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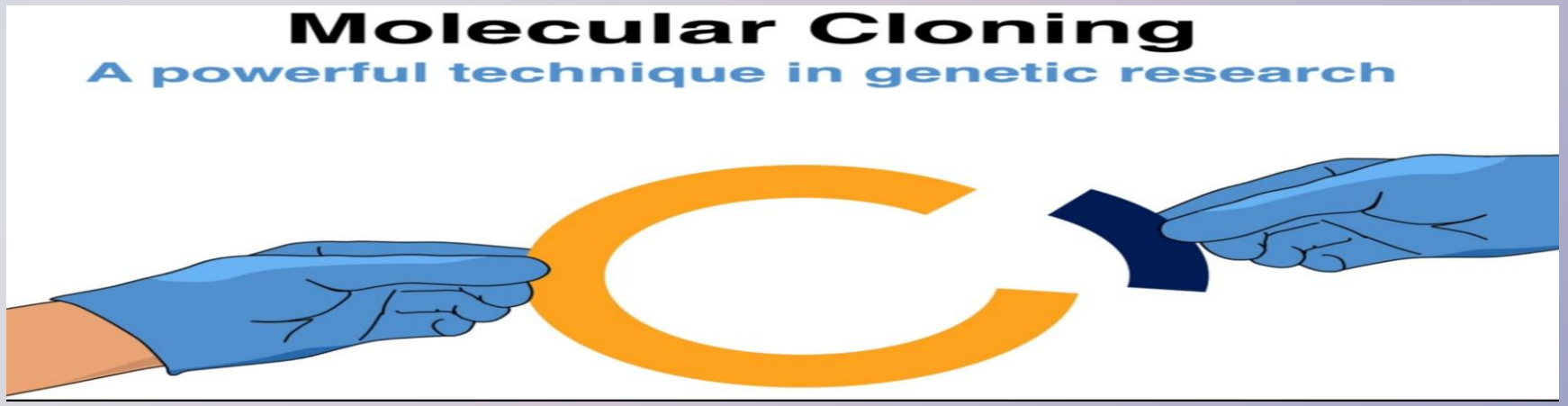
عنوان

"Cloning Workflow: Insertion of a Target Gene into the pUC19 Plasmid and Blue-White Screening"



- **Introduction**
- **The β-galactosidase gene (lacZ) encodes an enzyme that cleaves lactose and analogs such as X-gal. It is commonly used in molecular biology as a reporter gene for Blue/White colony screening. In this protocol, we clone the target gene into the pUC19 plasmid vector and transform it into competent E. coli cells. Successful recombinants are screened using the Blue/White selection method, followed by plasmid isolation and confirmation using PCR, restriction digestion, and sequencing.**

- **Materials:**
  - Source cells or plasmid and DNA target
  - Lysis buffer
  - Proteinase K
  - RNase A
  - Phenol:Chloroform or spin column kit
  - Ethanol/isopropanol

  Procedure:
  - Lyse cells using buffer and enzymes
  - Remove proteins by phenol-chloroform or column purification
  - Precipitate and wash DNA
  - Resuspend in TE or water

  Explanation:
  Obtain pure DNA containing for downstream amplification.

2. PCR Amplification of Target Gene

Materials:

- Template DNA
- Forward and reverse primers
- dNTPs
- Taq polymerase or high-fidelity enzyme
- Buffer and MgCl2
- Thermal cycler

Procedure:

- Prepare PCR mix with appropriate components
- Use program: Denaturation (94°C), Annealing (50–60°C), Extension (72°C)
- Confirm product on agarose gel

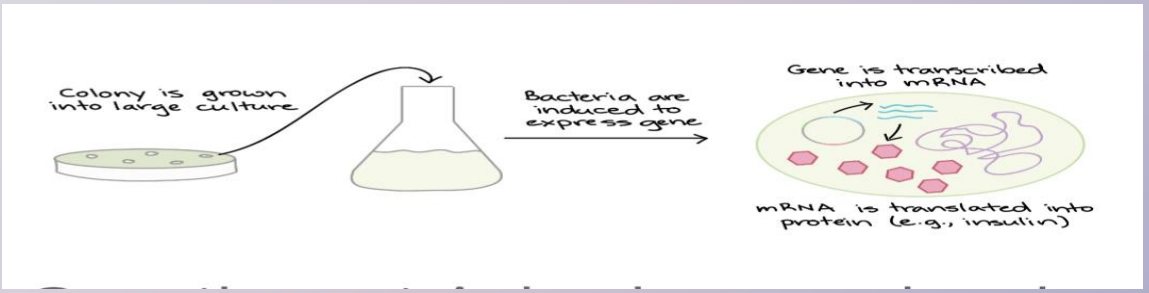
Explanation:

Amplify the gene with compatible restriction sites added via primers.

- **3. Digestion with Restriction Enzymes**
- **Materials:**
  - PCR product and pUC19 plasmid
  - Restriction enzymes (e.g., EcoRI, HindIII)
  - Buffer and BSA
  - Incubator or water bath

  Procedure:
  - Digest both insert and plasmid with same enzymes
  - Heat-inactivate enzymes or purify products

  Explanation:
  Create compatible ends for ligation and prevent vector self-ligation



- **4. Ligation into Plasmid Vector**
- **Materials:**
  - Digest gene insert
  - Digested pUC19 vector
  - T4 DNA ligase and buffer
  - Ice

  Procedure:
  - Mix insert and vector at 3:1 molar ratio
  - Add ligase and buffer
  - Incubate at 16°C overnight or room temp for 1–2 h

  Explanation:
  Insert the gene into MCS of pUC19 vector.

- **5. Preparation of Competent E. coli and Transformation**
- **Materials:**
  - E. coli strain (e.g., DH5α)
  - CaCl2 or MgCl2 buffer for chemical method OR electroporation cuvettes a
  - Ligation mixture

  Procedure (Heat Shock):
  - Incubate cells on ice with plasmid
  - Heat shock at 42°C for 45 sec
  - Recover on ice, add SOC medium, shake for 1 h at 37°C

  Procedure (Electroporation):
  - Mix DNA with electrocompetent cells
  - Electroporate (e.g., 1.8 kV)
  - Add SOC, incubate 1 h at 37°C

  Explanation:
  Introduce recombinant plasmid into bacterial host.

- **6. Selection and Screening of Colonies**
- **Materials:**
  - LB agar plates with ampicillin, X-gal, and IPTG

  Procedure:
  - Plate transformed cells
  - Incubate overnight at 37°C
  - Identify white colonies (successful clones), blue ones are non-recombinant

  Explanation:
  Disruption of target gene prevents β-galactosidase activity → white colonies

- **7. Plasmid Miniprep and Verification**
- **Materials:**
  - White colonies
  - LB broth + ampicillin
  - Miniprep kit
  - Nanodrop or gel electrophoresis system

  Procedure:
  - Grow selected colonies overnight
  - Isolate plasmid DNA using kit
  - Measure concentration and check on gel

  Explanation:
  Obtain pure recombinant plasmid DNA for verification.

- **8. Confirmation of Cloning Success**
- **Materials:**
  - Isolated plasmid
  - PCR primers for insert or vector
  - Restriction enzymes
  - DNA ladder
  - Sequencing service

  Procedure:
  - (a) PCR to check presence of insert
  - (b) Restriction digestion to release insert
  - (c) Sequence plasmid for exact confirmation

  Explanation:
  Each method ensures that the desired gene is correctly inserted in the right orientation.

