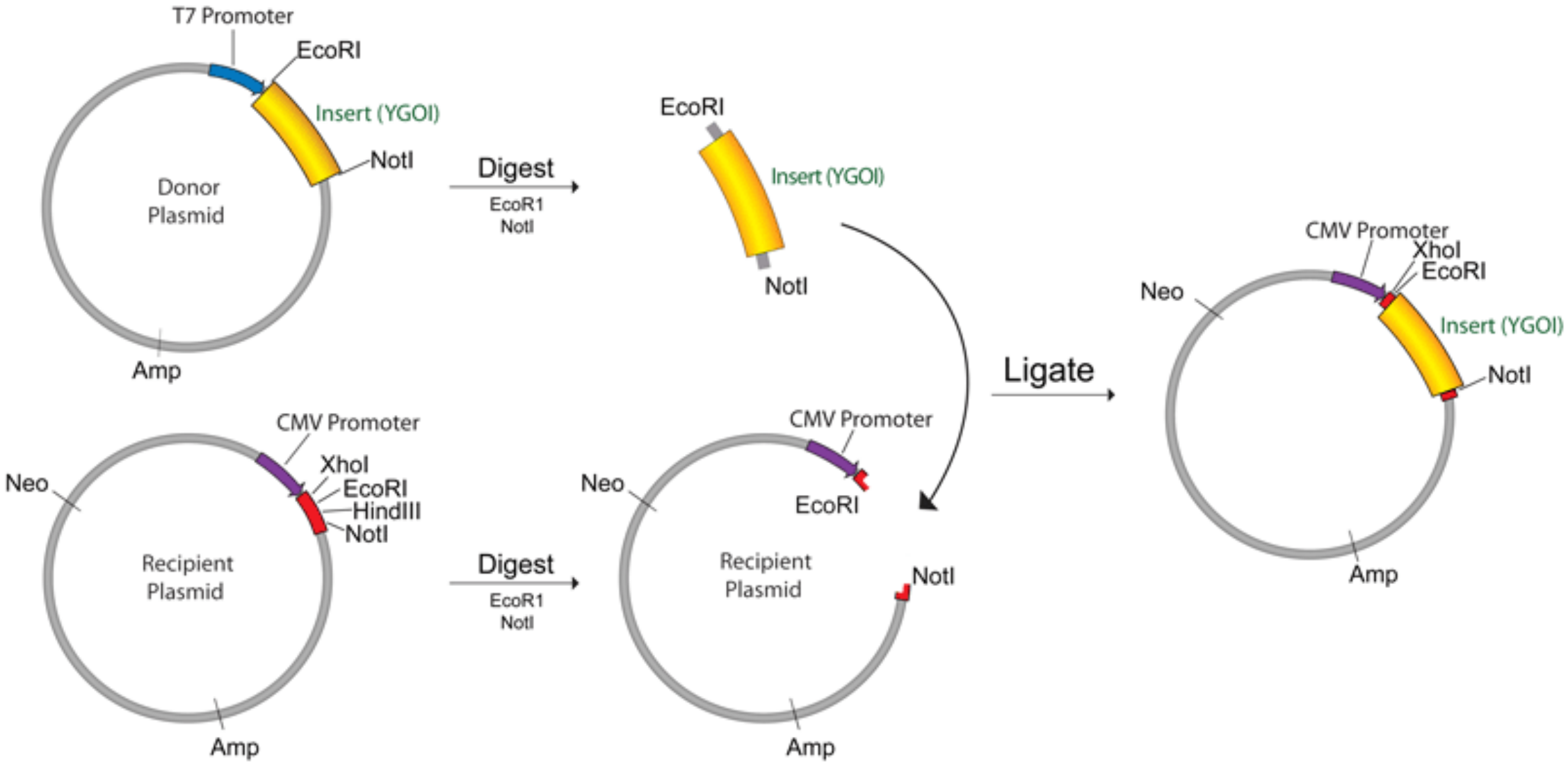
2.The cut DNA fragment is then inserted into the vector, and **DNA ligase** seals the nicks in the sugar-phosphate backbone.

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.

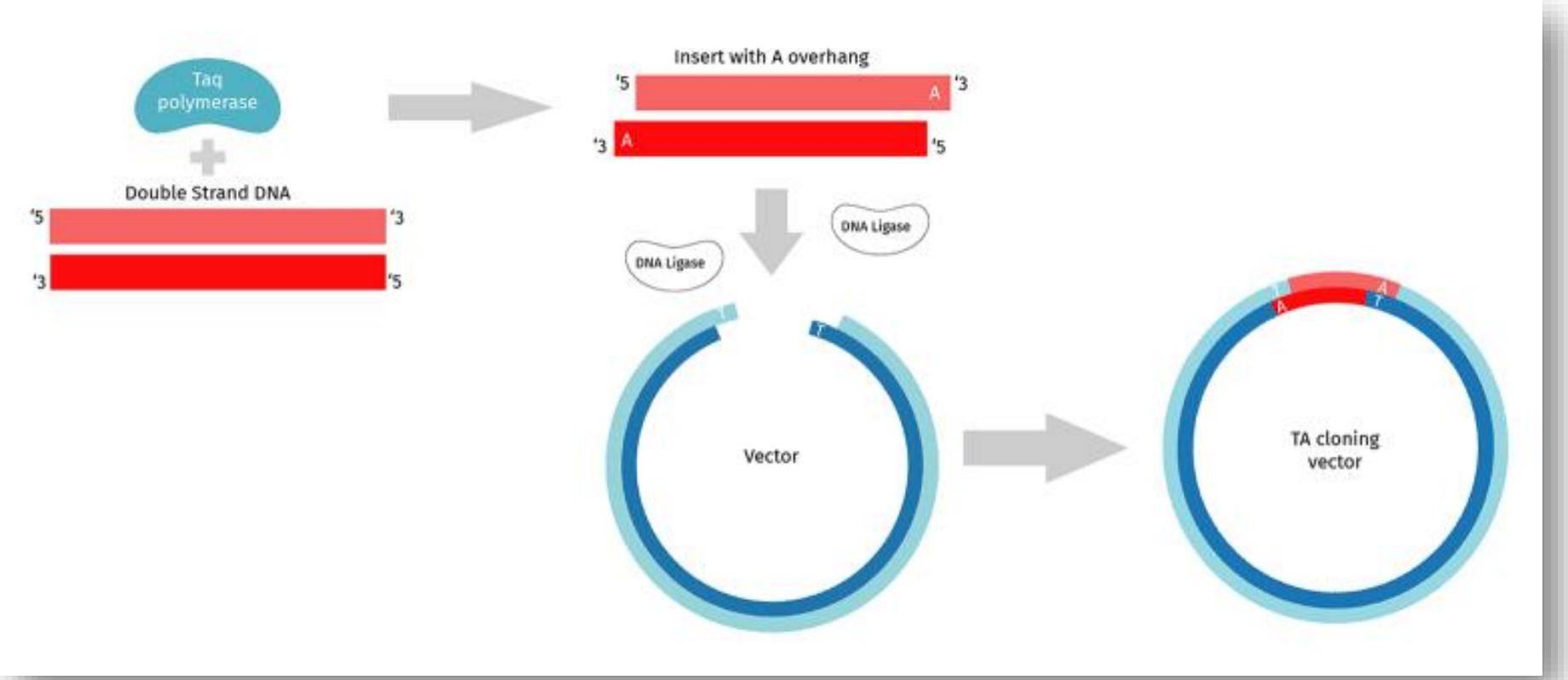
Advantages: Simple and fast; no need for restriction enzymes.

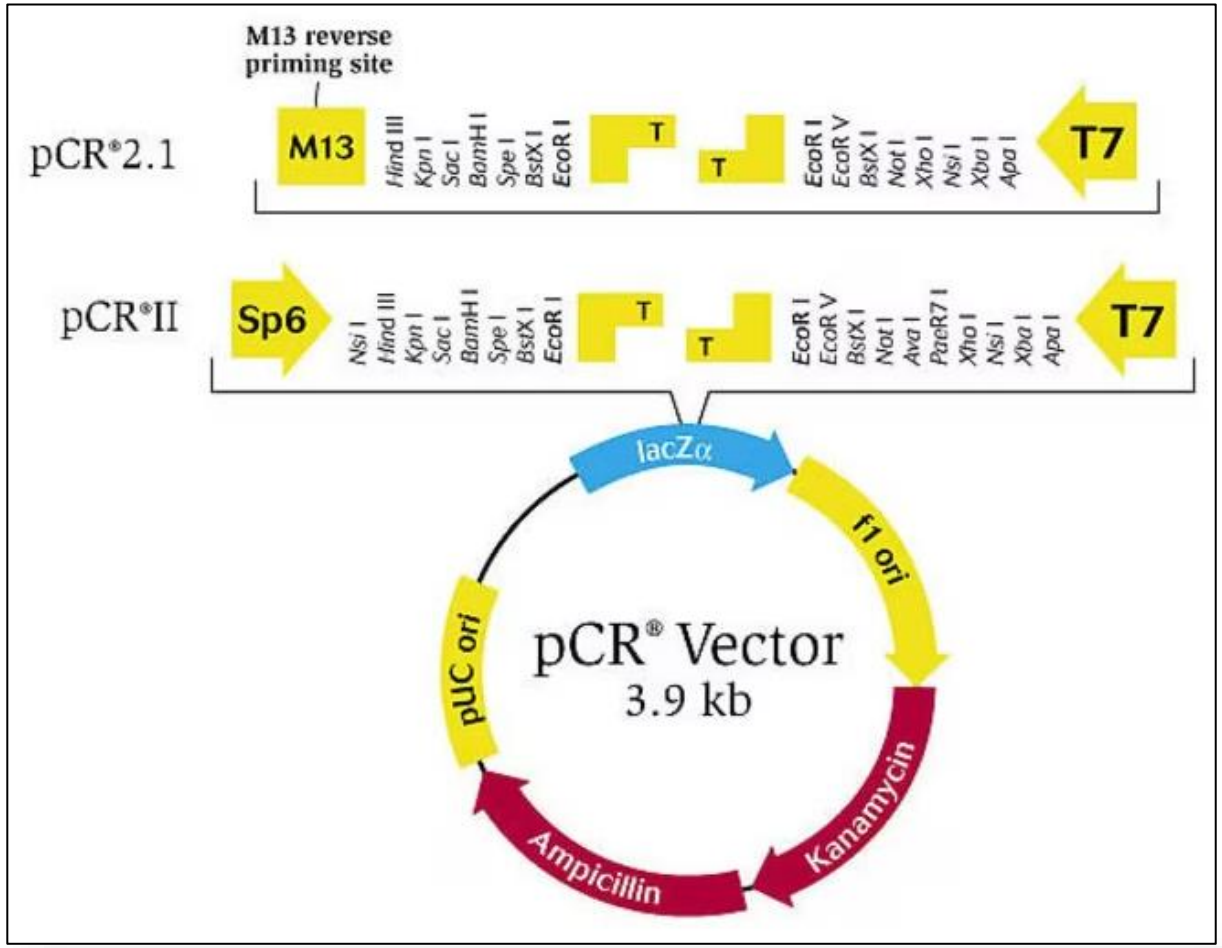
Limitations: Suitable mainly for PCR-amplified products.

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.

