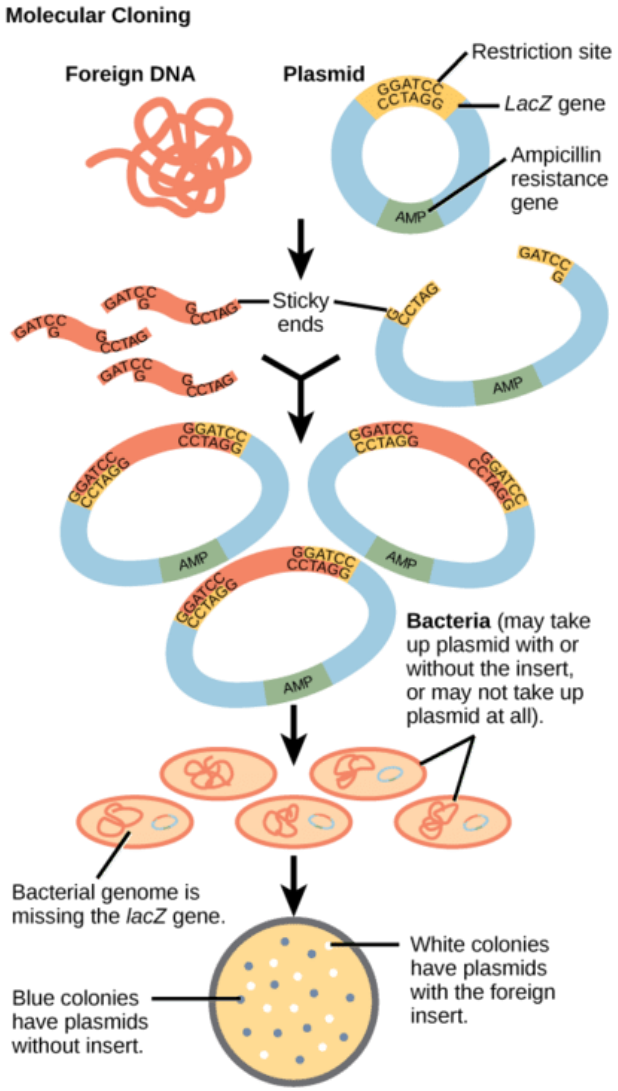
DNA cloning



Matsapume Detcharoen
2/2566

DNA cloning

- DNA cloning is a technique for **reproducing DNA fragments**.
- A **vector** is required to carry the DNA fragment of interest into the **host cell**.
- DNA cloning allows a copy of any specific part of a DNA sequence to be selected among many others and produced in an unlimited amount.

DNA cloning

- A single DNA molecule can be amplified allowing it to be:
  - Studied – sequenced, cloning vector
  - Manipulated - mutagenized or engineered
  - Expressed - generation of protein

Importance and applications

- **Gene Function Studies**
  By cloning a gene of interest into a suitable host organism, researchers can analyze the effects of altered gene expression or mutations on an organism's phenotype.

- **Genetic Engineering**
  Used in agriculture to create genetically modified crops with improved yield, pest resistance, or nutritional content. It's also used in medicine to produce recombinant proteins and pharmaceuticals.

- **Biotechnology and Protein Production**
  Cloning allows for the production of large quantities of specific proteins, such as insulin, growth factors, and enzymes, for medical and industrial purposes.

Importance and applications

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  By cloning a gene of interest into a suitable host organism, researchers can analyze the effects of altered gene expression or mutations on an organism's phenotype.

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Historical context and significance

- Discovery of DNA Structure: James Watson and Francis Crick in 1953.
- Discovery of restriction enzymes in the early 1970s.
- Recombinant DNA Technology in 1973 by Paul Berg and his team. They created the first recombinant DNA molecule by combining DNA from different sources.
- DNA Cloning Techniques in 1977. Using restriction enzymes and DNA ligase to insert specific DNA fragments into bacterial plasmids, creating recombinant DNA molecules.