pUC19 Vector

Joachim Messing

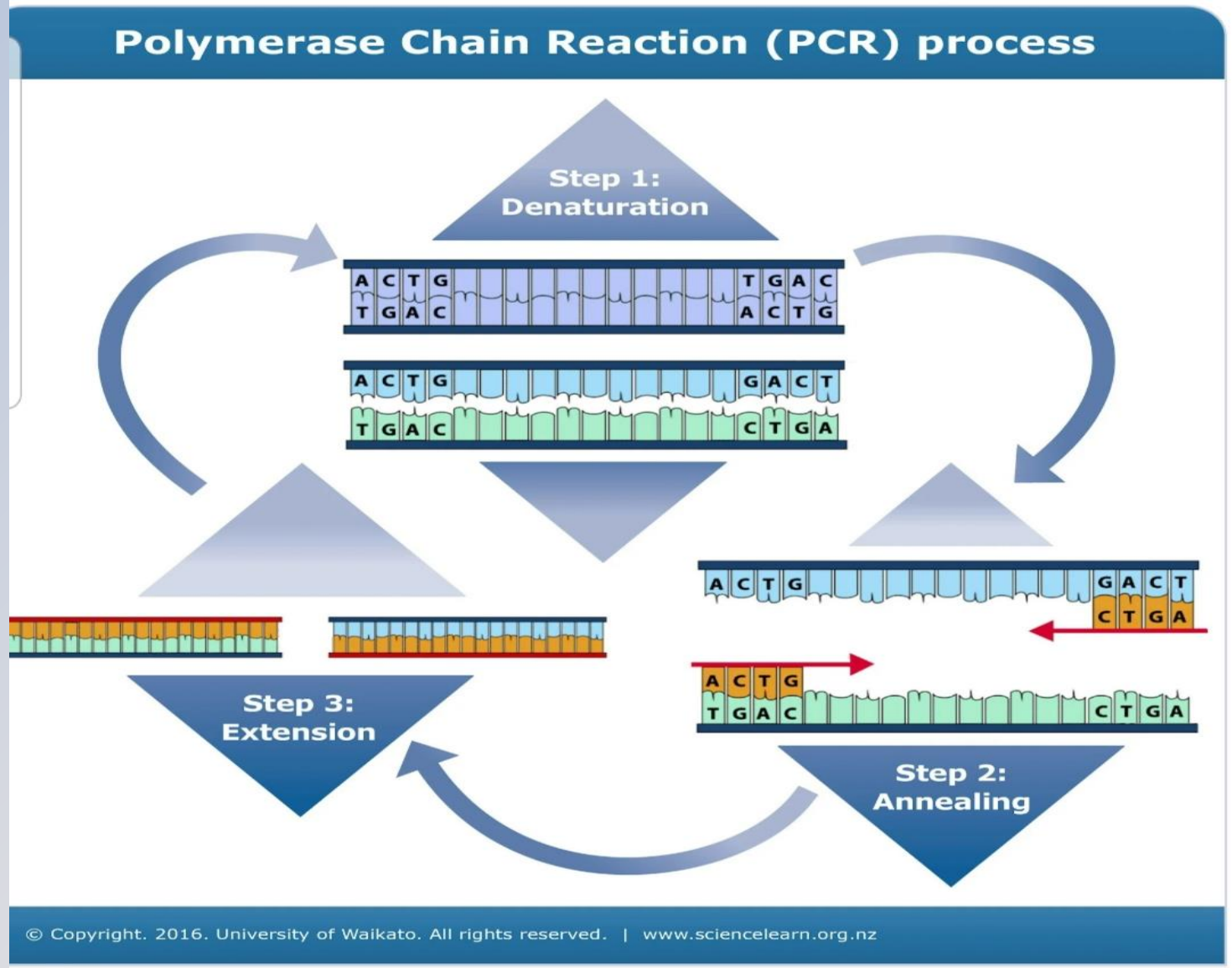
p - plasmid

UC -

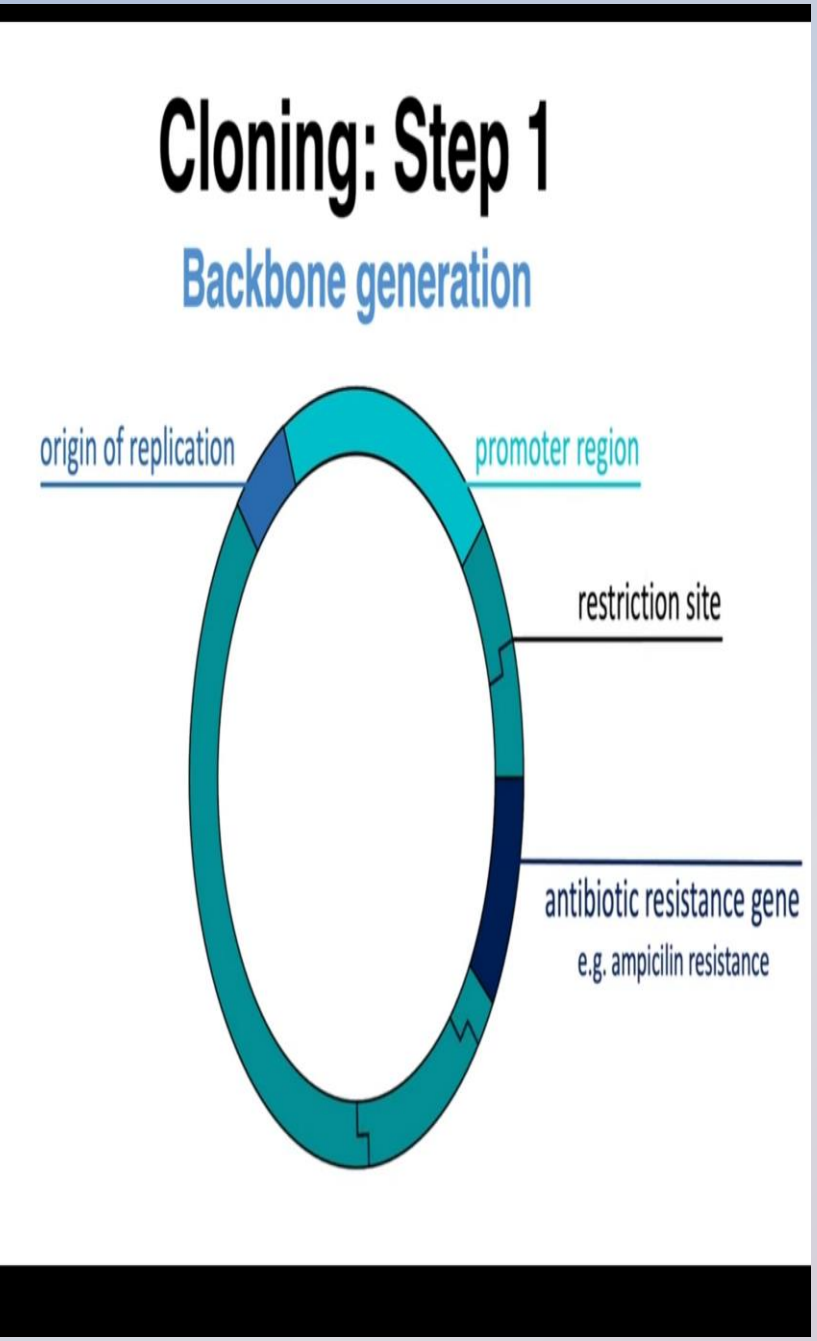
19 - Numerical Designation

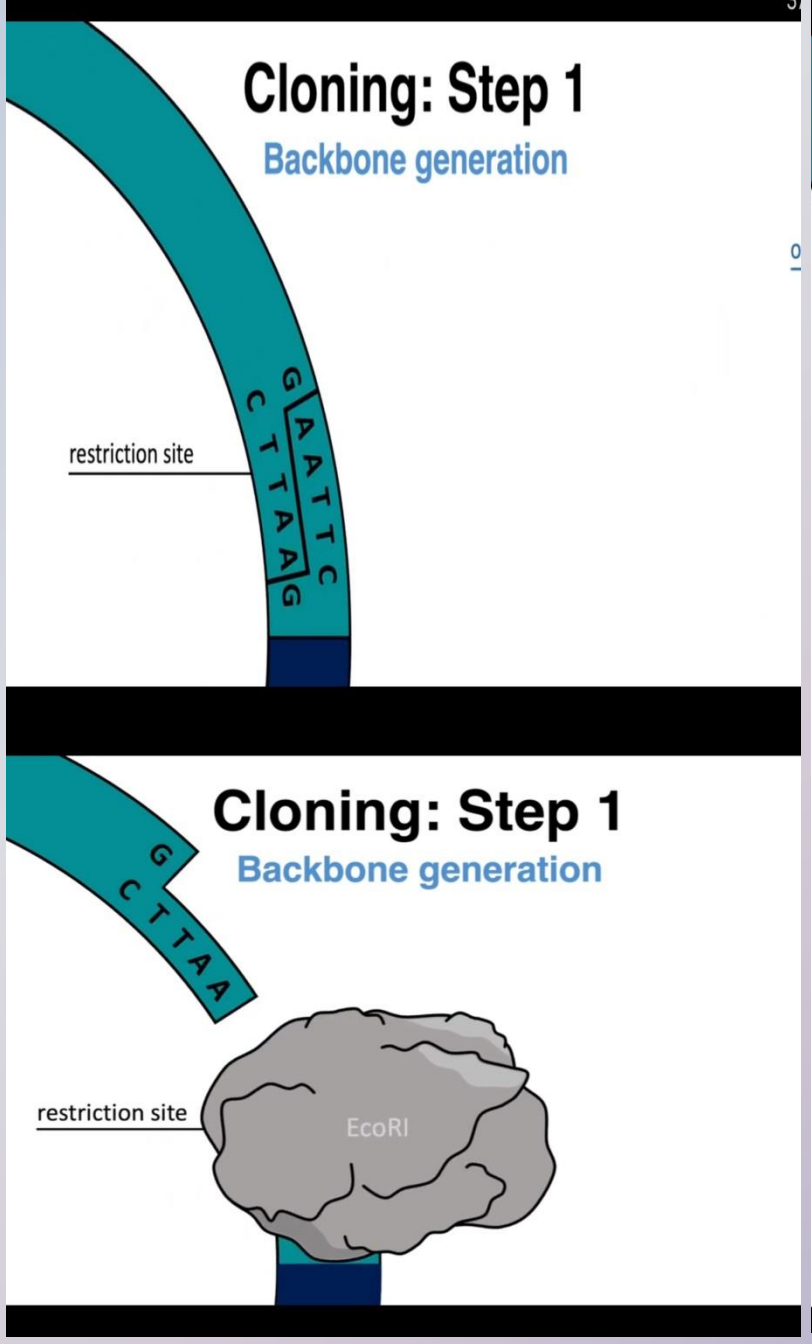
Application of pUC19 Vector

1. Easy to do cloning and subcloning. Since, pUC19 plasmid is compatible with a wide range of bacterial strains.
2. Have a high copy number. About 500 to 700 copies per cell. While for pBR322 it is only 20 copies of plasmid per cell.
3. The compact size of pUC19 makes it suitable for sequencing cloned inserts.
4. pUC19 can be used for preliminary studies involving gene expression or promoter analysis.
5. The screening can be done in a single step.