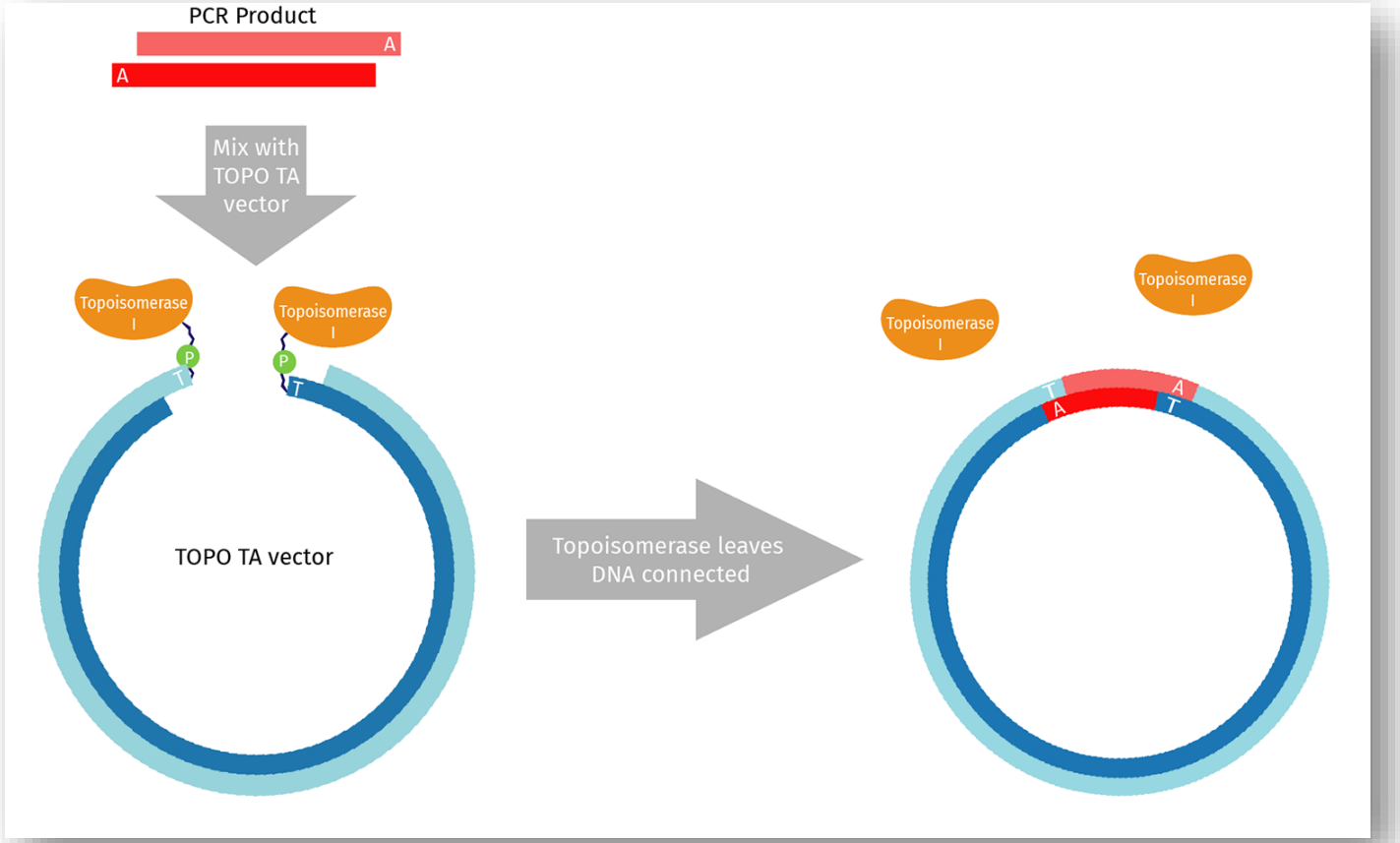
Advantages: Highly efficient and quick, no need for restriction enzymes or ligase.

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).



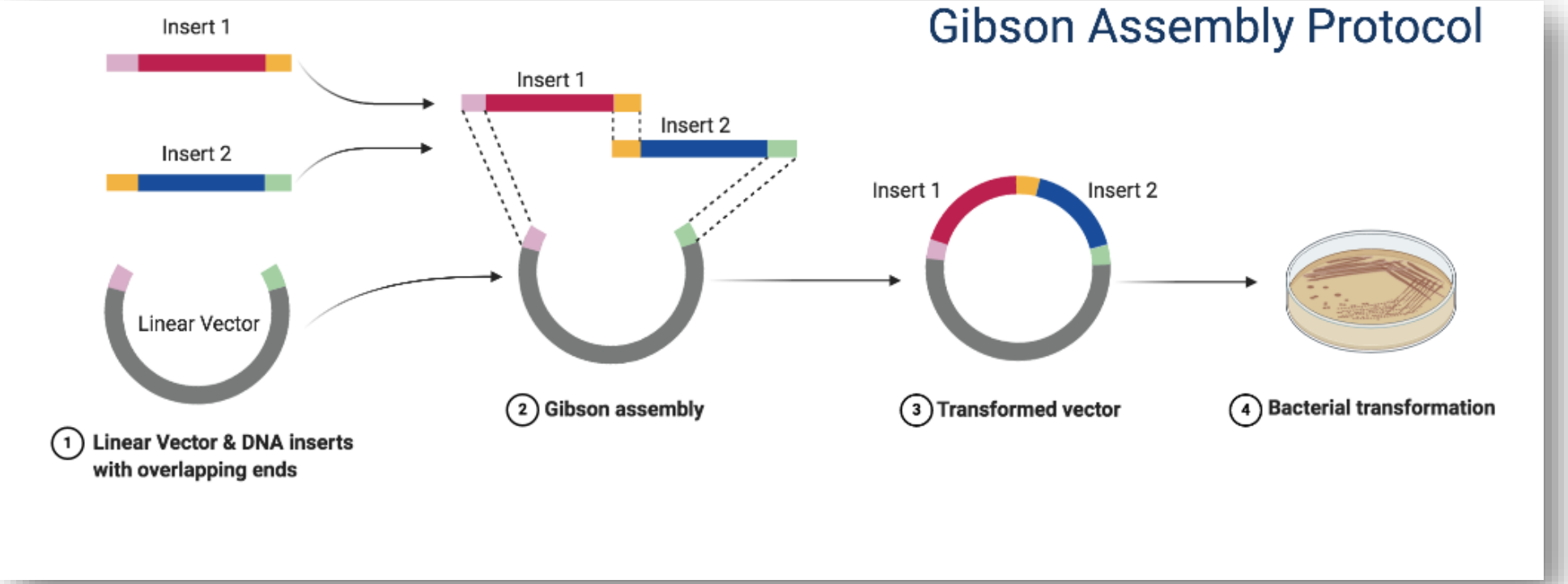
4. Gibson Assembly

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).

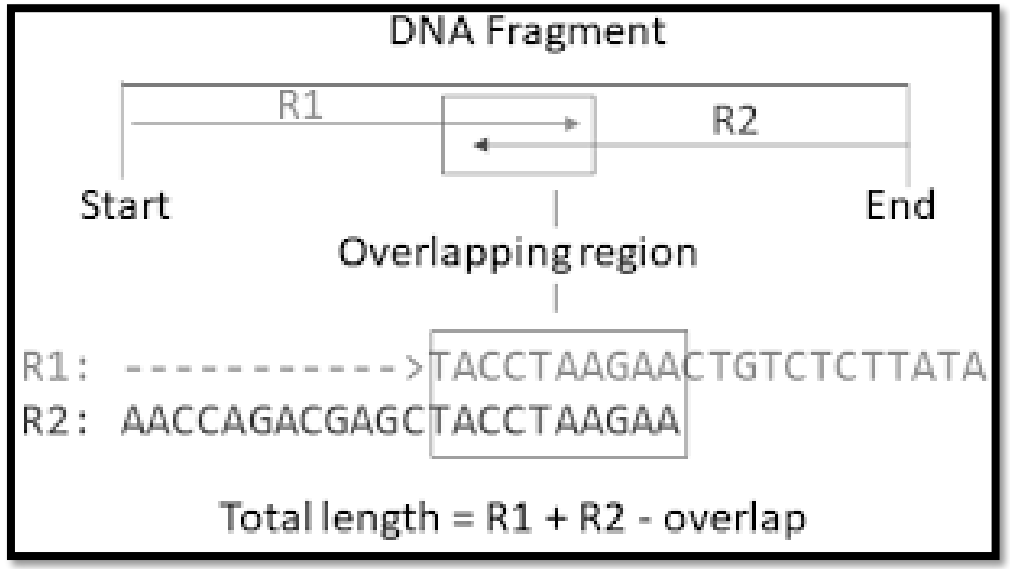
Advantages: Can assemble multiple DNA fragments in one reaction, no need for restriction sites.

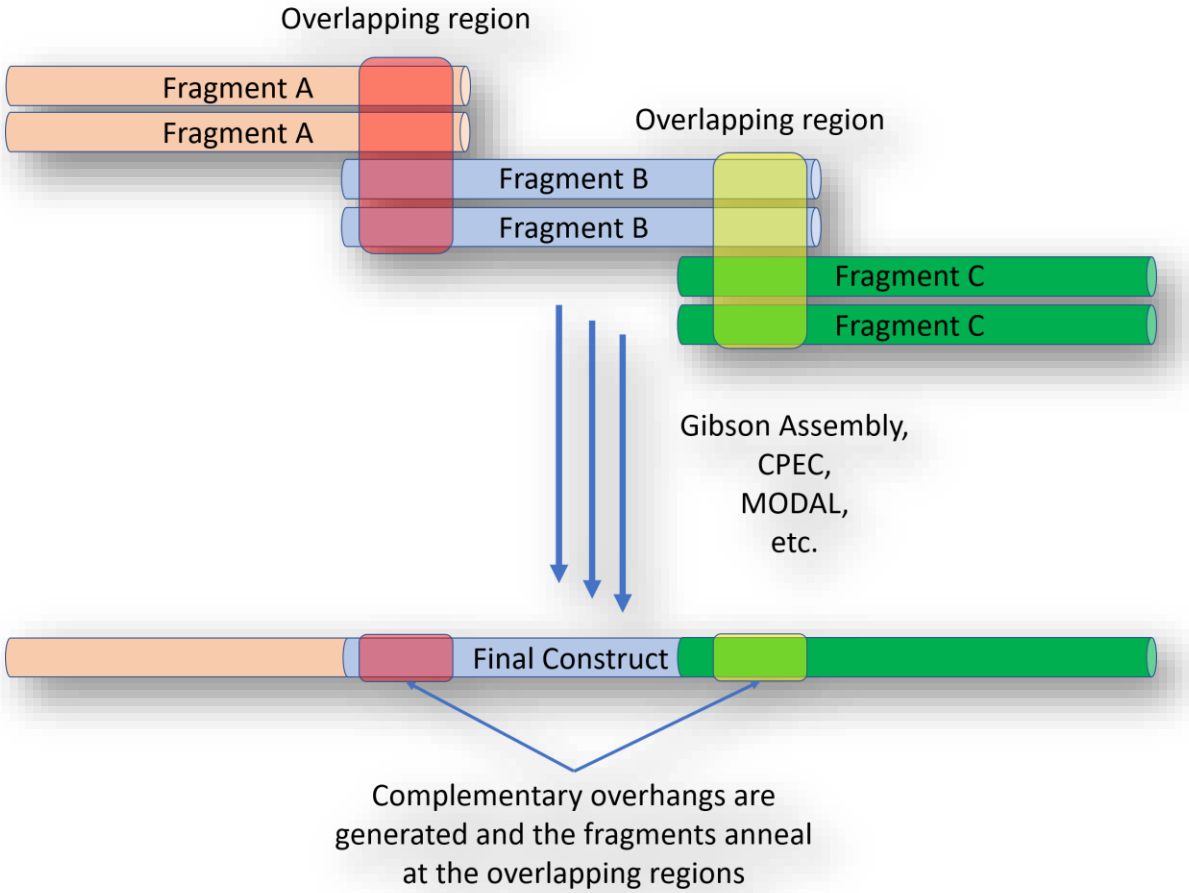
Limitations: Requires precise design of overlapping sequences.



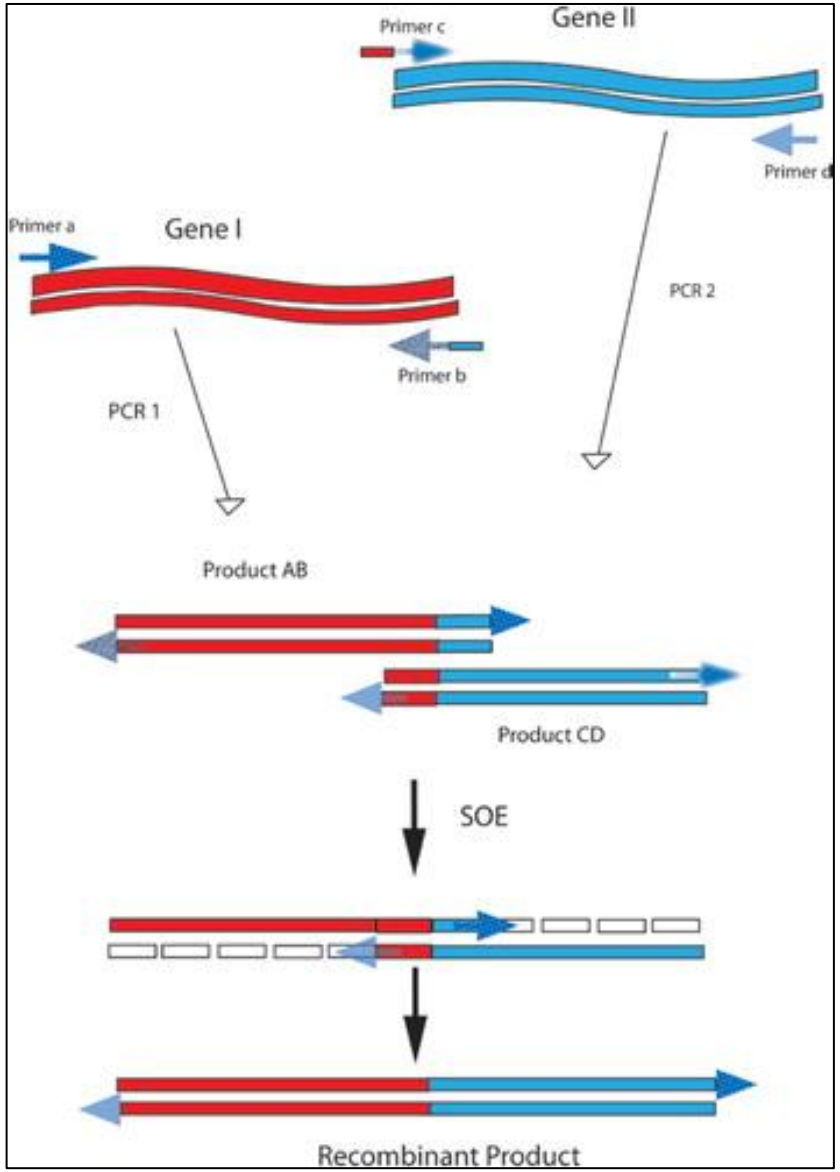
Steps:

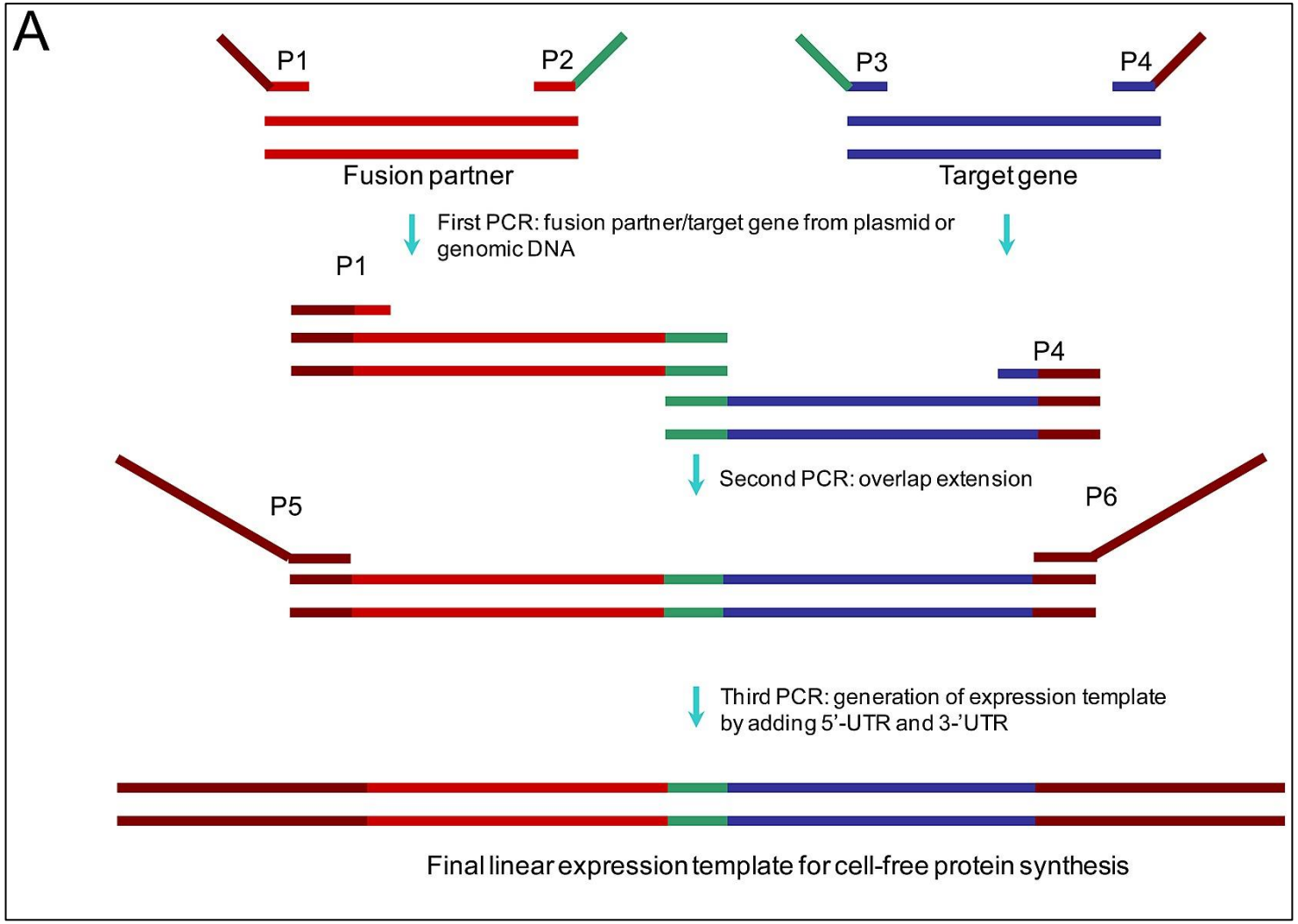
1. Design your plasmid and order primers;
2. Generate DNA segments by PCR. DNA fragments with overlapping regions (20-40 bp) are joined together. Exonuclease chews back the 5' ends to expose complementary sequences, polymerase fills in the gaps, and ligase seals the nicks.



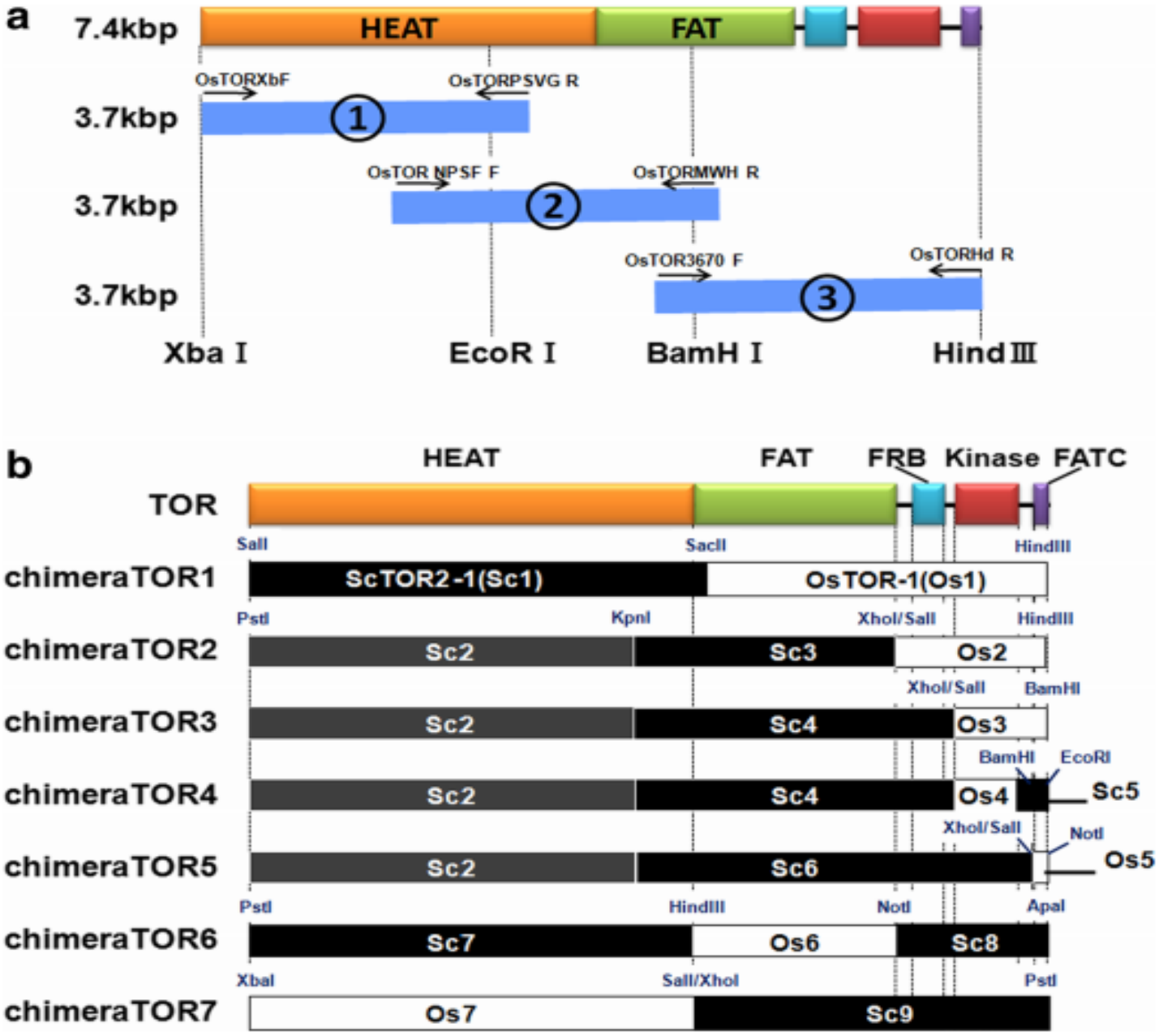


Overlapping extinction PCR





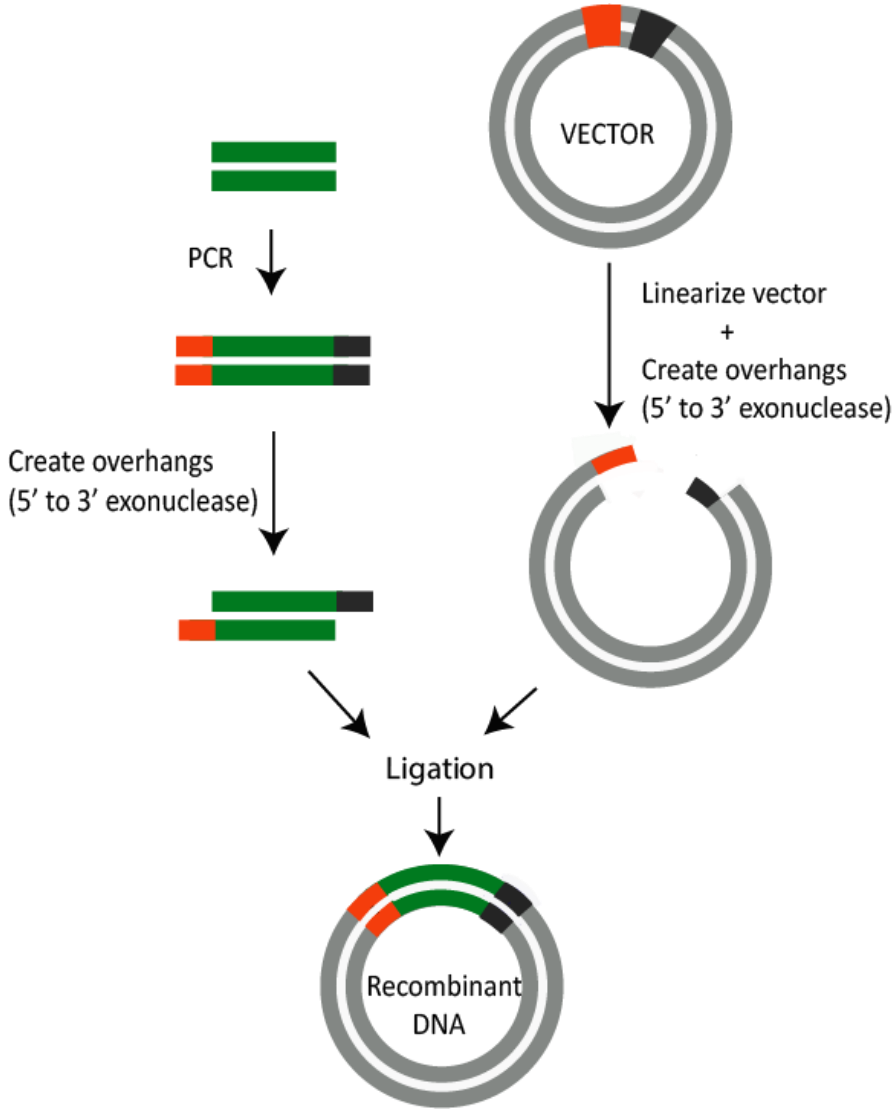
Creation of chimeric genes



4. PCR Cloning Method

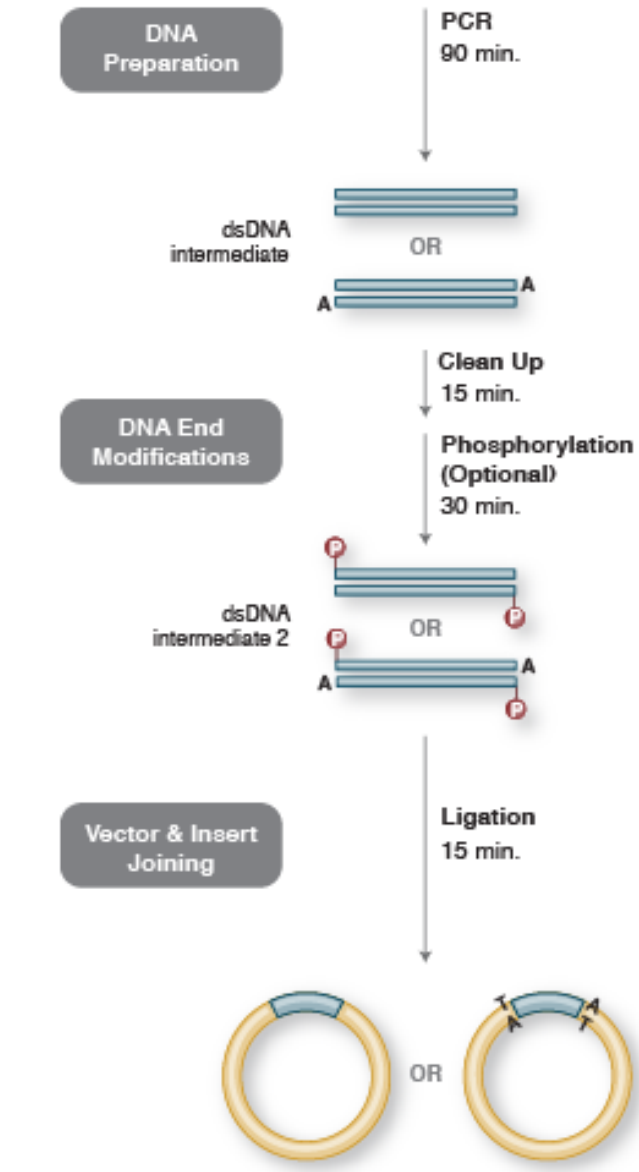
Overview: PCR cloning differs from traditional cloning in that the DNA fragment of interest, and even the vector, can be amplified by the Polymerase Chain Reaction (PCR) and ligated together, without the use of restriction enzymes.