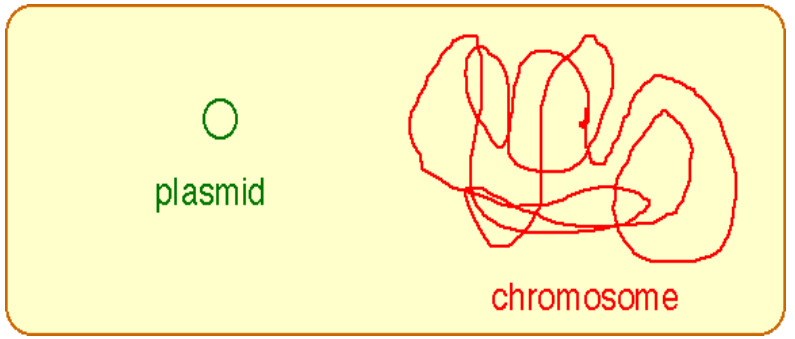
Historical context and significance

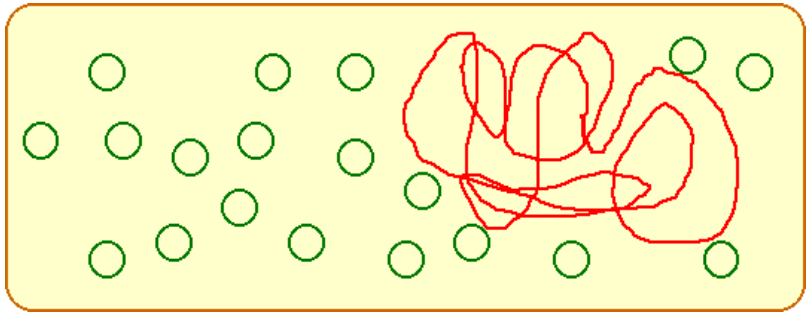
- The invention of PCR in the 1980s, making it possible to generate millions of copies of a DNA fragment from just a small amount of starting material.

- Human Genome Project in the 1990s. By breaking the genome into smaller fragments and cloning them into bacterial or other suitable host cells.

Plasmids

- Bacterial cells may contain extra-chromosomal DNA called plasmids.
- Plasmids are usually represented by small, circular DNA.
- Some plasmids are present in multiple copies in the cell





Vectors

- **Vectors** are DNA molecules that are used to "transport" cloned sequences between biological hosts and the test tube.
- Vectors share four common properties
  1. Ability to promote autonomous replication.
  2. Contain a genetic marker (usually dominant) for selection.
  3. Unique restriction sites to facilitate cloning of insert DNA.
  4. Minimum amount of nonessential DNA to optimize cloning.

Plasmid vectors

- Plasmid vectors are ≈1.2–3 kb and contain:
  - origin of replication (ORI) sequence
  - a gene that permits selection,
  - Here the selective gene is *amp*^{r}; it encodes the enzyme b-lactamase, which inactivates ampicillin.