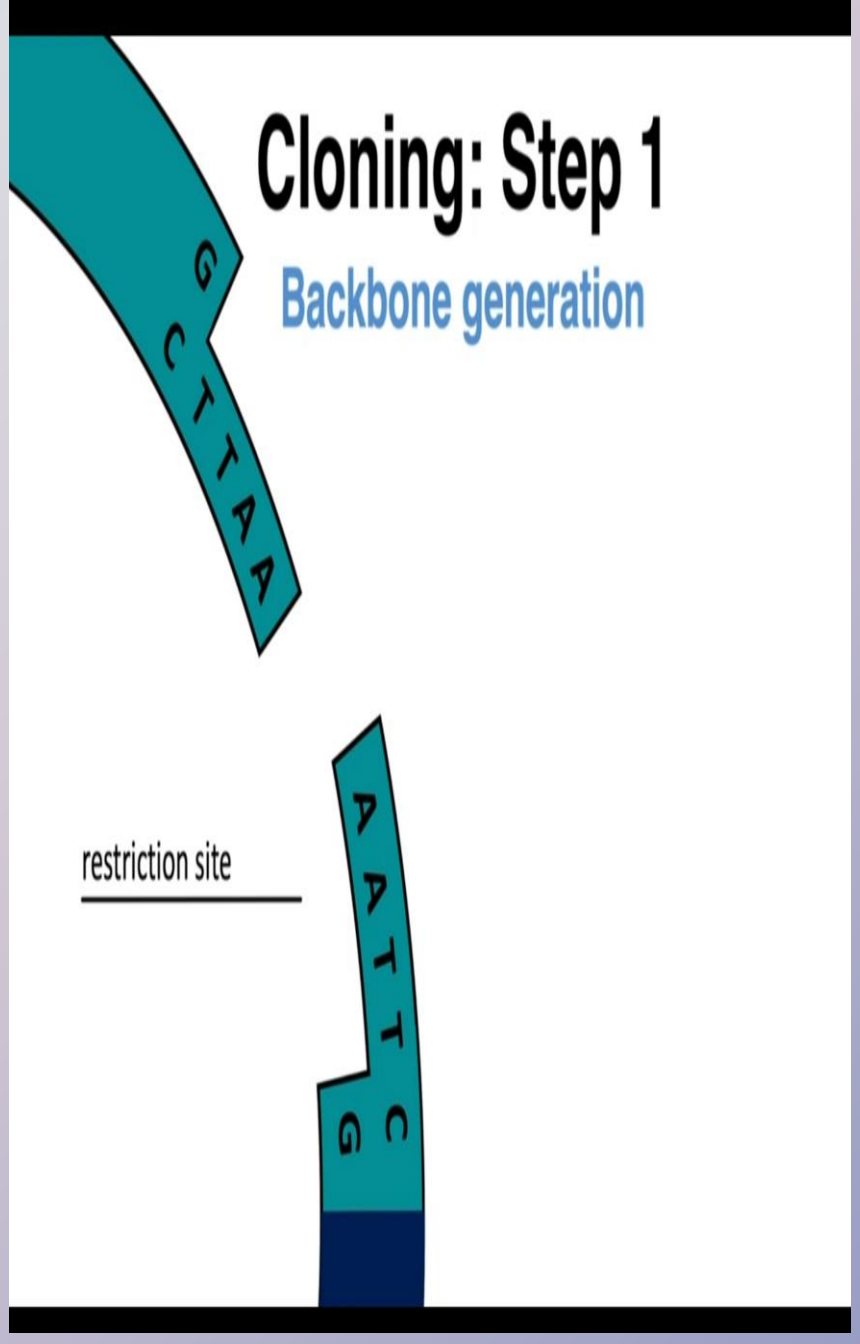


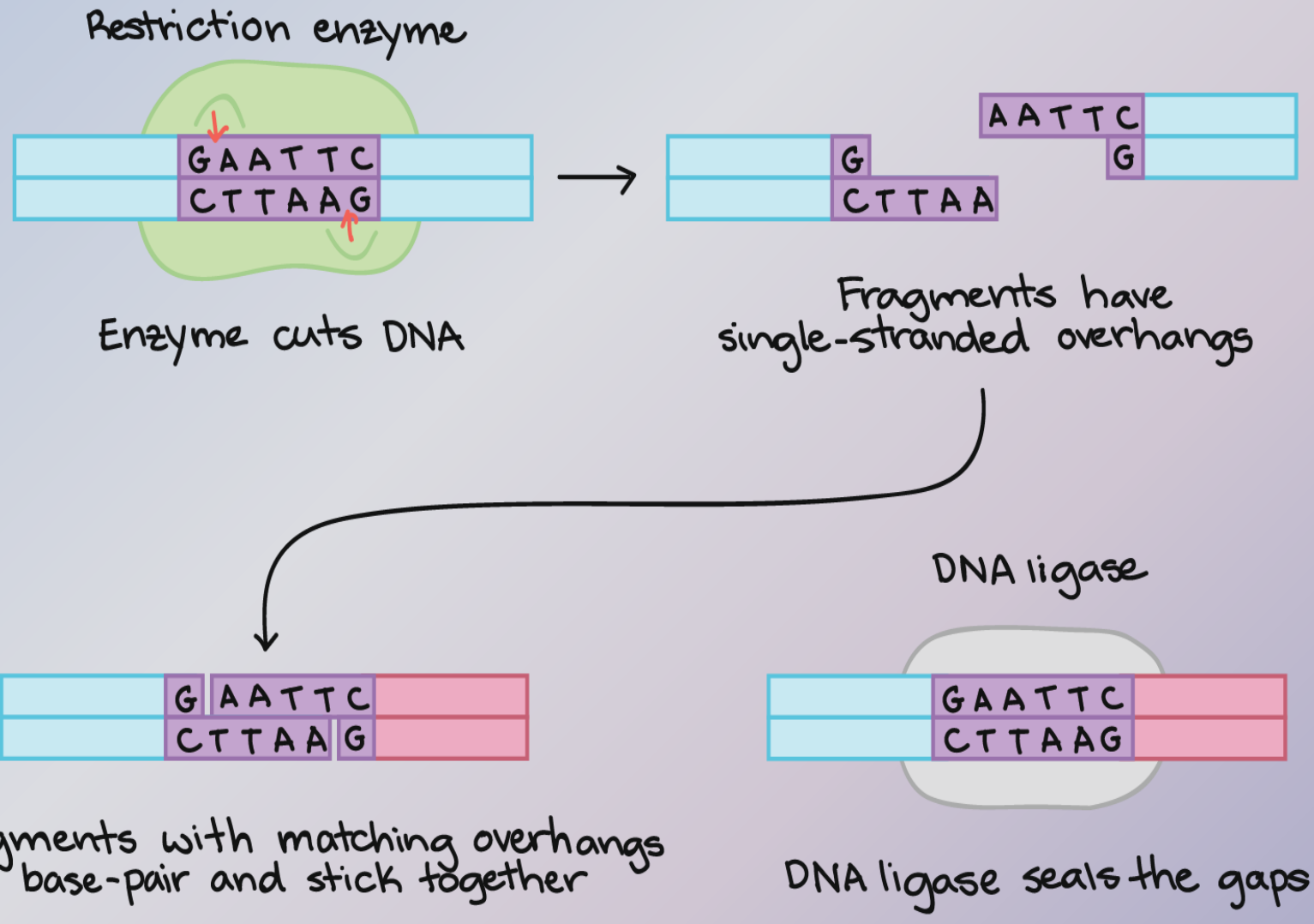
Three steps of PCR

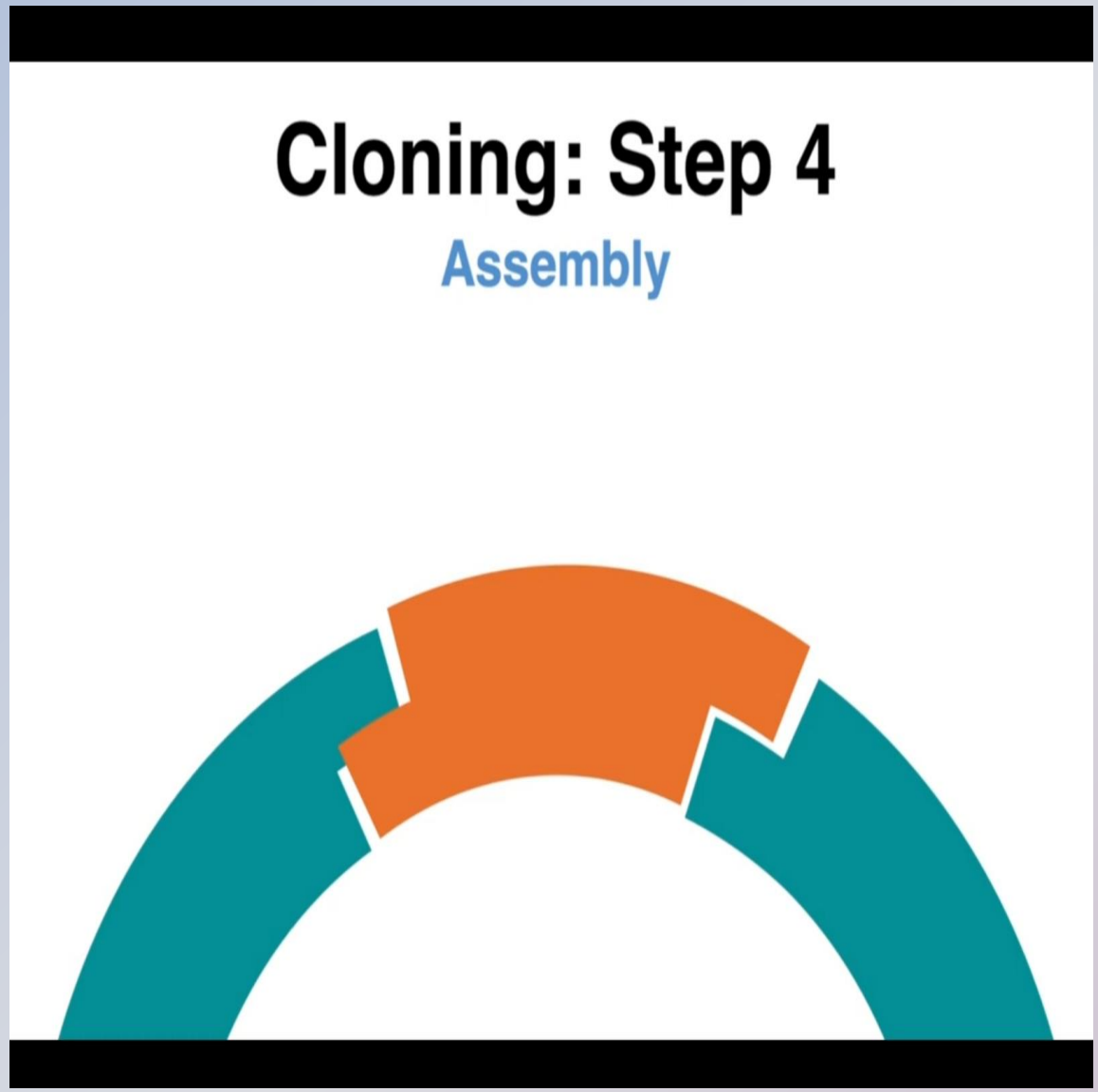

Cloning: Step 1
Backbone generation
origin of replication
promoter region
restriction site
antibiotic resistance gene
e.g. ampicilin resistance