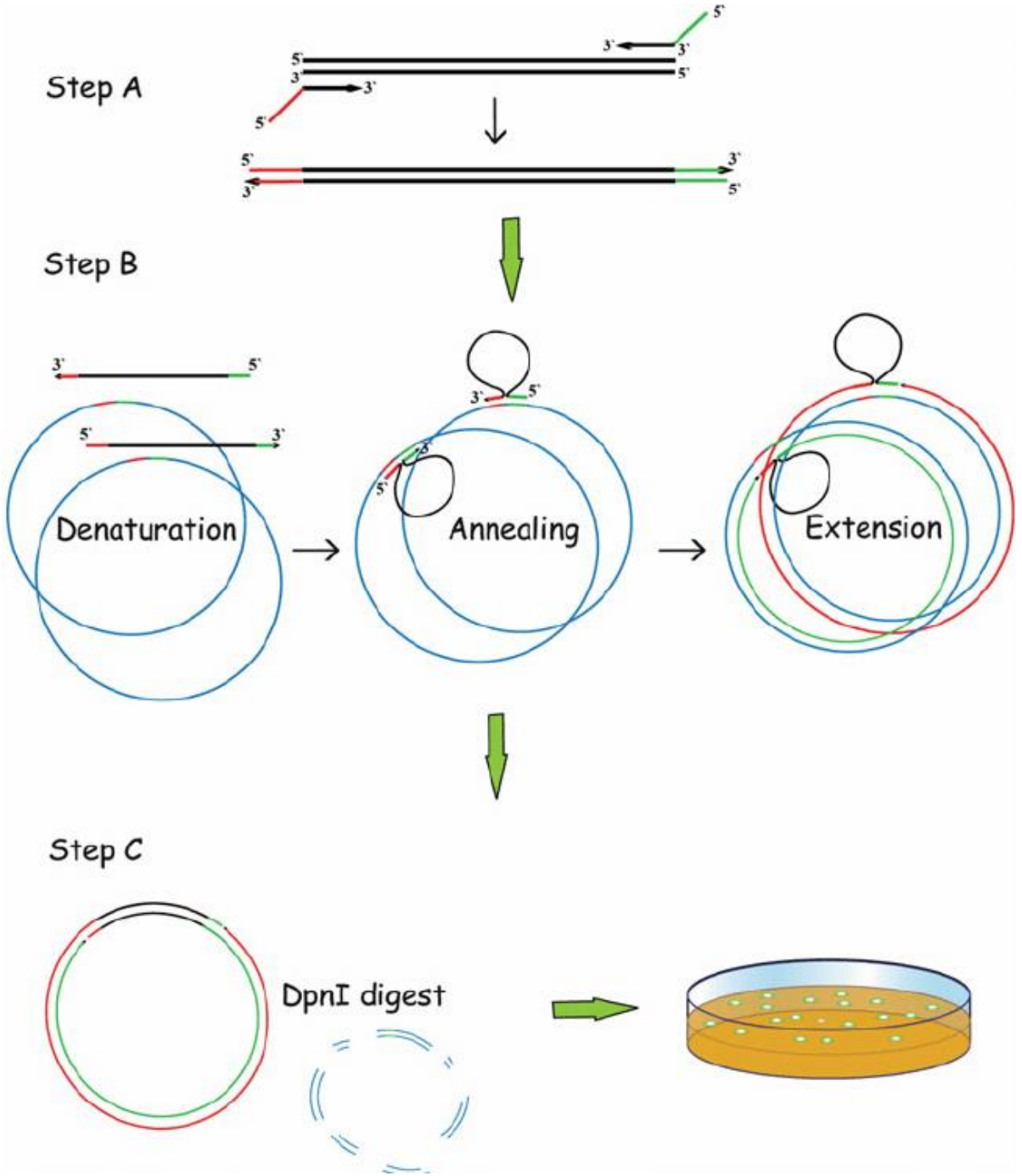
PCR cloning is a rapid method for cloning genes, and is often used for projects that require higher throughput than traditional cloning methods can accommodate. It allows for the cloning of DNA fragments that are not available in large amounts.



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
P
P
A
A
P
P
Vector & Insert Joining
Ligation
15 min.
OR
T
A
A
T
Estimated total time
2 hr. – 2 hr., 30 min.


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

Selective and reporter genes.

Lecture 5-2

Selective Genes (Selection Markers)

Selective genes, or selection markers, are genes introduced into a host organism to help distinguish cells that have successfully taken up a genetic construct (e.g., plasmid) from those that have not.

These genes often provide resistance to antibiotics or other toxic compounds.

Common Characteristics:
Function: The selective gene allows only the cells that have successfully incorporated the gene to survive or grow in the presence of a selective agent.

It is possible to distinguish 2 groups of marker genes that allow us to distinguish transformed cells (Types of Selective Markers):

1. Selective genes responsible for antibiotic resistance.

✓ Provide resistance to antibiotics like ampicillin, kanamycin, or tetracycline. These antibiotics are added to the growth medium, and only the transformed cells with the resistance gene survive.

In bacteria: kanamycin, tetracycline, ampicillin, neomycin, etc.

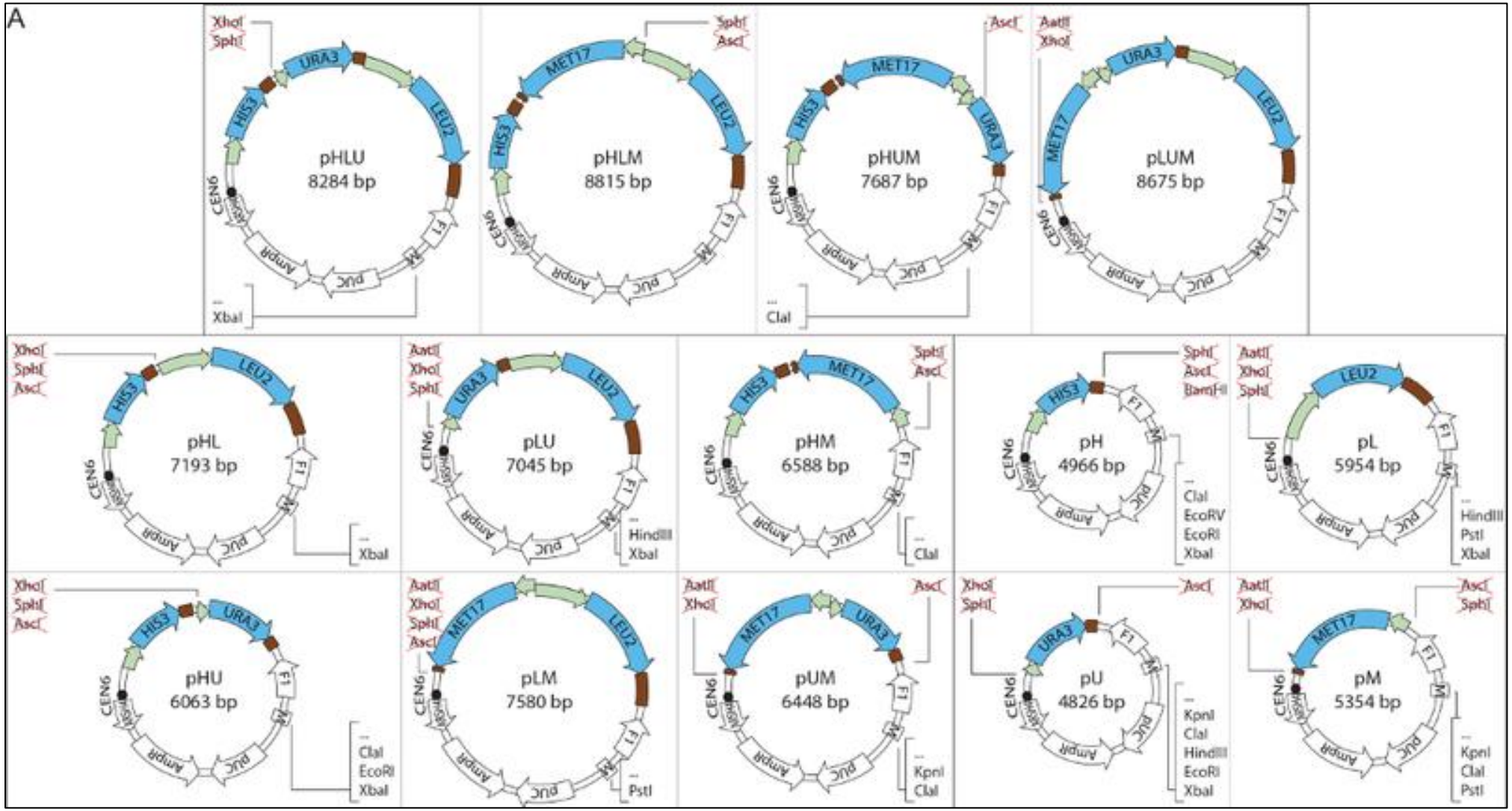
In plants: herbicides and genes presented in the table.

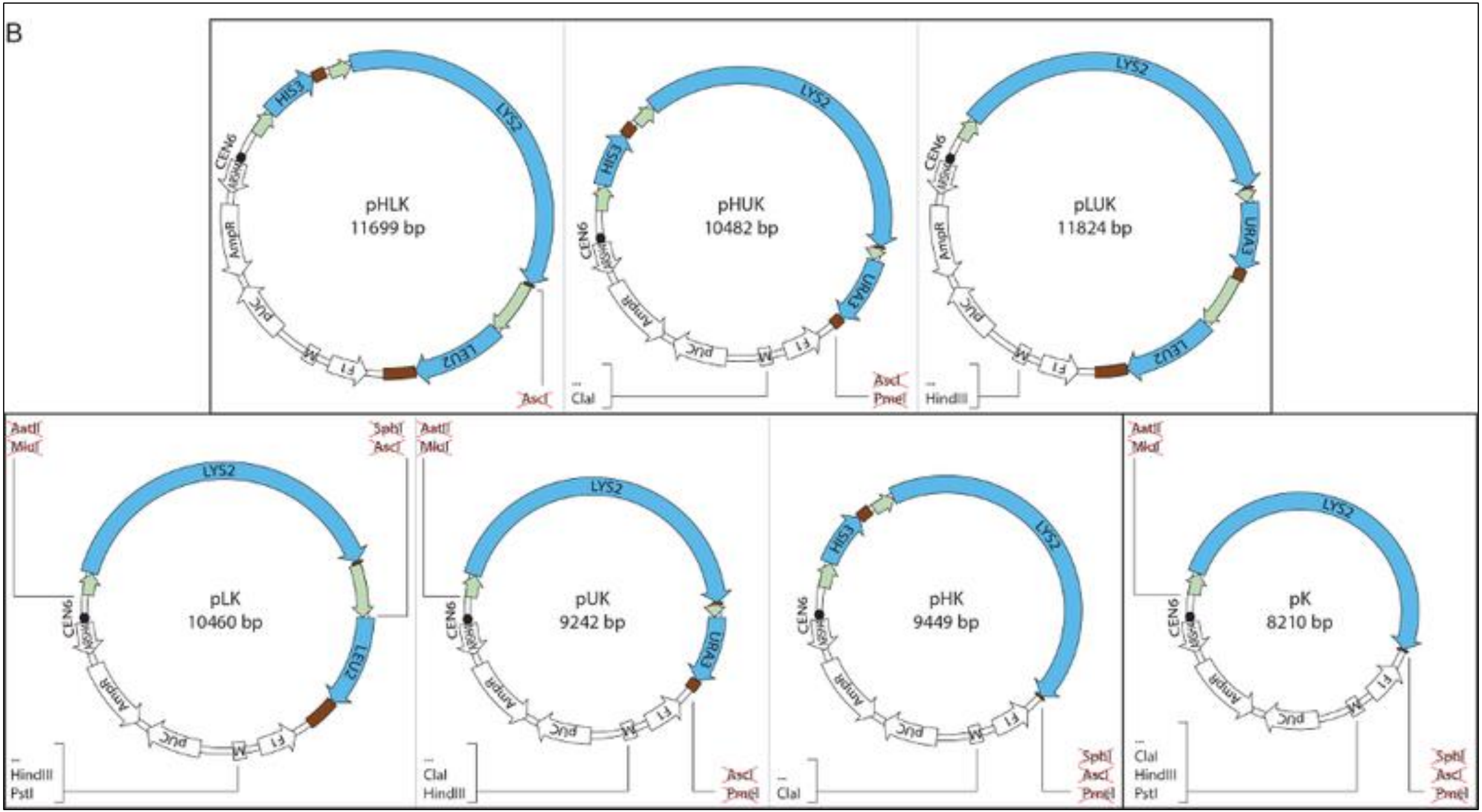
2. Nutritional Markers (auxotrophy).

✓ Often used in yeast and bacteria, these genes allow cells to grow in media lacking a specific nutrient (e.g., histidine, leucine).

Sl. no.	*Substrates*	*Marker genes*	*Enzyme produced*
1.	Antibiotics	Bleomycin	Gene ble (unknown enzyme)
		G418, Kanamycin, Neomycin	Neomycin phosphotransferese (nptll)
		Gentamycin	Gentamycin acetyl transferase (gat)
		Hygromycin B	Hygromycin phosphotransferase (hpt)
		Methotrexate trimethoprim	Dihydrofoate reductase (dfr)
		Streptomycin	Streptomycin phosphotransferase (spt)
2.	Herbicides	Chlorosulfuron imidazolinones	Mutant form of acetolactase synthase (als)
		Bromoxynil	Bromoxynil nitrilase (bnl)
		Glyphosate	5-enolpyruvate shikimate-3 - phosphate (EPSP)-synthase (aroA)
		PPT (L-phosphino-thricin, also called bialaphos)	Phosphinothricin acetyltransferase (bar)

The basic principle of operation of such a marker is the ability of transformed cells to grow on a selective nutrient medium, with the addition of certain substances that inhibit the growth and division of non-transformed, normal cells or the selection of mutant transformants.



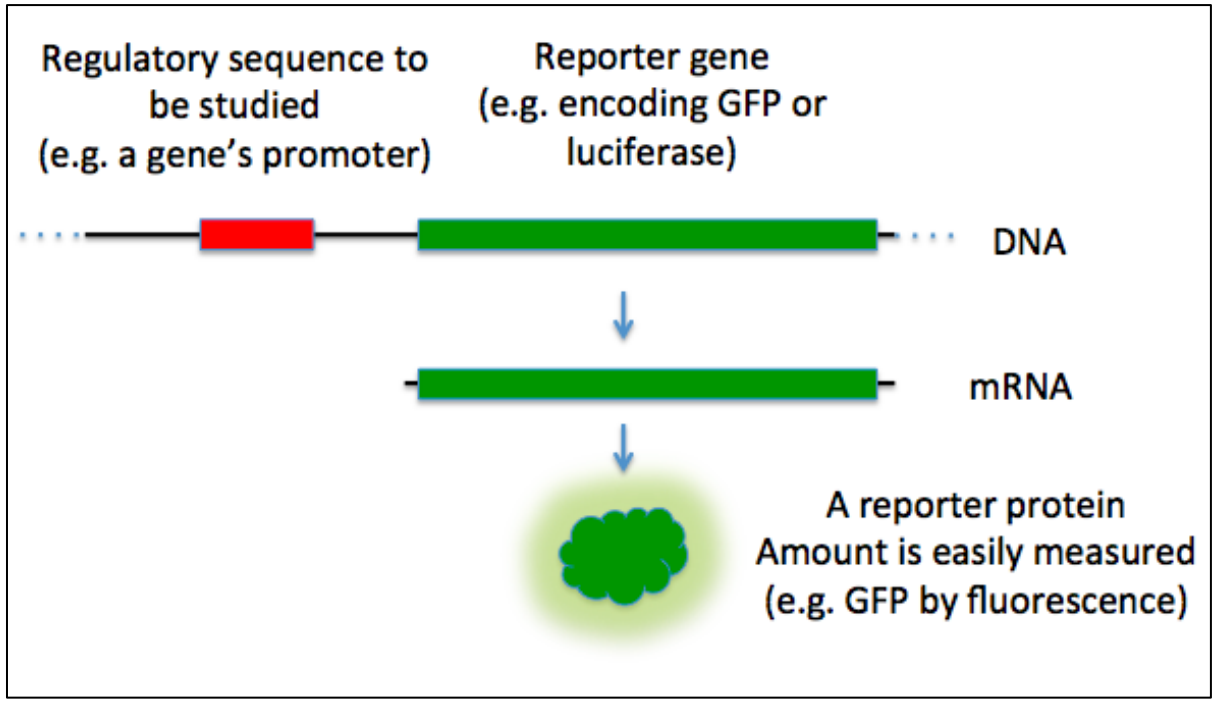

B
pHLK
11699 bp
HIS3
LYS2
LEU2
CEN6
ARSH4
AmpR
PUC
M
F1
AscI
pHUK
10482 bp
URA3
ClaI
AscI
PmeI
pLUK
11824 bp
HindIII
pLK
10460 bp
AatII
MluI
SphI
pUK
9242 bp
pHK
9449 bp
pK
8210 bp
PstI

REPORTER GENES encoding cell-neutral proteins whose presence in tissues can be easily tested.

- The most commonly used reporter genes are β-glucuronidase (**GUS**), green fluorescent protein (**GFP**), luciferase (**LUC**), and chloramphenicol acetyltransferase (**CAT**).
- To date, the most commonly used genes from this arsenal are **GUS** and **GFP**, and to a lesser extent, **LUC** and **CAT**.

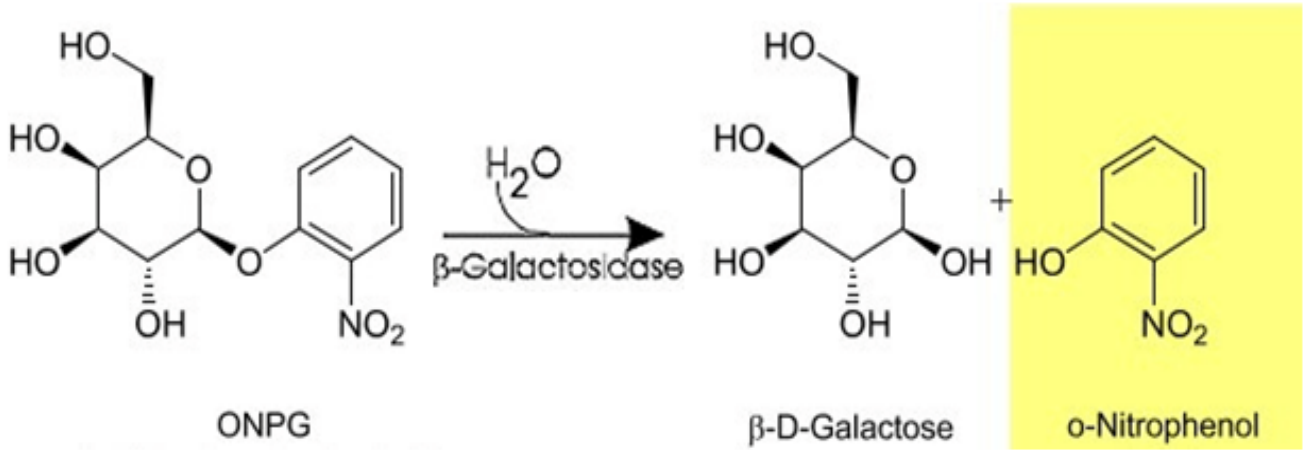
✓ **Reporter genes** encode proteins that have unique enzymatic activity and are used to assess the **transcriptional properties** of DNA elements.

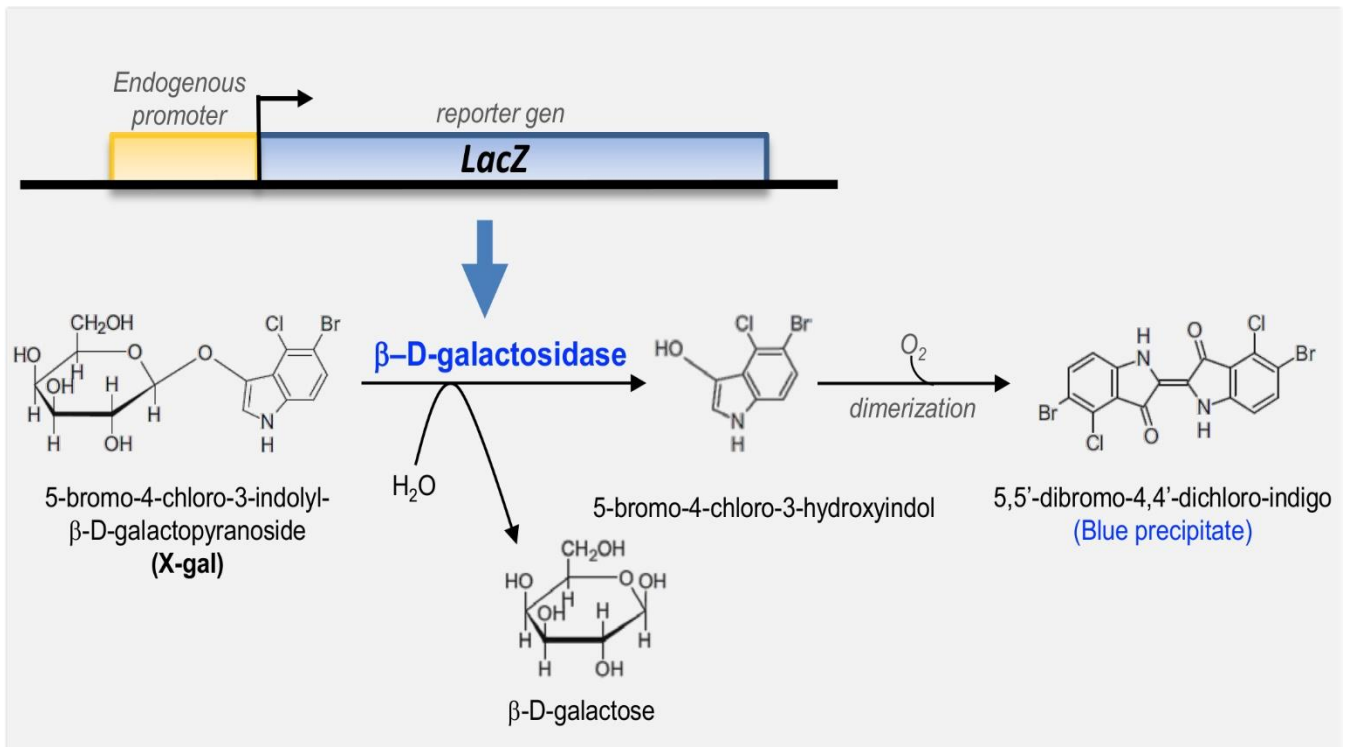
✓ The use of **reporter genes** in transgenic animals provides a rapid method for **detecting transgene expression** that can be easily distinguished from the expression of the corresponding endogenous gene of the animal.