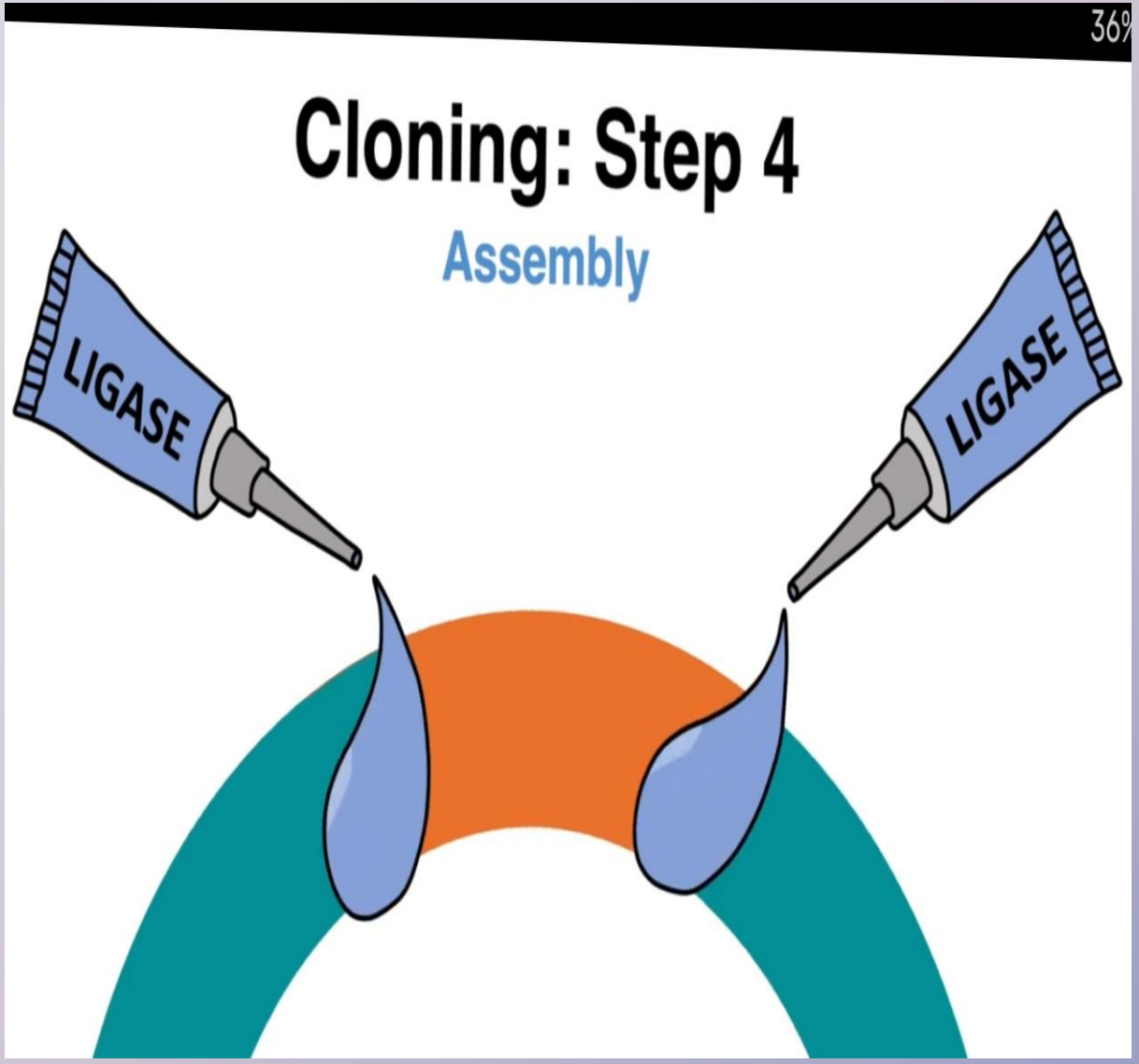

Cloning: Step 1
Backbone generation
restriction site
G
C T T A A G
A A T T C

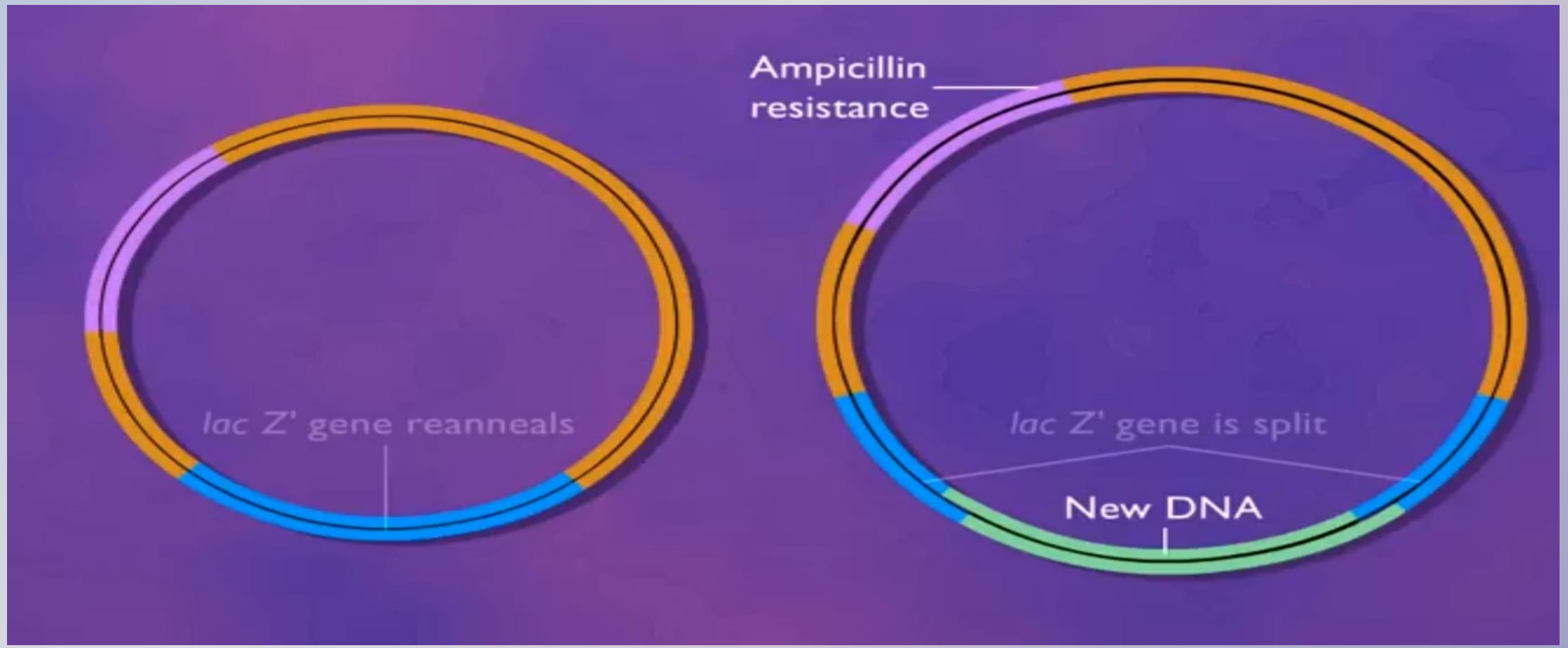
Cloning: Step 1
Backbone generation
G
C T T A A
restriction site
EcoRI


Cloning: Step 1
Backbone generation
G
C T T A A
restriction site
A A T T C
G


Restriction enzyme
GAATTC
CTTAAG
Enzyme cuts DNA
G
CTTAA
AATTC
G
Fragments have single-stranded overhangs
DNA ligase
GAATTC
CTTAAG
DNA ligase seals the gaps
GAATTC
CTTAAG
Fragments with matching overhangs base-pair and stick together