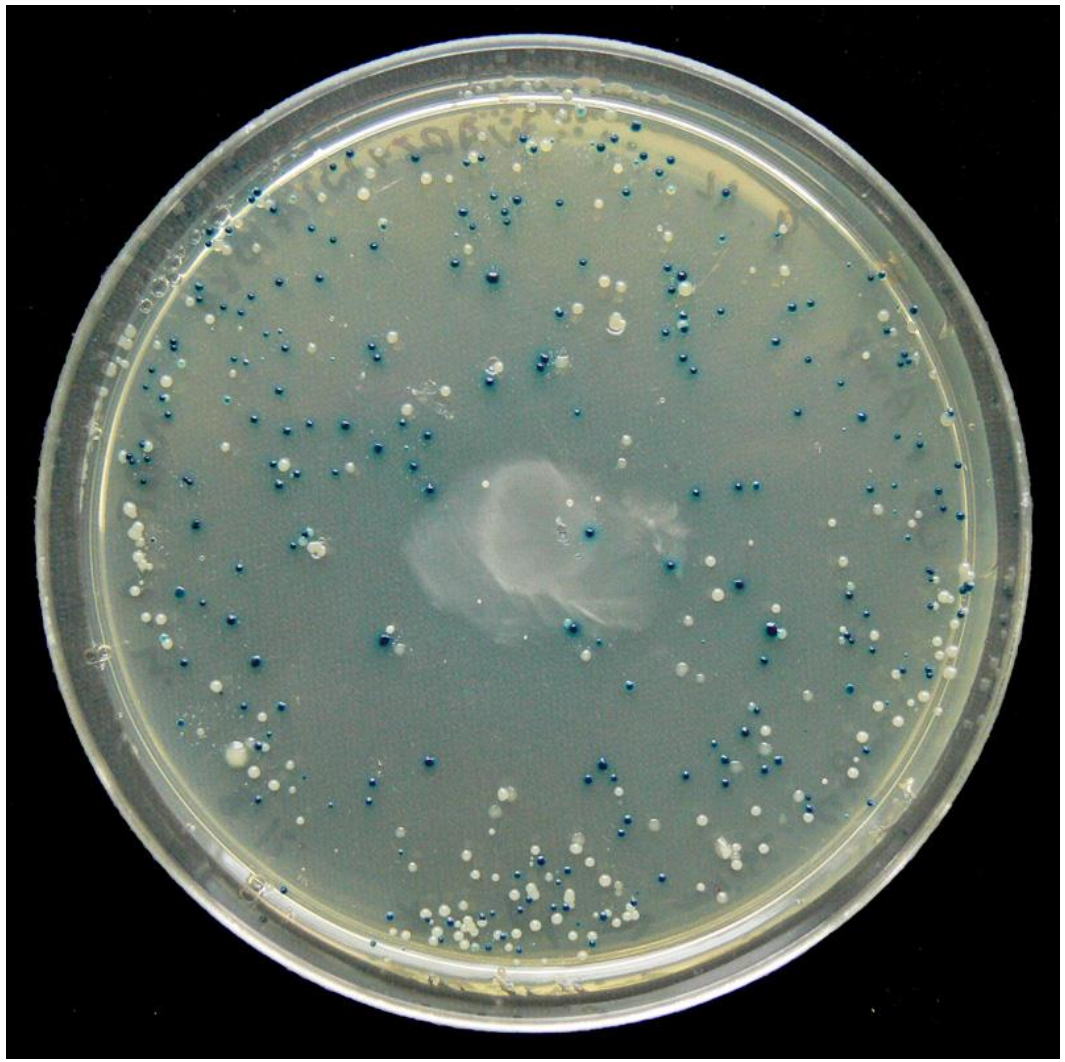


β-galactosidase

- The enzyme **β-galactosidase**, which catalyzes the hydrolysis of β-galactosides including **lactose**, is encoded by the **LacZ gene** of E. coli.
- Enzyme activity is measured using a simple **photometric assay** that measures the hydrolysis of the substrate **o-nitrophenyl-P-o-galactopyranoside** (ONPG) by β-galactosidase in cell-free extracts.
- β-galactosidase can also be monitored **histochemically** using the substrate **X-Gal** (5-bromo-4-chloro-3-indoyl β-D-galactoside).
- A potential drawback of using β-galactosidase as a reporter enzyme is the presence of endogenous β-galactosidase activity in some mammalian tissues, including the brain.
- However, the **pH optimum** for this enzyme is low (pH 3.5), whereas the pH optimum for the *E. coli* enzyme is 7.3. False positive results can be minimized by performing the assay at pH 7.5 and including normal tissue extract as a control.


HO
HO
HO
O
O
OH
NO2
H2O
β-Galactosidase
HO
HO
HO
O
OH
OH
+
HO
NO2
ONPG
β-D-Galactose
o-Nitrophenol

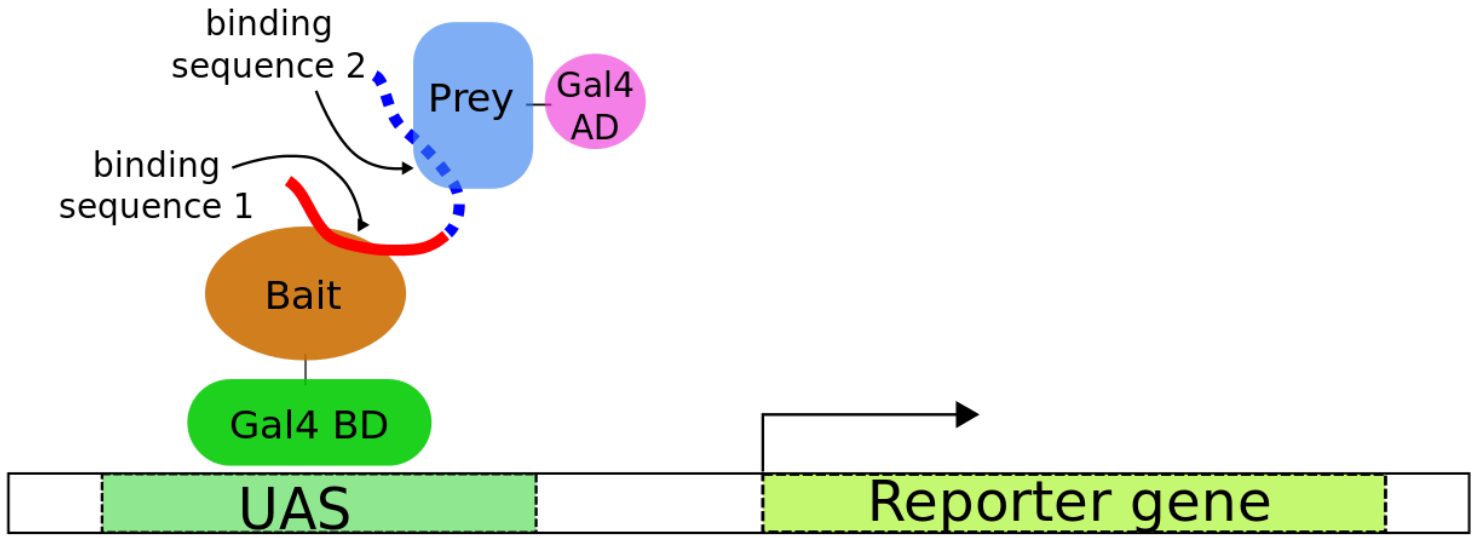

Endogenous promoter
reporter gen
LacZ
β–D-galactosidase
H2O
O2
dimerization
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
(X-gal)
5-bromo-4-chloro-3-hydroxyindol
5,5'-dibromo-4,4'-dichloro-indigo
(Blue precipitate)
β-D-galactose

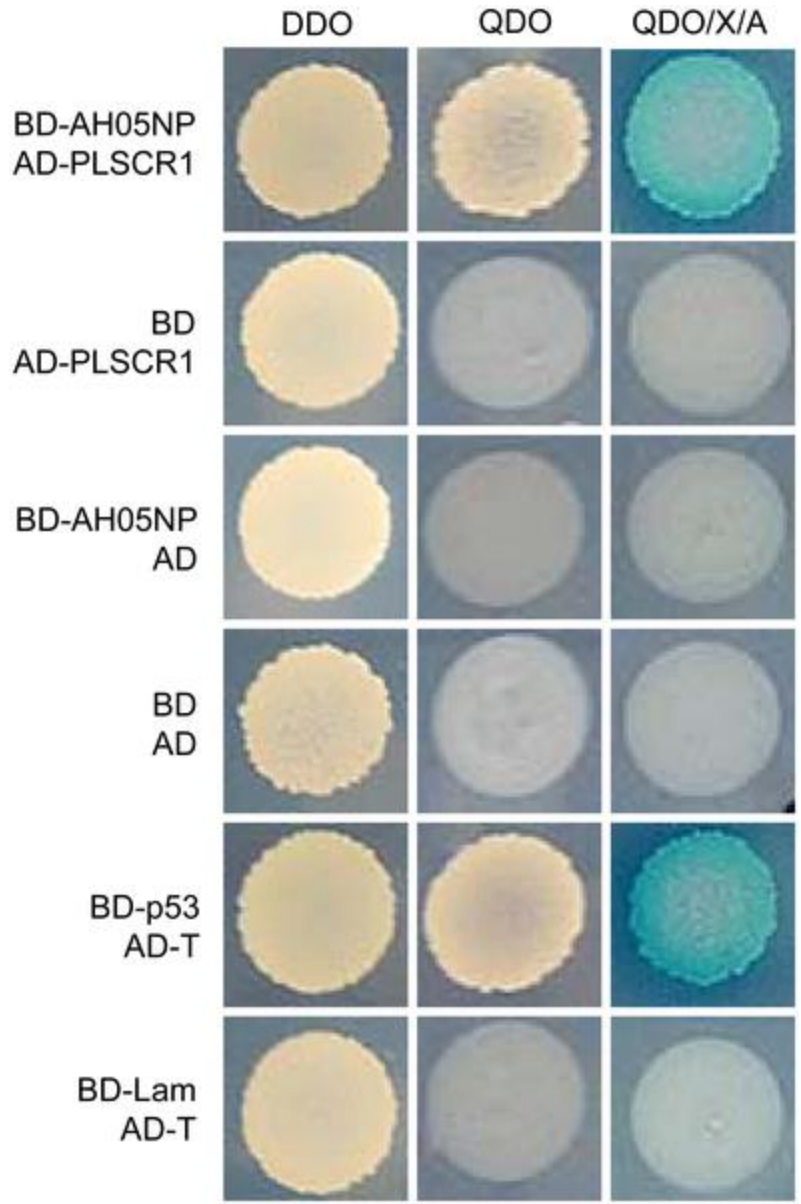


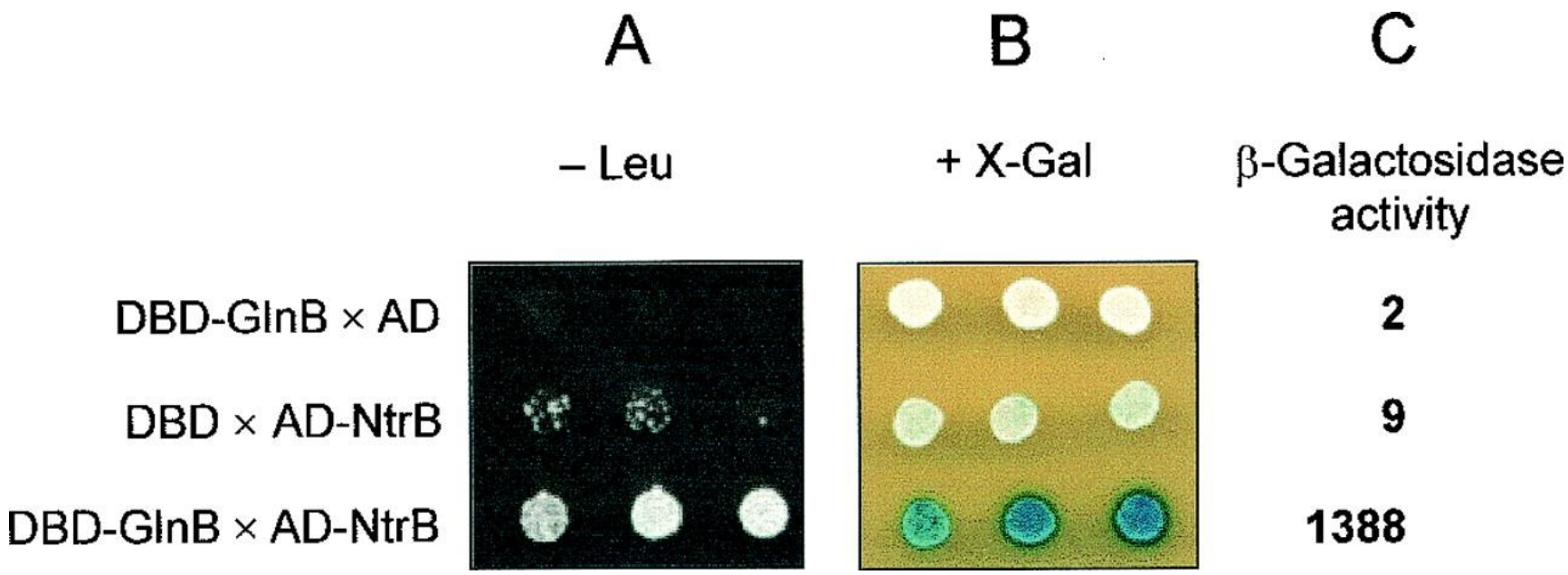
- Genetic engineering technologies allow the creation of genetic chimeras between the promoter region of a gene of interest and a reporter gene as a means of studying the regulation of eukaryotic gene expression at the transcriptional level.

A good reporter gene product has the following characteristics:

1. the enzymatic activity is thermostable and resistant to proteases and corresponds well to the strength of the promoter;
2. background and/or interfering enzymatic activities are absent in cells;
3. simple, sensitive, reproducible, and convenient enzymatic or immunoassays of the reporter gene product are available to assess promoter activity.