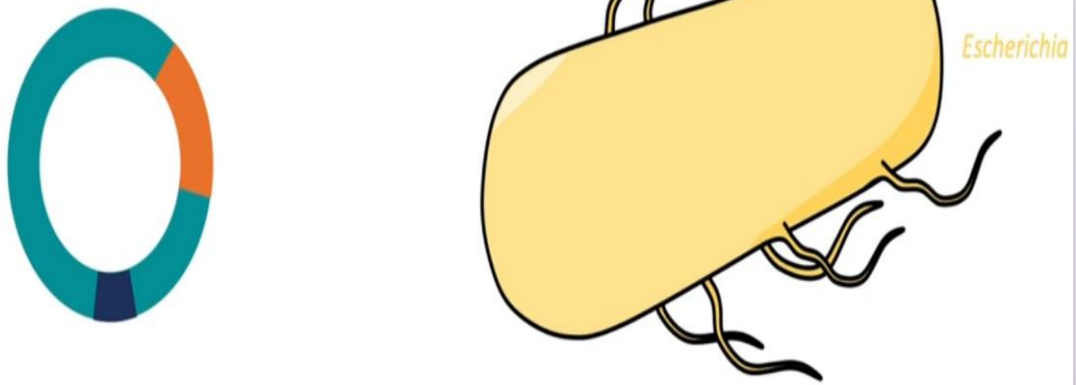

Cloning: Step 4
Assembly


36%
Cloning: Step 4
Assembly
LIGASE
LIGASE


Ampicillin
resistance
lac Z' gene reanneals
lac Z' gene is split
New DNA

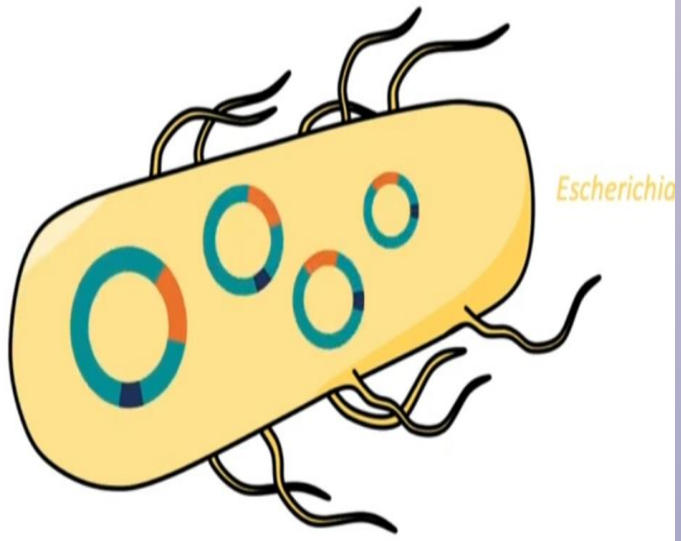
Cloning: Step 5

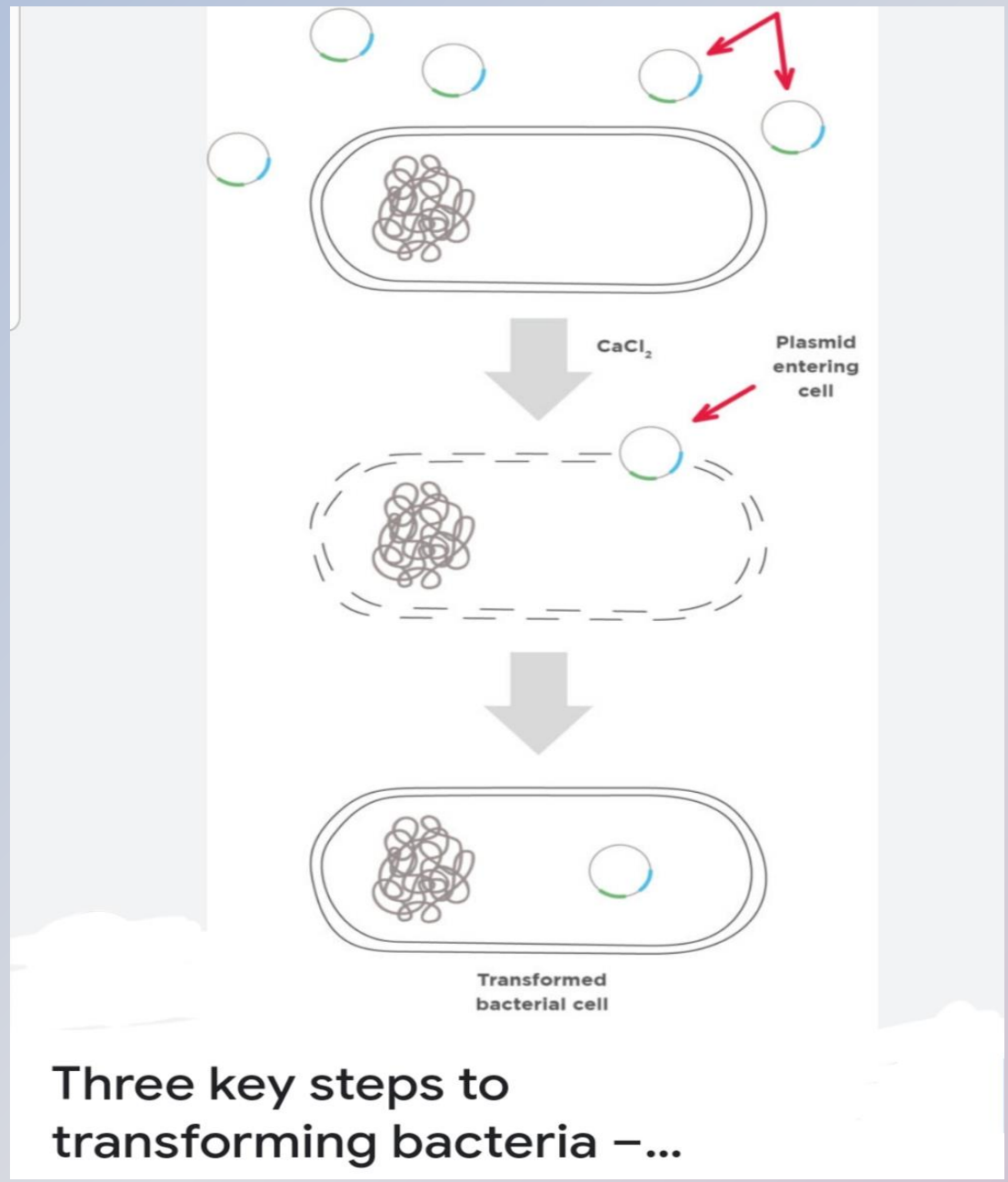
Transformation



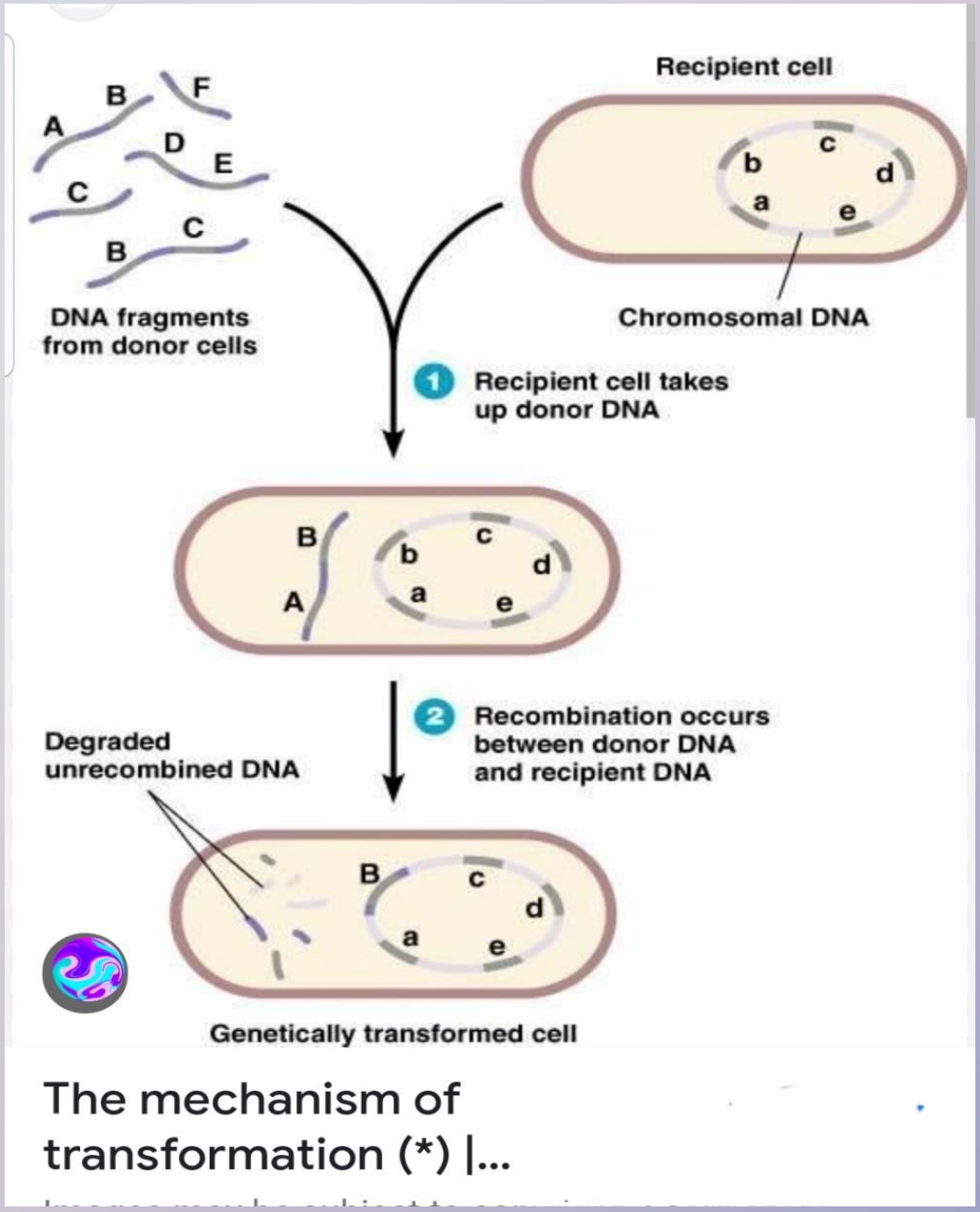
Cloning: Step 5

Transformation

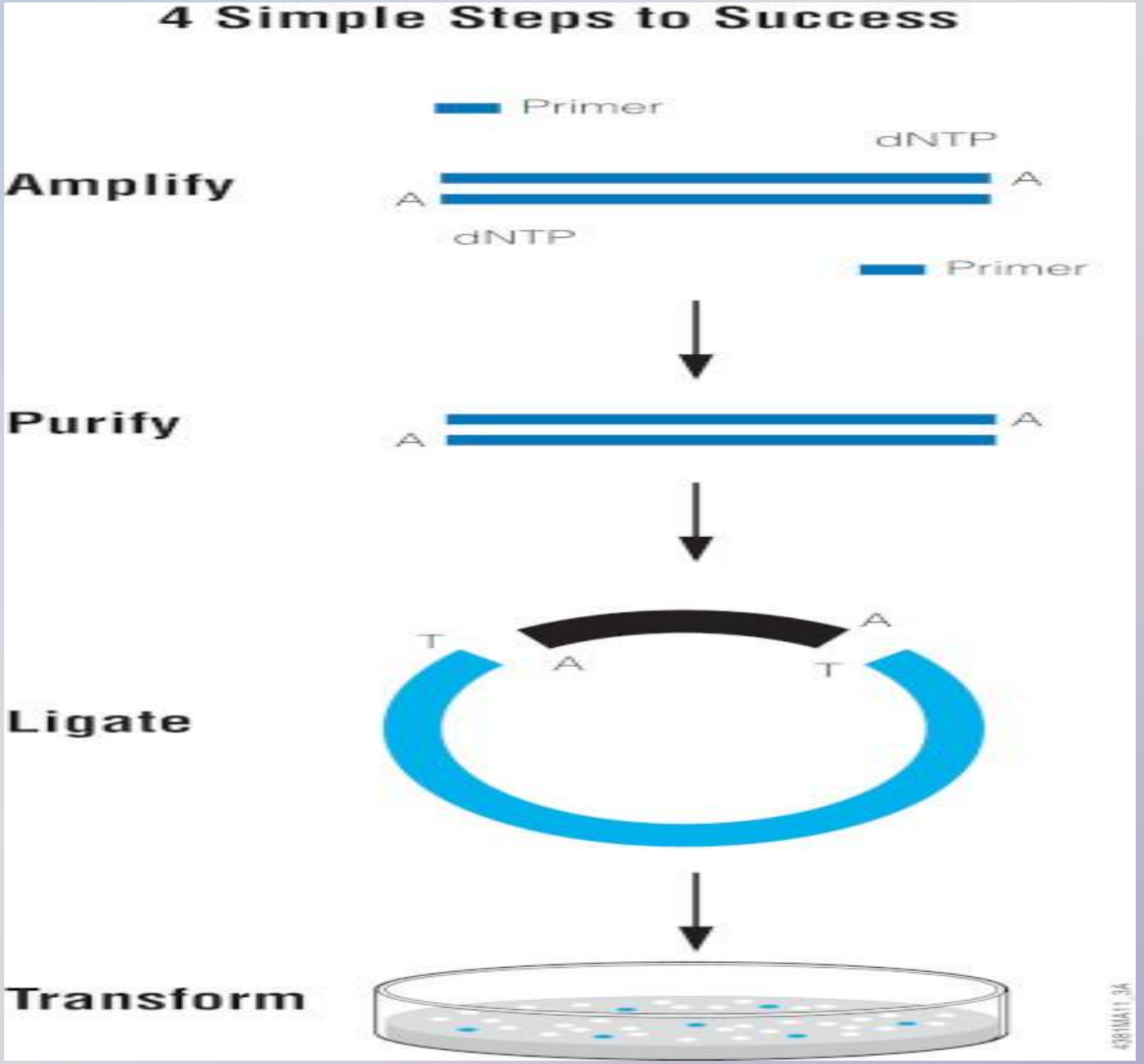




Three key steps to transforming bacteria –...



The mechanism of transformation (*) |...



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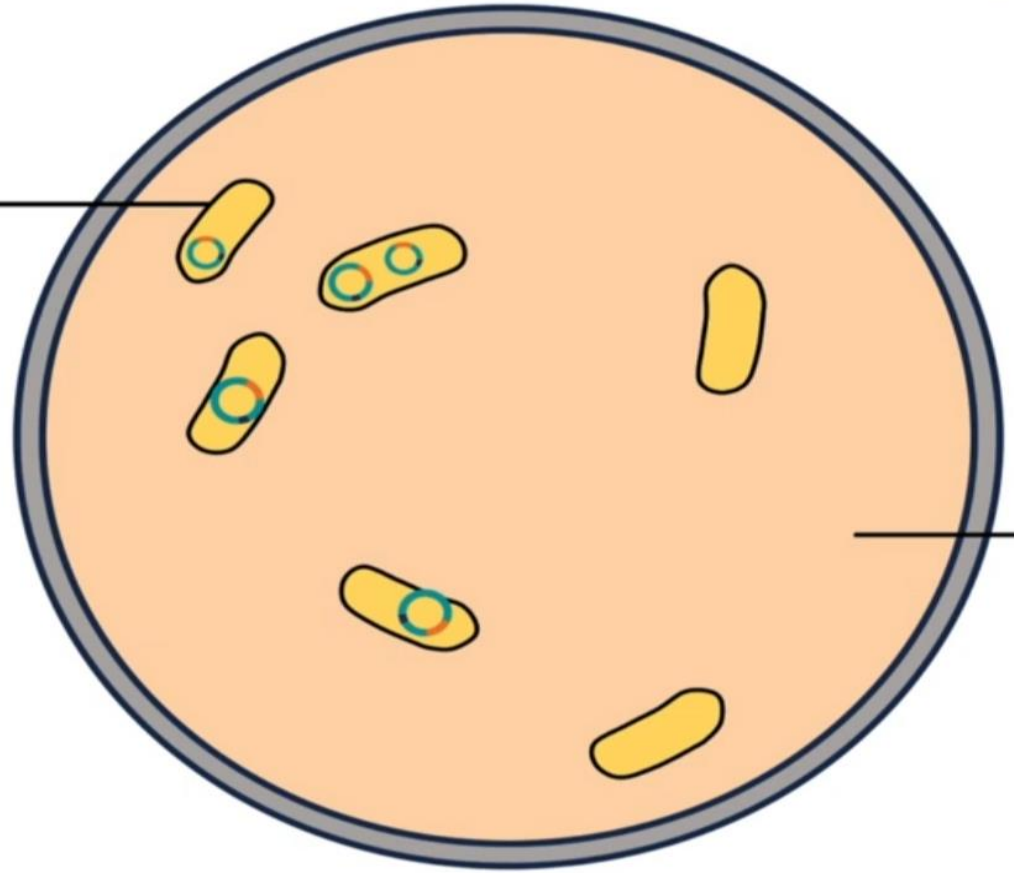
Cloning: Step 6

Selection and screening

recombinant cell:
contains plasmid with the antibiotic resistant gene



agar plate:
contains antibiotic medium (only bacteria with resistance gene grow)

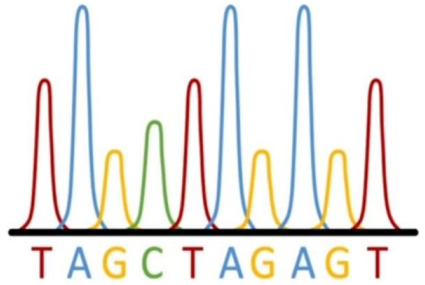
Cloning: Step 7

Verification

PCR

Cloning: Step 7

Verification



DNA sequencing

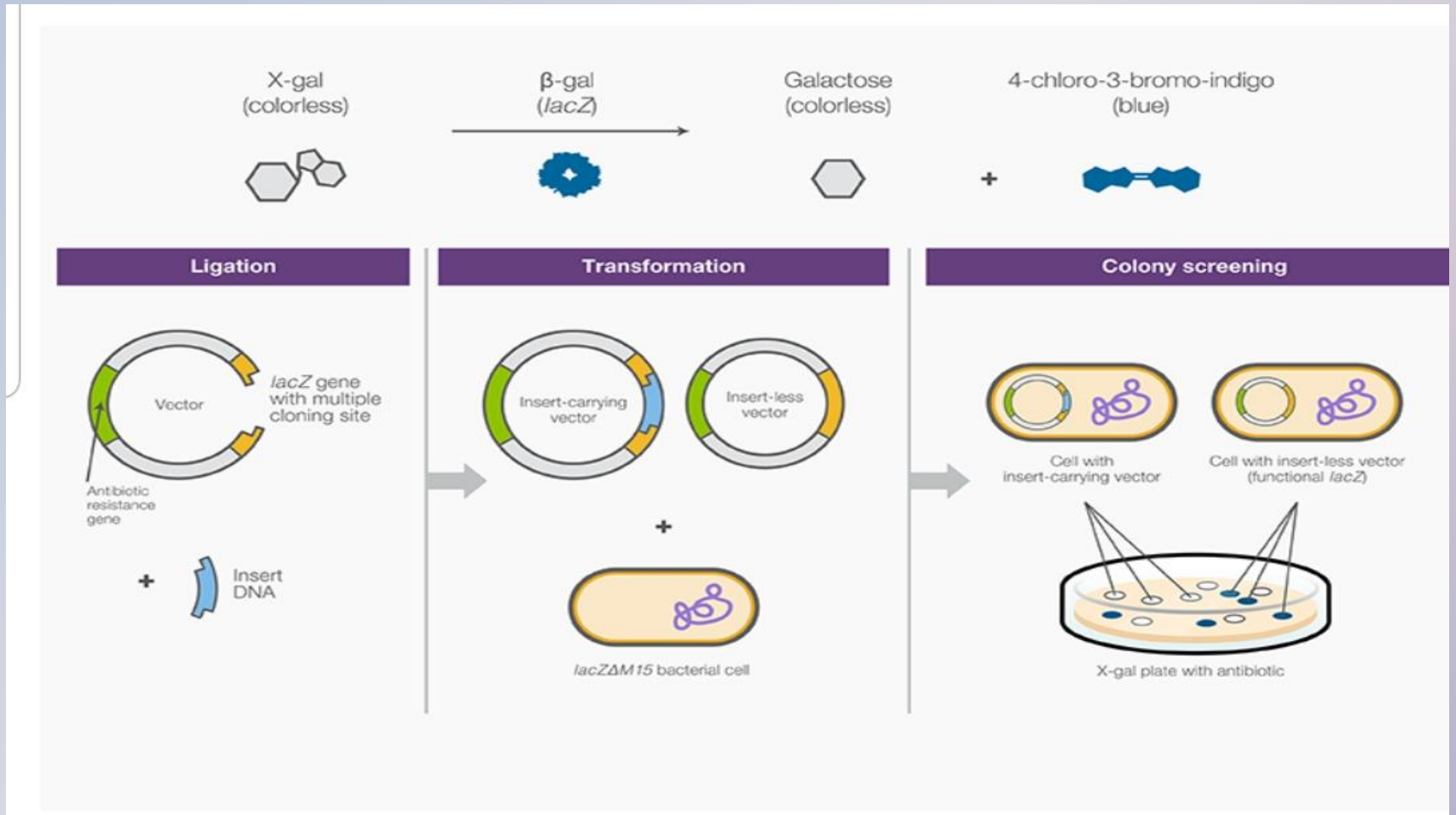
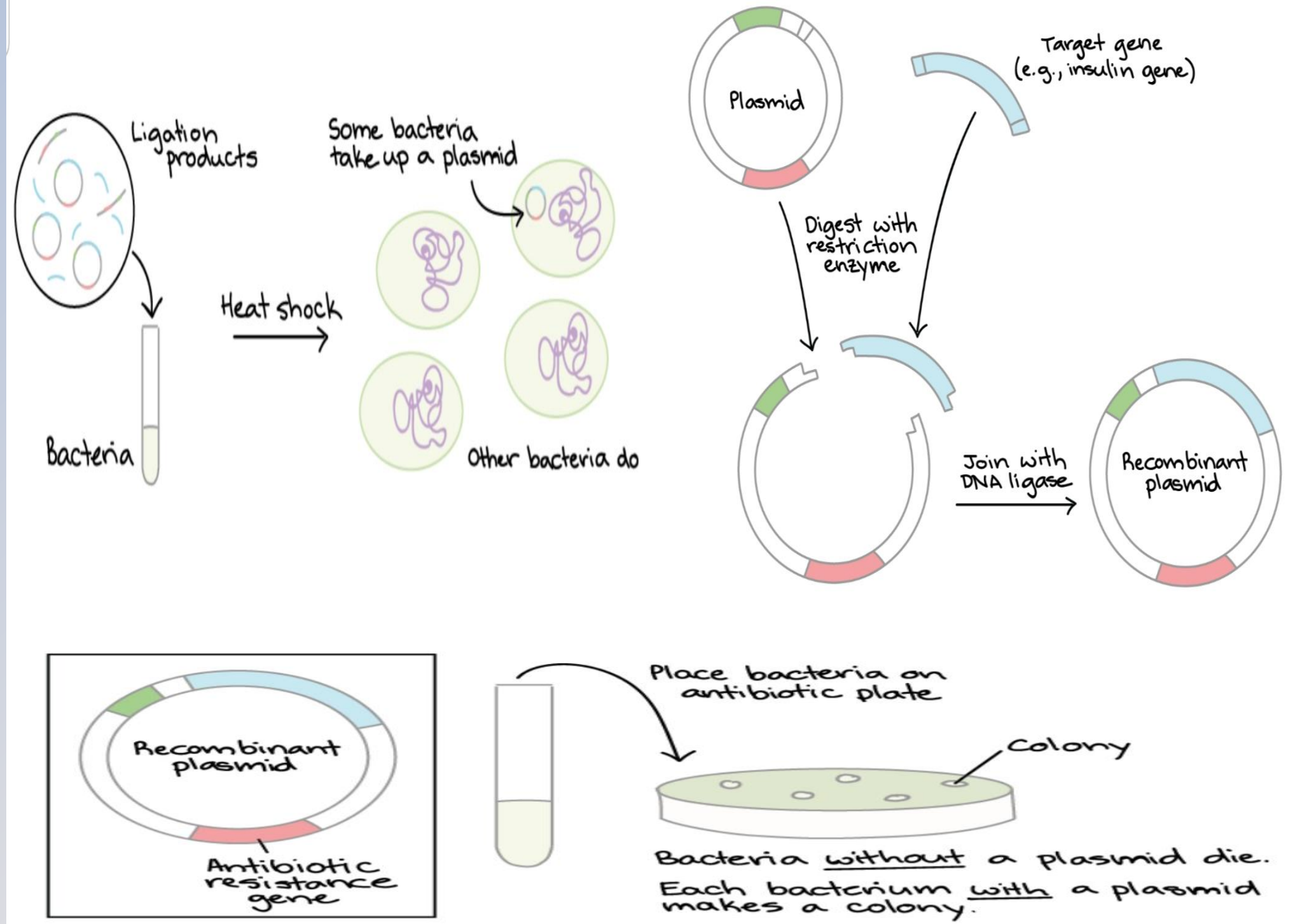
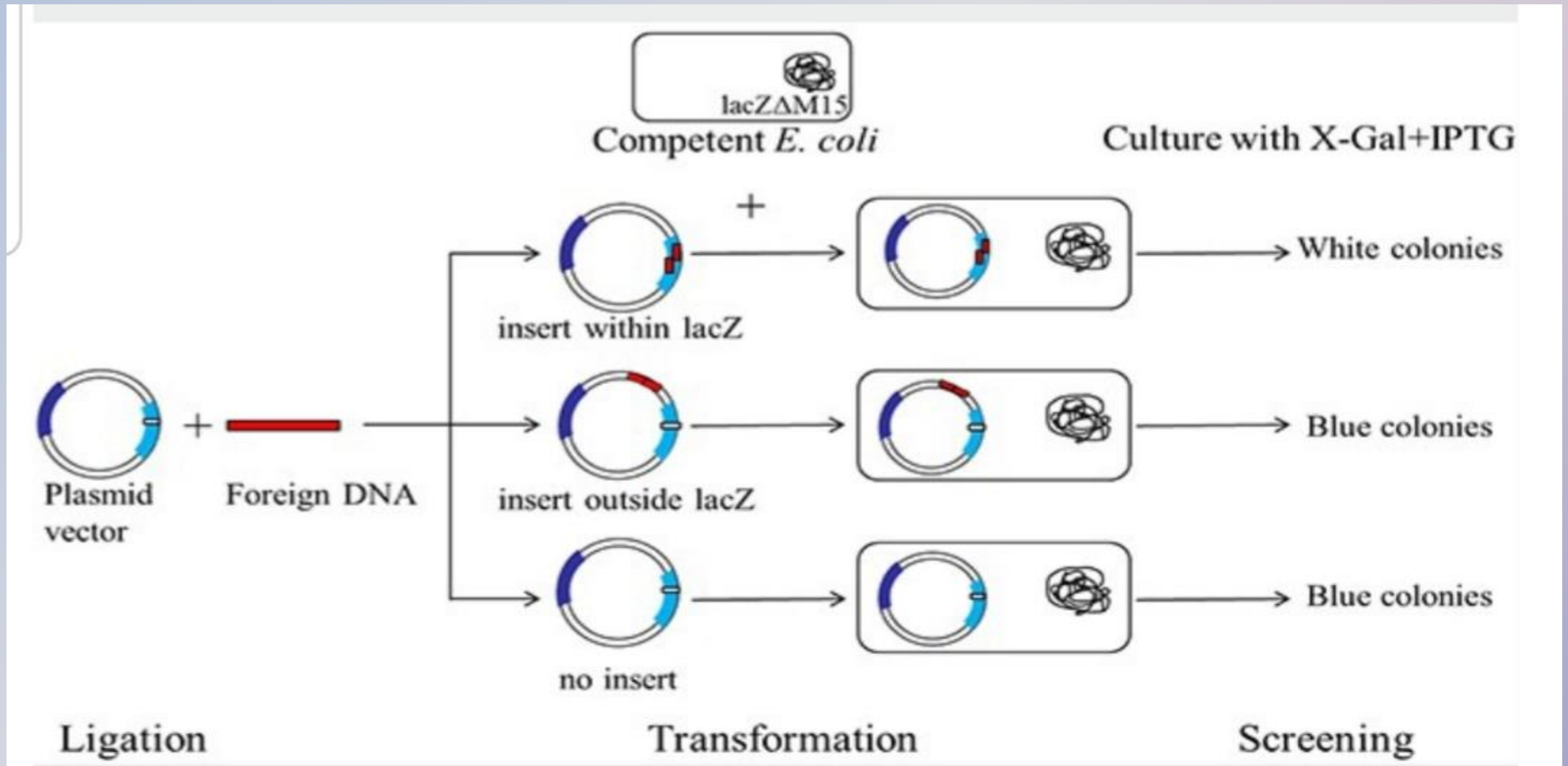