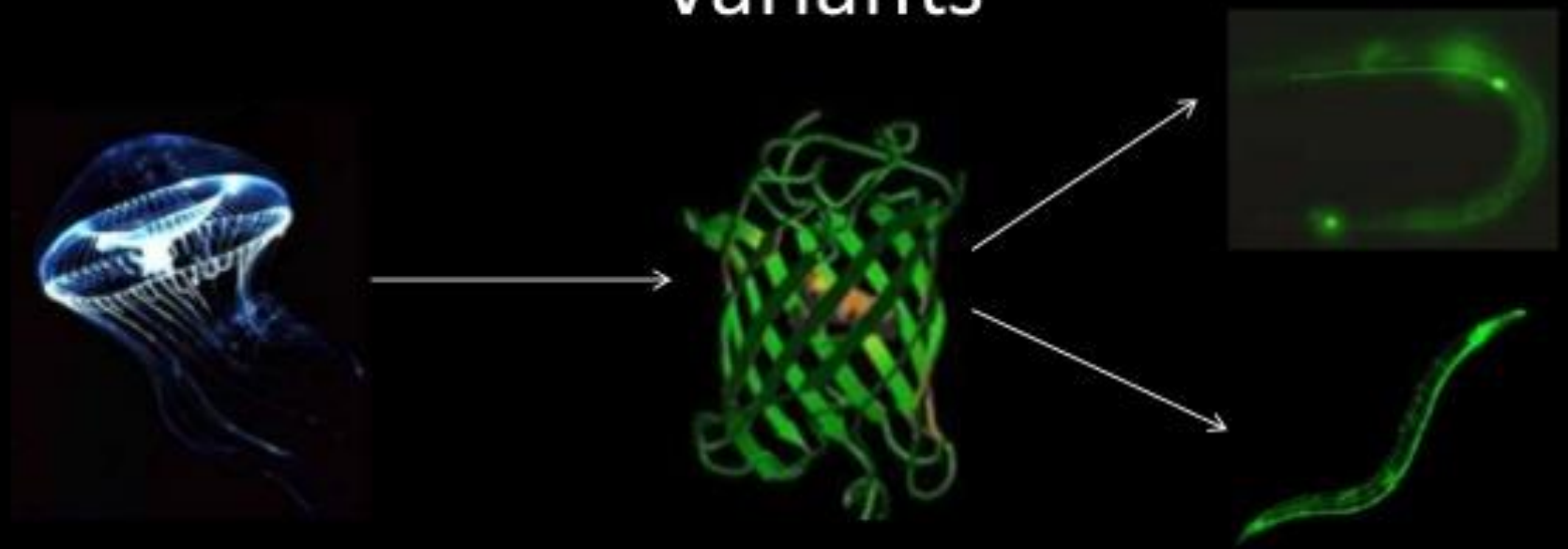

binding
sequence 2
Prey
Gal4
AD
binding
sequence 1
Bait
Gal4 BD
UAS
Reporter gene

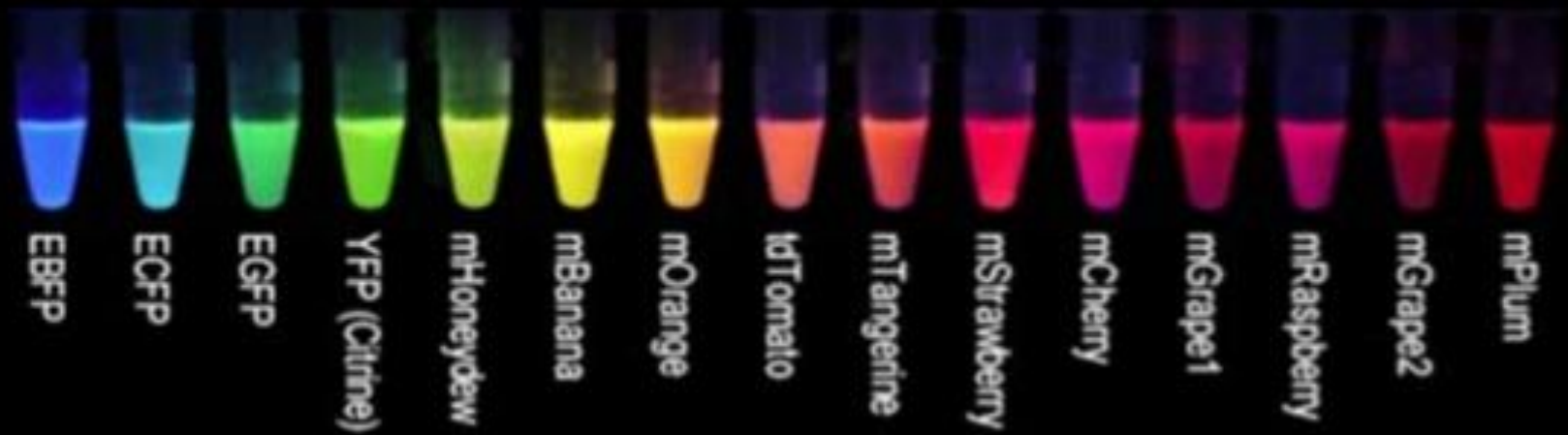

DDO
QDO
QDO/X/A
BD-AH05NP
AD-PLSCR1
BD
AD-PLSCR1
BD-AH05NP
AD
BD
AD
BD-p53
AD-T
BD-Lam
AD-T


A
B
C
– Leu
+ X-Gal
β-Galactosidase
activity
DBD-GlnB × AD
DBD × AD-NtrB
DBD-GlnB × AD-NtrB
2
9
1388

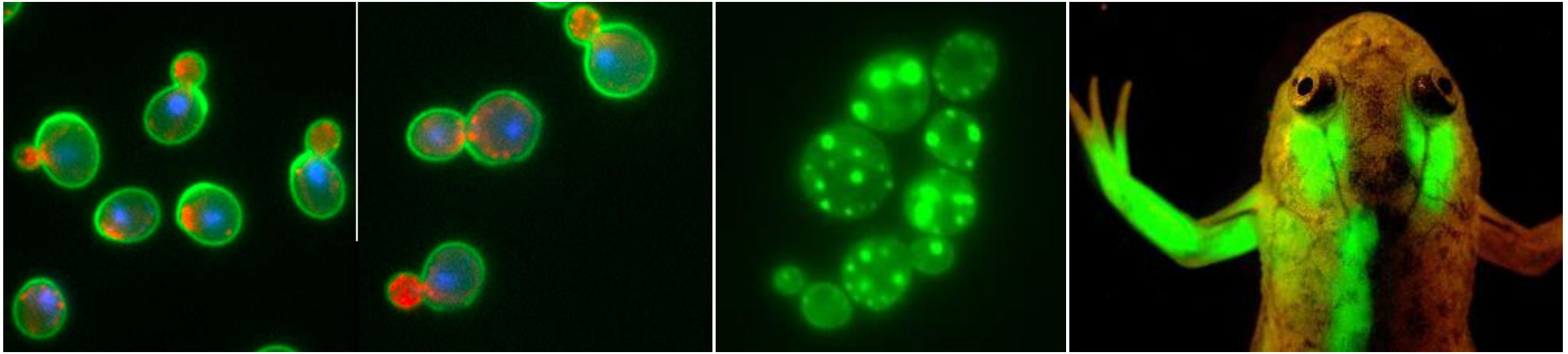
- **GFP** (green fluorescent protein) was **discovered** by Shimomura et al. in 1962 in the luminescent jellyfish *Aequorea victoria*.
- The **GFP** gene **was cloned** in 1992 by Prasher et al., and within a few years, this gene began to be actively used as a reporter in studies with a wide variety of pro- and eukaryotic organisms.
- Currently, the GFP gene is used in hundreds of studies worldwide, and their number is rapidly growing. This rapid growth is due to the special properties of the GFP protein, namely its ability to **fluoresce in the visible** (**green**) **region** of the spectrum when irradiated with long-wave UV. This fluorescence is caused directly by the protein; it does not require substrates or cofactors for its manifestation.
- Due to this property, the GFP gene is a very **promising reporter gene**, allowing for a variety of intravital (non-destructive) studies with transgenic organisms.
- Another protein, DsRed, which fluoresces in red light, has recently been isolated from the sea anemone *Discosoma* sp.

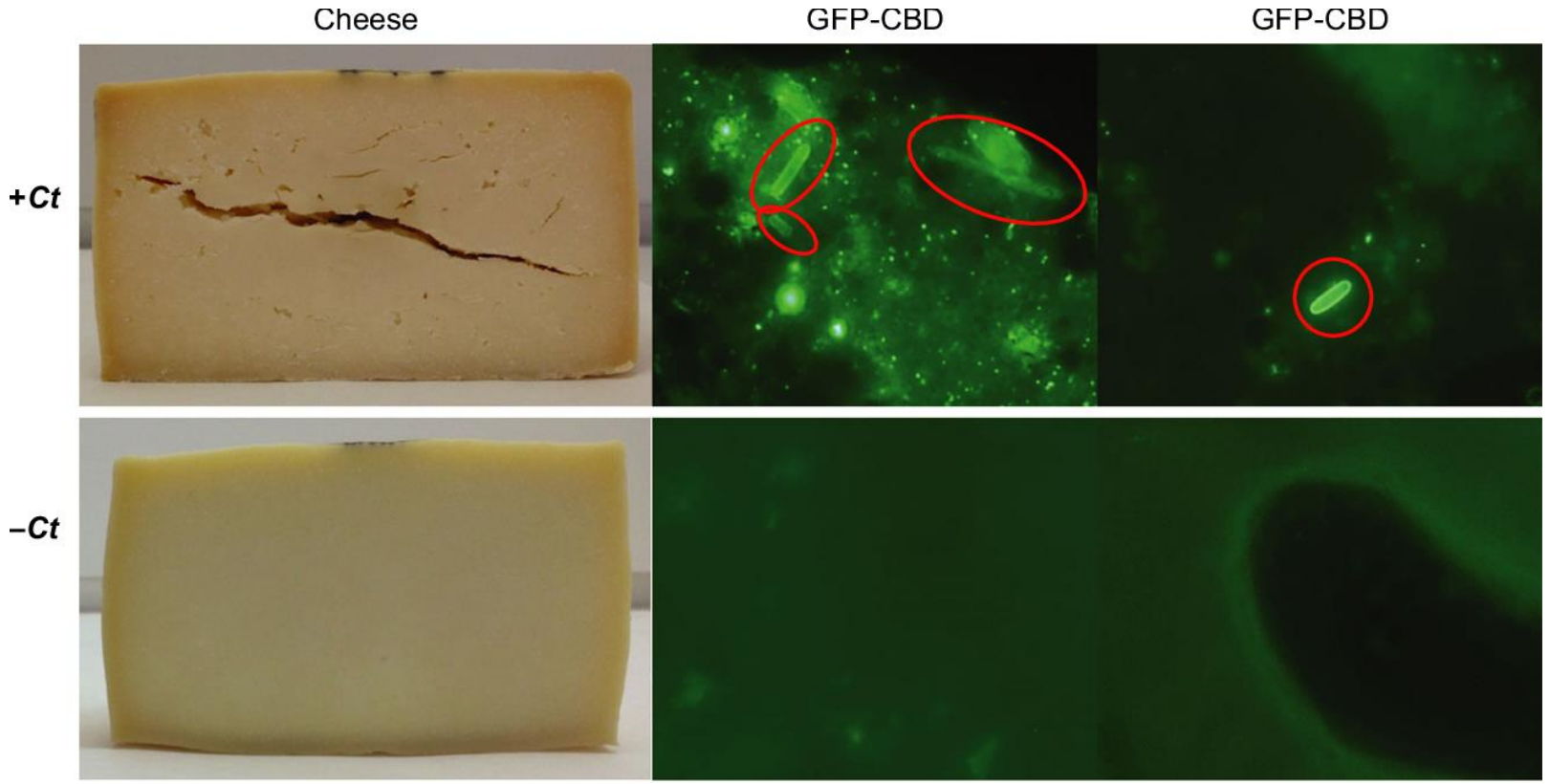
The Green Fluorescent Protein and Variants



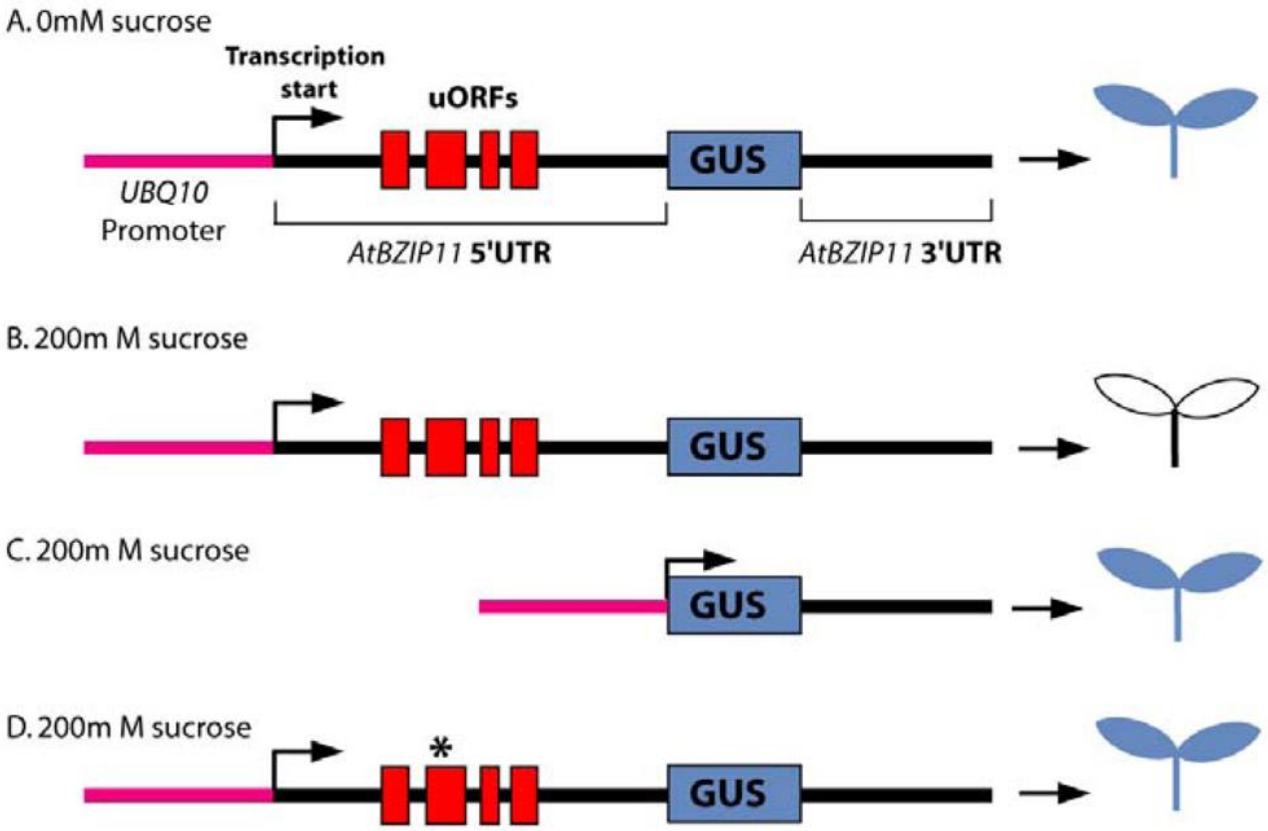


life.bio.sunysb.edu; brainwindows.wordpress.com; Chalfie et al., 1994; bcgsc.ca; Zimmer, 2009




Cheese
GFP-CBD
GFP-CBD
+Ct
–Ct

- Используемый в настоящее время как **репортерный ген GUS** является модифицированным геном из *Escherichia coli*, кодирующим **β-глюкуронидазу** с молекулярной массой **68 кД.**
- GUS активен в широком диапазоне условий среды с оптимумом при pH 5-8 и 37°C. Он может гидролизовать обширный спектр природных и синтетических глюкуронидов, что позволяет подбирать соответствующие субстраты для спектрофотометрического или флюориметрического определения активности фермента, а также для гистохимического окрашивания тканей *in situ* (например, в синий цвет).
- В живых клетках белок GUS также весьма стабилен и активен от нескольких часов до нескольких суток.



Enzyme activity is determined by absorbance, fluorescence or chemiluminescence.

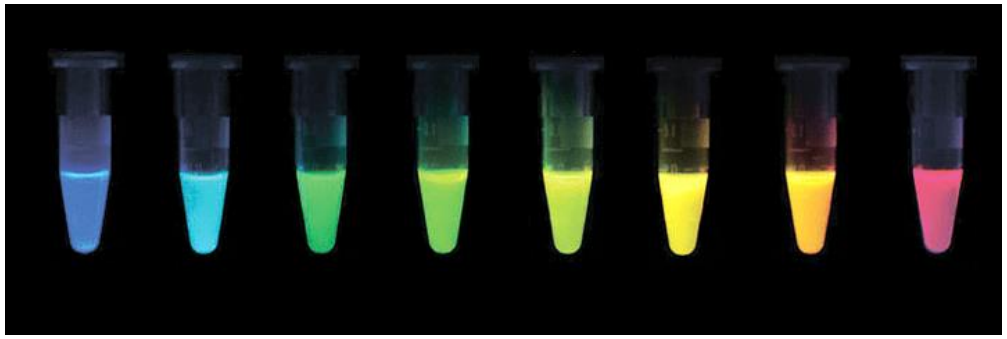
Fluorometric

Fluorescence is when a molecule emits light at one wavelength after absorbing light at another wavelength. Fluorometric assays use the difference in substrate-product fluorescence to measure an enzymatic reaction. These assays are generally much more sensitive than spectrophotometric assays, but can suffer from interference from impurities and the instability of many fluorescent compounds when exposed to light.

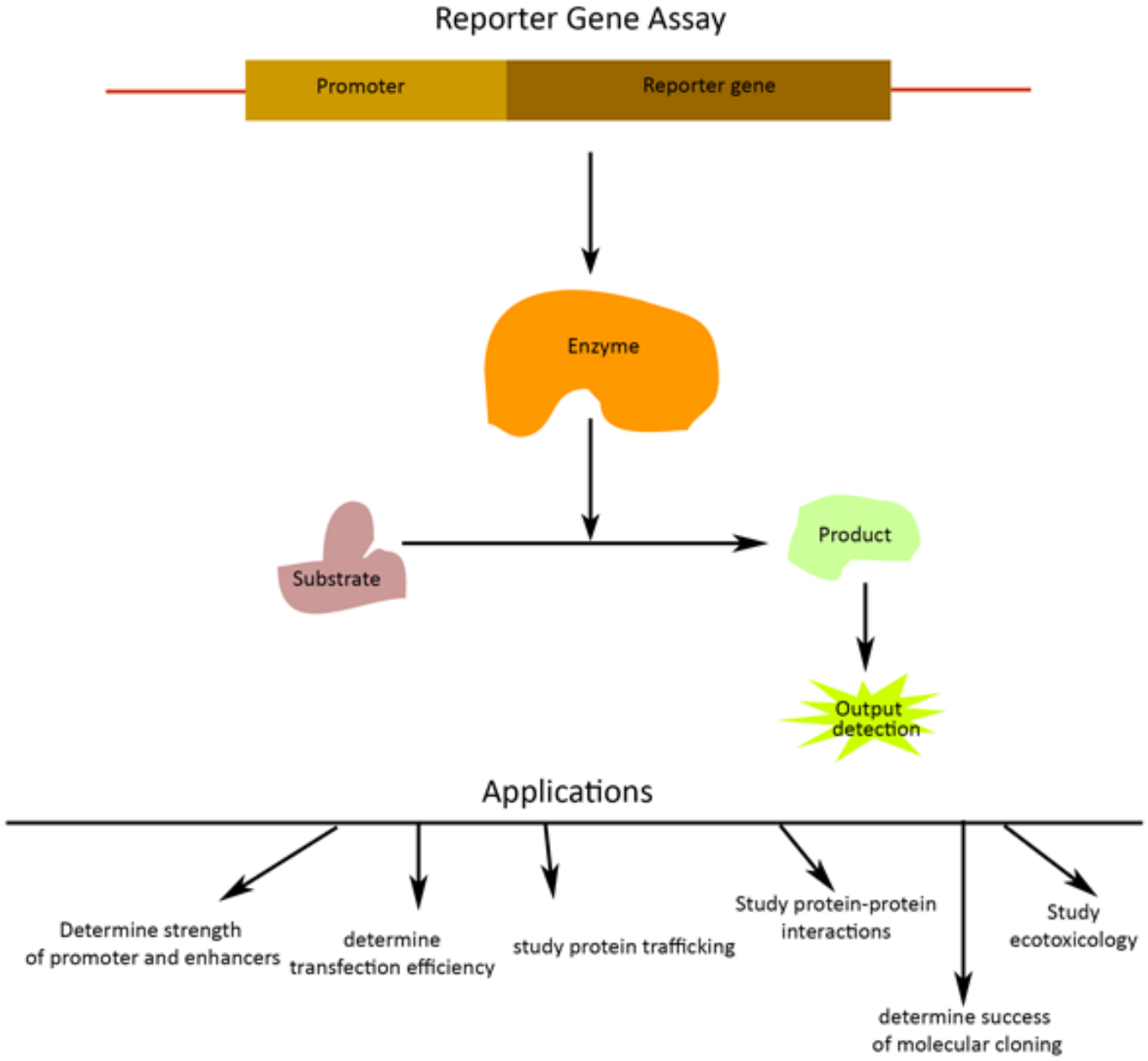
Chemiluminescent

Chemiluminescence is the emission of light as a result of a chemical reaction. Some enzymatic reactions produce light, and this can be measured to detect the formation of a product. These types of assays can be extremely sensitive because the light produced can be captured by photographic film for days or weeks, but they are difficult to quantify because not all of the light emitted by the reaction will be detected.

Fluorescence

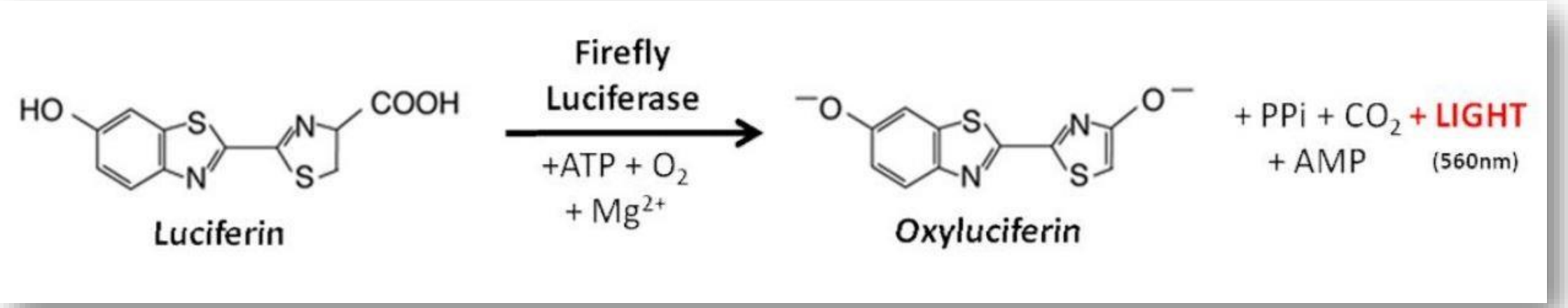


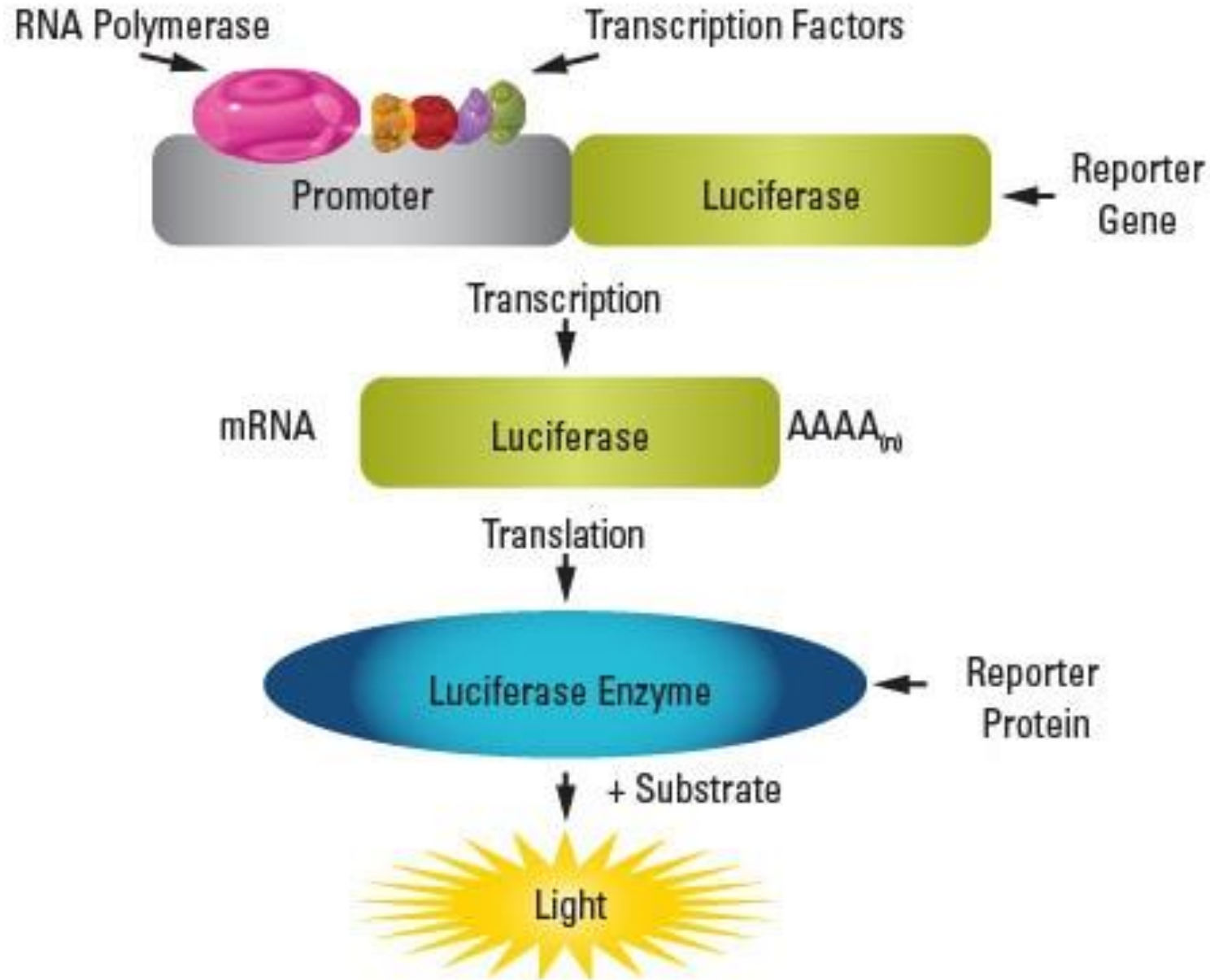
Chemiluminescence



Bioluminescent reporter probes for imaging

- More recently, new imaging techniques have emerged that use the **firefly bioluminescent protein**, **luciferase**. The main advantage of using bioluminescence reporters is their high sensitivity combined with minimal background.





Light Signal = Luciferase Expression = Promoter Activity

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