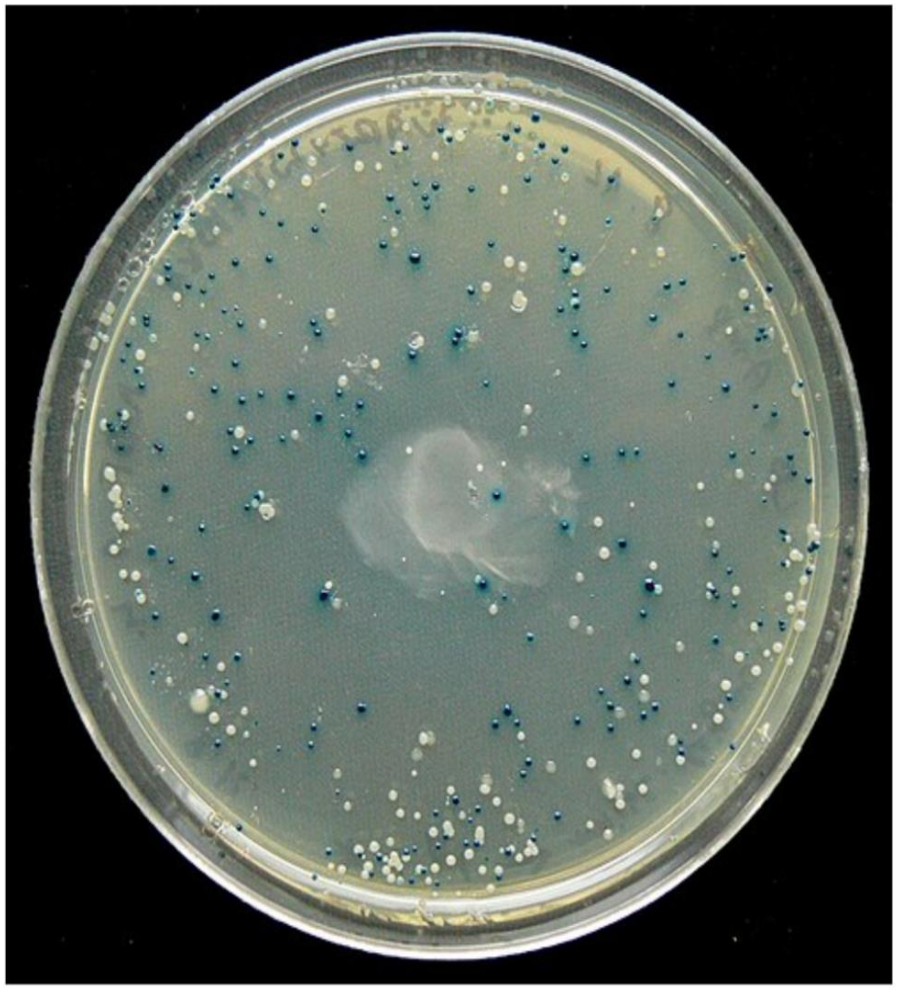
Figure 7. Color formation by beta-galactosidase (β-gal) activity on X-gal, and its application in blue/white screening.


Ligation products
Some bacteria take up a plasmid
Heat shock
Bacteria
Other bacteria do
Plasmid
Target gene (e.g., insulin gene)
Digest with restriction enzyme
Join with DNA ligase
Recombinant plasmid
Recombinant plasmid
Antibiotic resistance gene
Place bacteria on antibiotic plate
Colony
Bacteria without a plasmid die.
Each bacterium with a plasmid makes a colony.

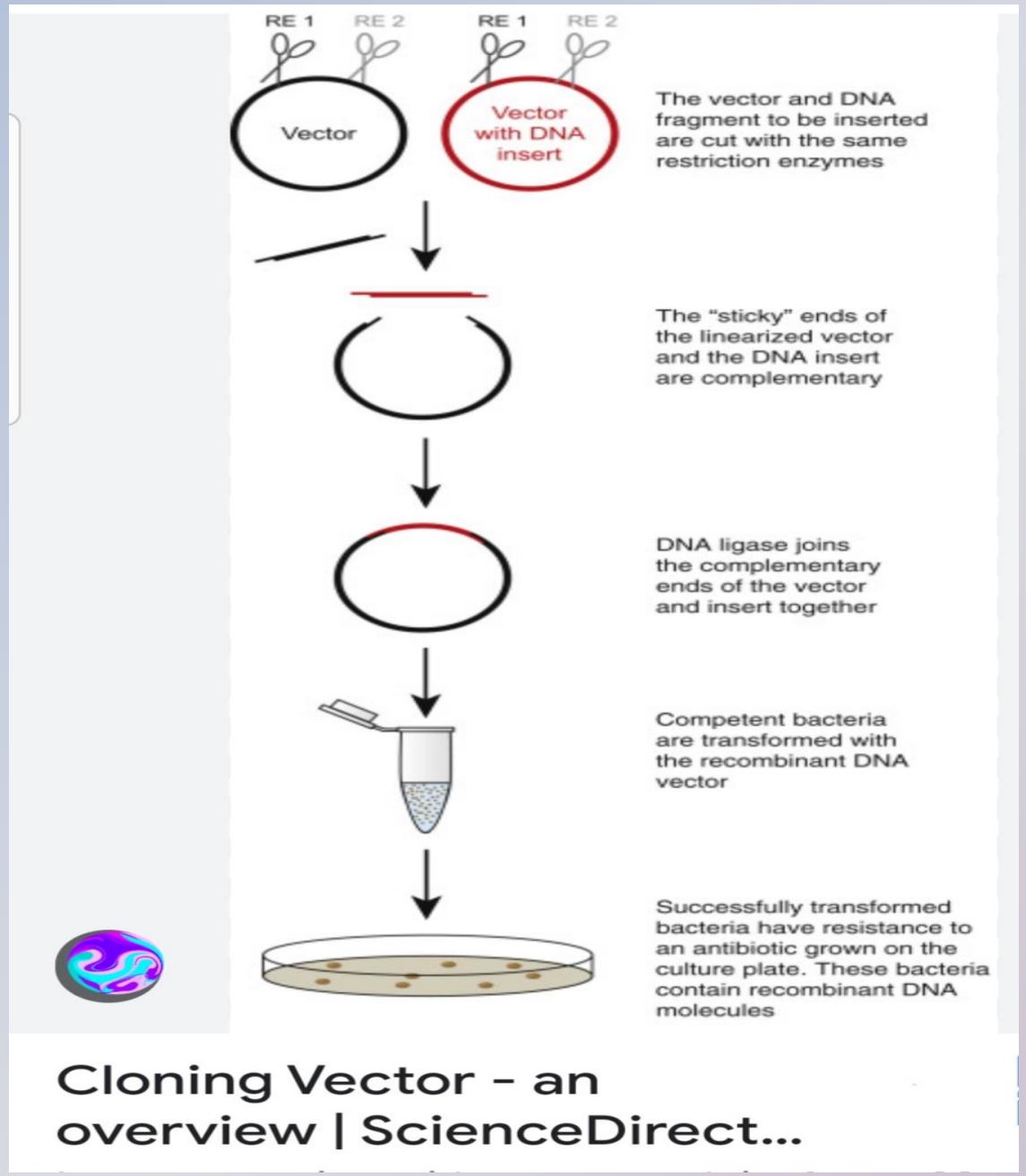


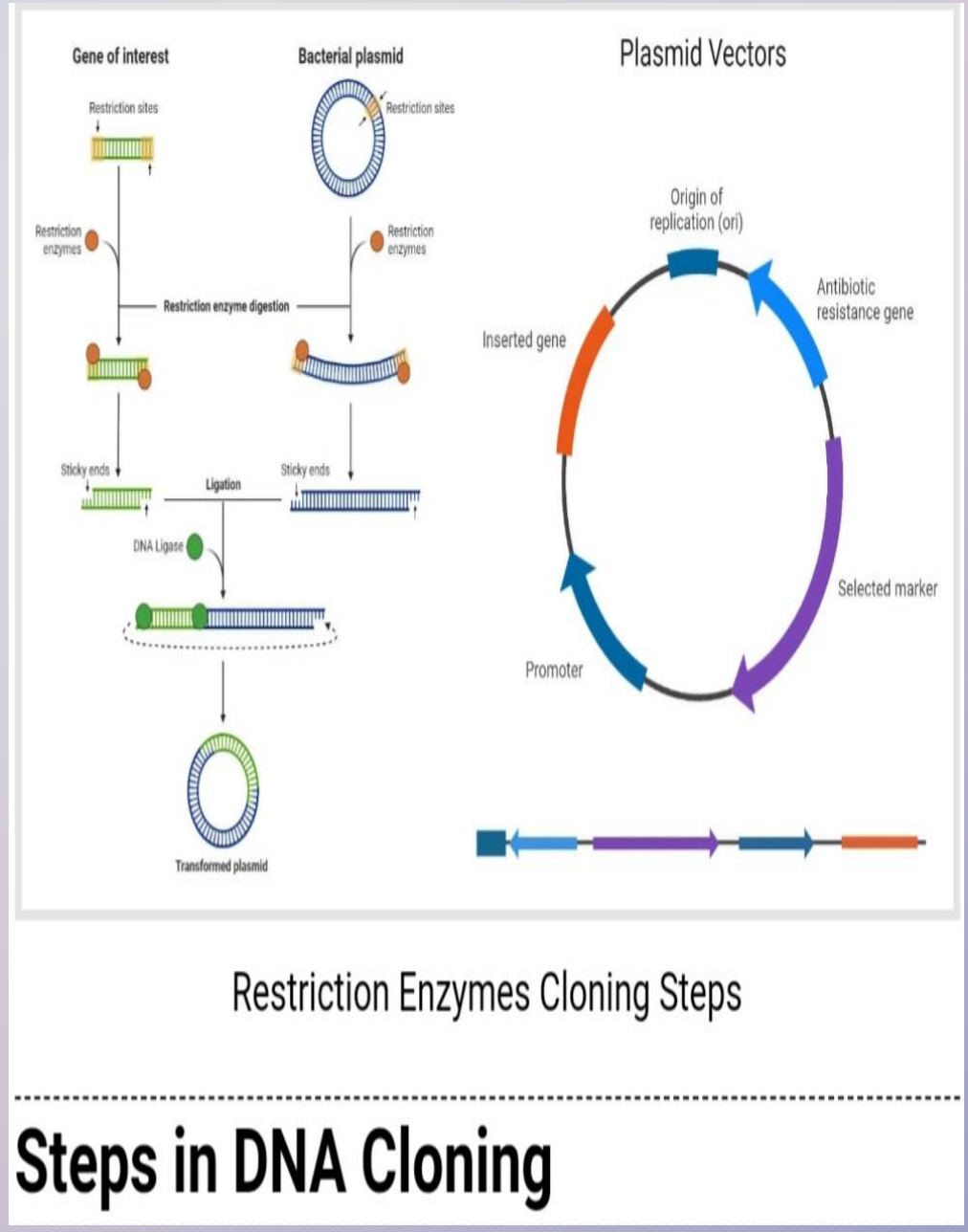
تصویری شماتیک از پروسه انتخاب کلونی

Blue white screening of
DNA clones



An LB agar plate showing the result of a blue–white screen.


RE 1
RE 2
RE 1
RE 2
Vector
Vector with DNA insert
The vector and DNA fragment to be inserted are cut with the same restriction enzymes
The "sticky" ends of the linearized vector and the DNA insert are complementary
DNA ligase joins the complementary ends of the vector and insert together
Competent bacteria are transformed with the recombinant DNA vector
Successfully transformed bacteria have resistance to an antibiotic grown on the culture plate. These bacteria contain recombinant DNA molecules
Cloning Vector – an overview | ScienceDirect...


Gene of interest
Bacterial plasmid
Plasmid Vectors
Restriction sites
Restriction sites
Restriction enzymes
Restriction enzymes
Restriction enzyme digestion
Sticky ends
Sticky ends
Ligation
DNA Ligase
Transformed plasmid
Origin of replication (ori)
Antibiotic resistance gene
Inserted gene
Selected marker
Promoter
Restriction Enzymes Cloning Steps
Steps in DNA Cloning

A general workflow for traditional cloning includes the following steps (Figure 1):

1. Vector preparation › 2. Insert preparation › 3. Ligation › 4. Transformation › 5. Colc screeni

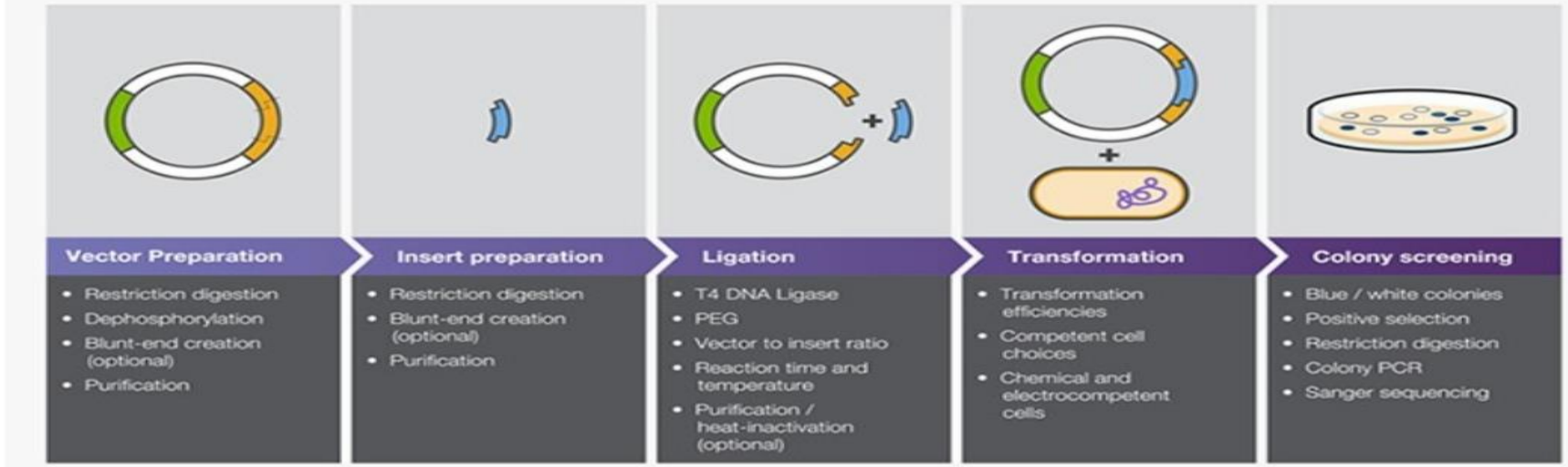
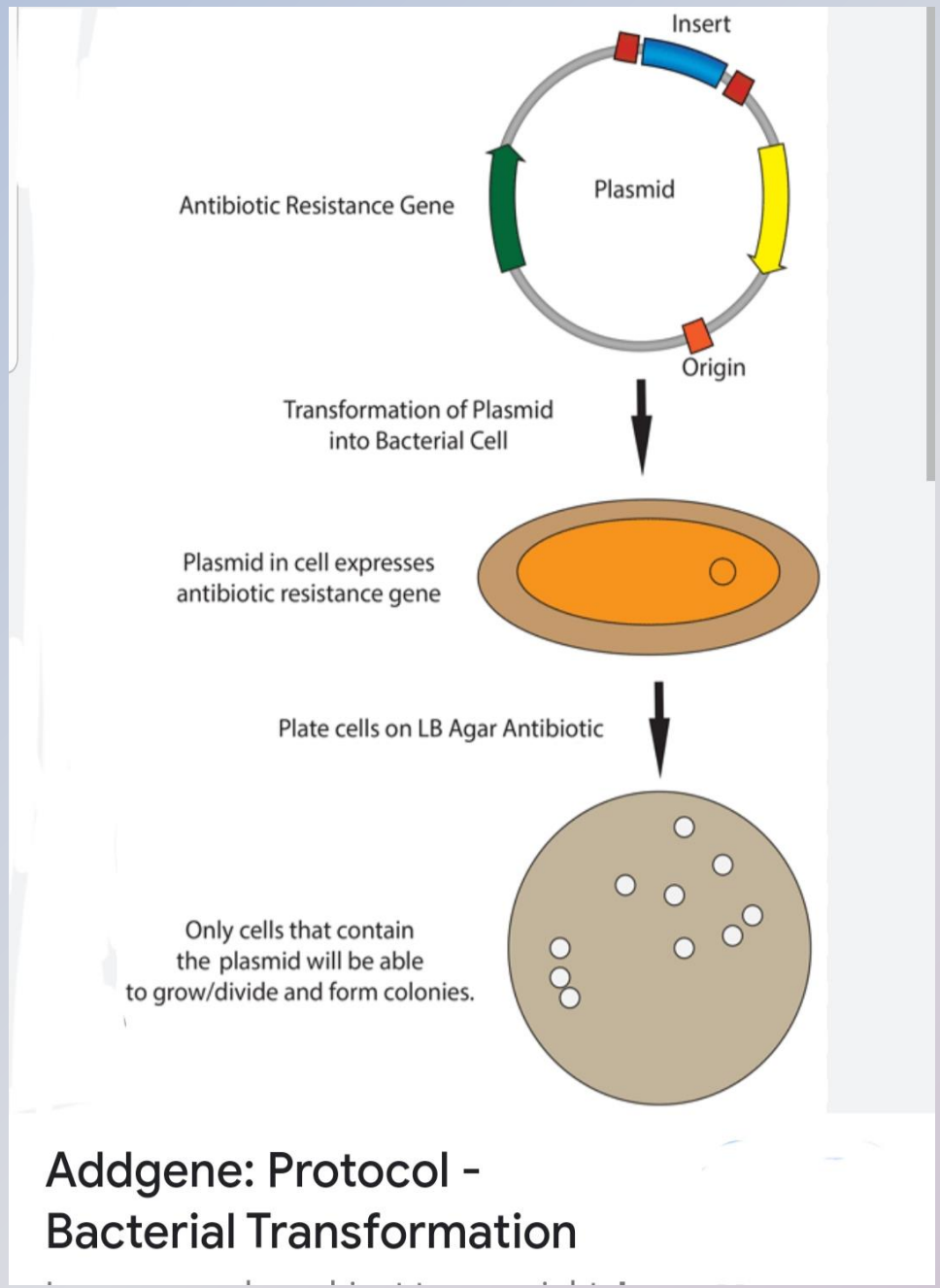
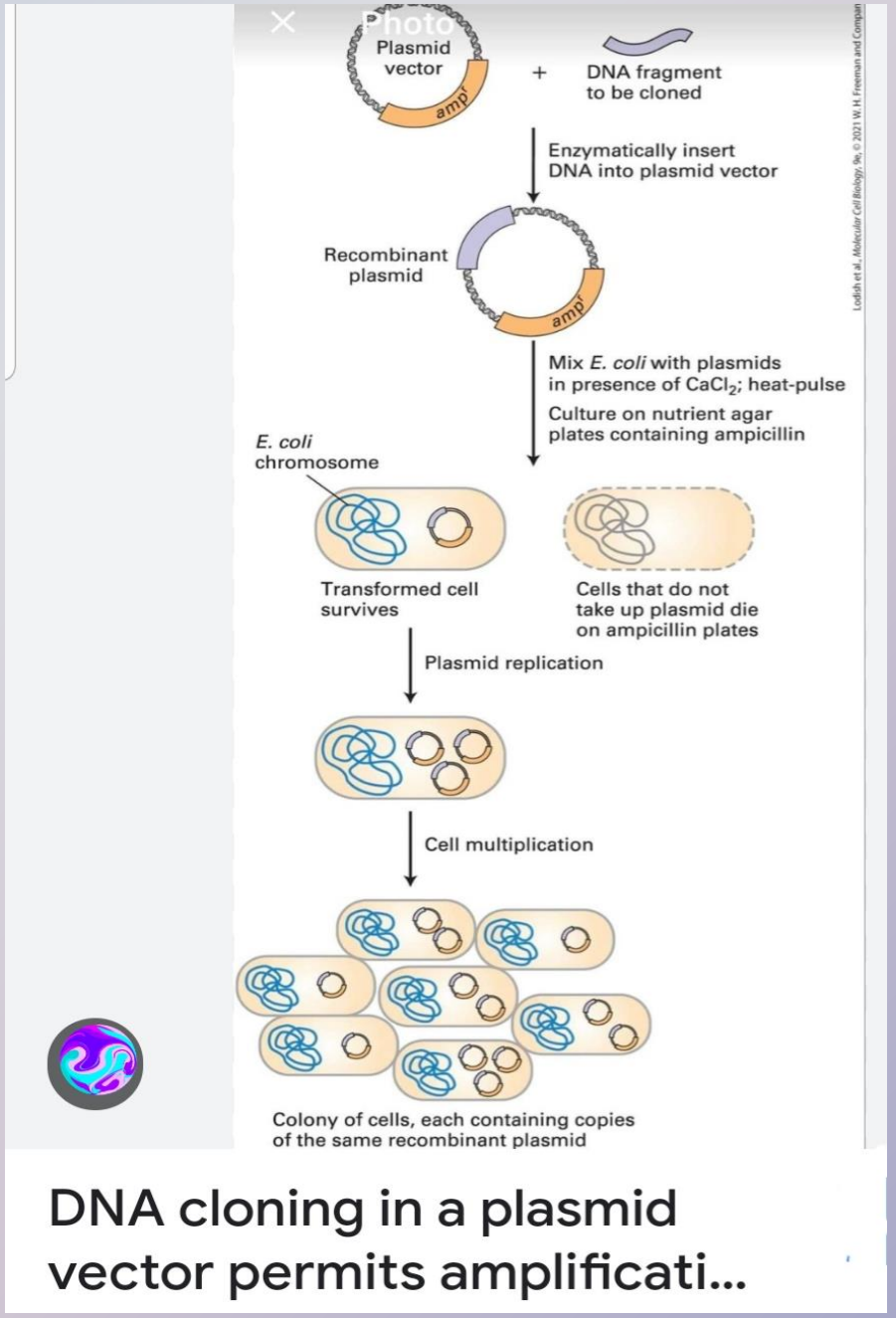


Figure 1. Traditional cloning workflow.



Addgene: Protocol - Bacterial Transformation



DNA cloning in a plasmid vector permits amplificati...