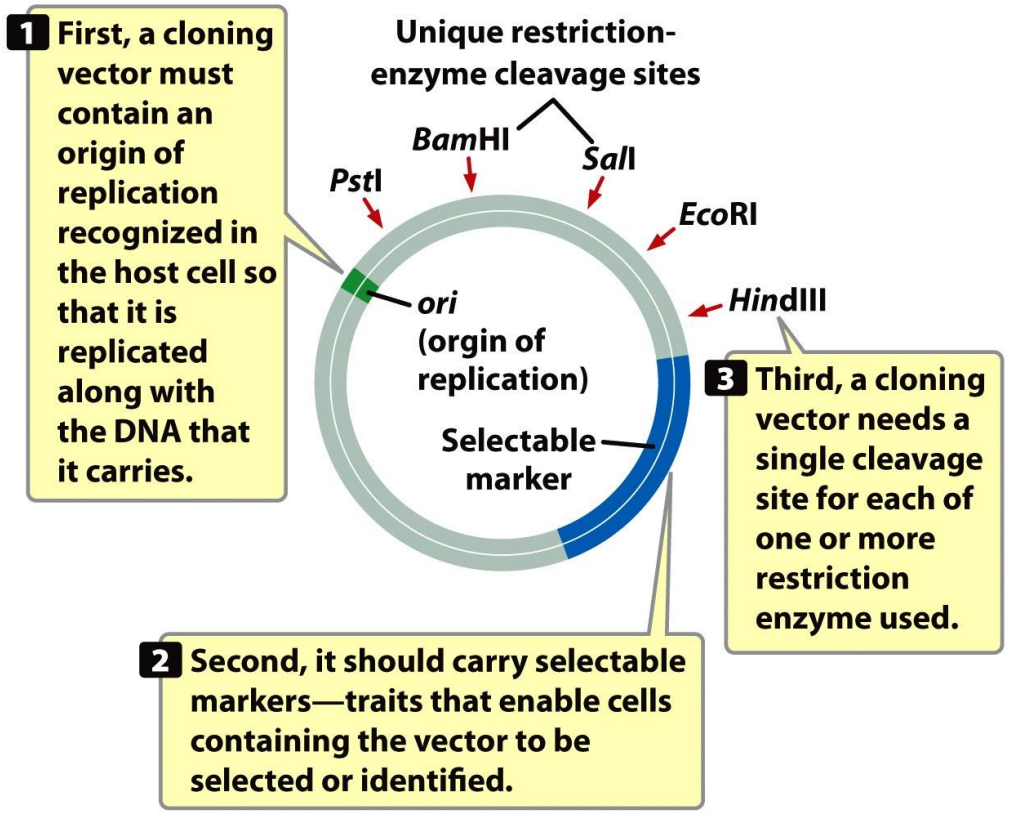
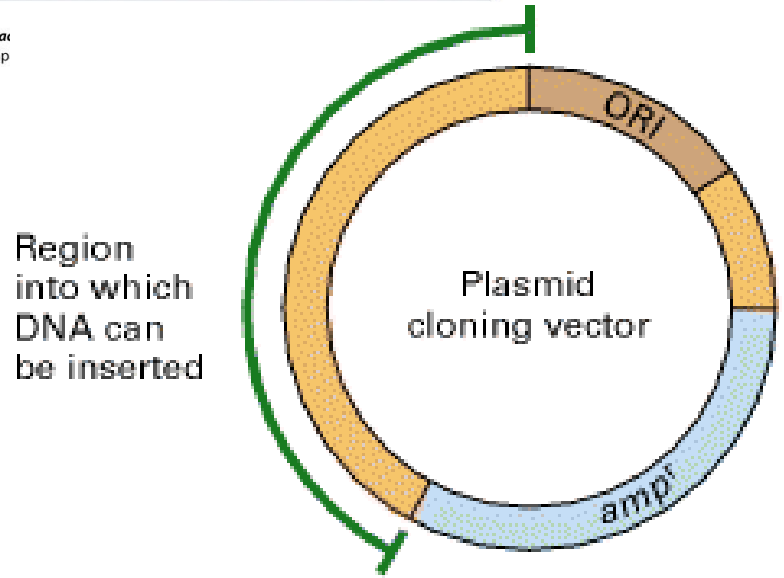
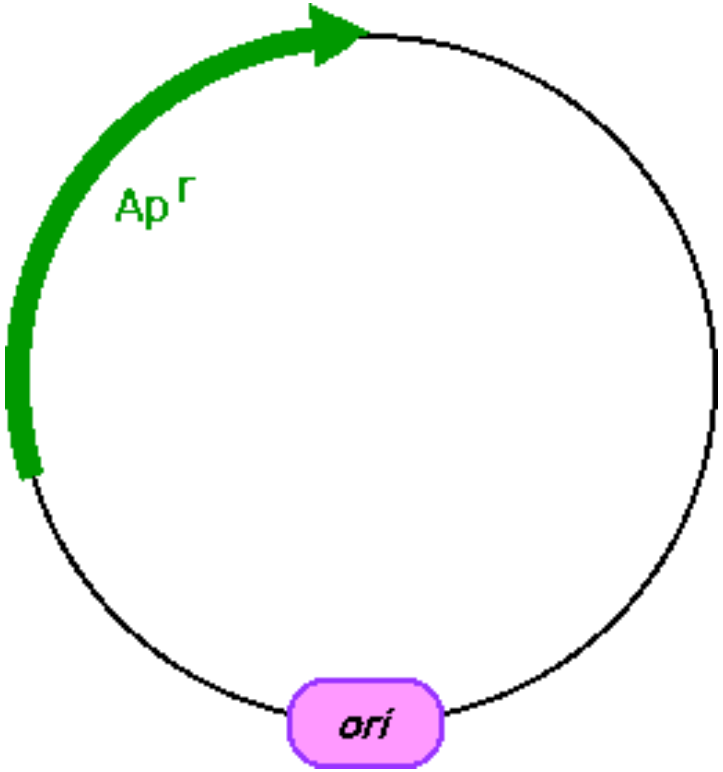


Figure 19-5
Genetics: A Conceptual Approac
© 2009 W. H. Freeman and Comp



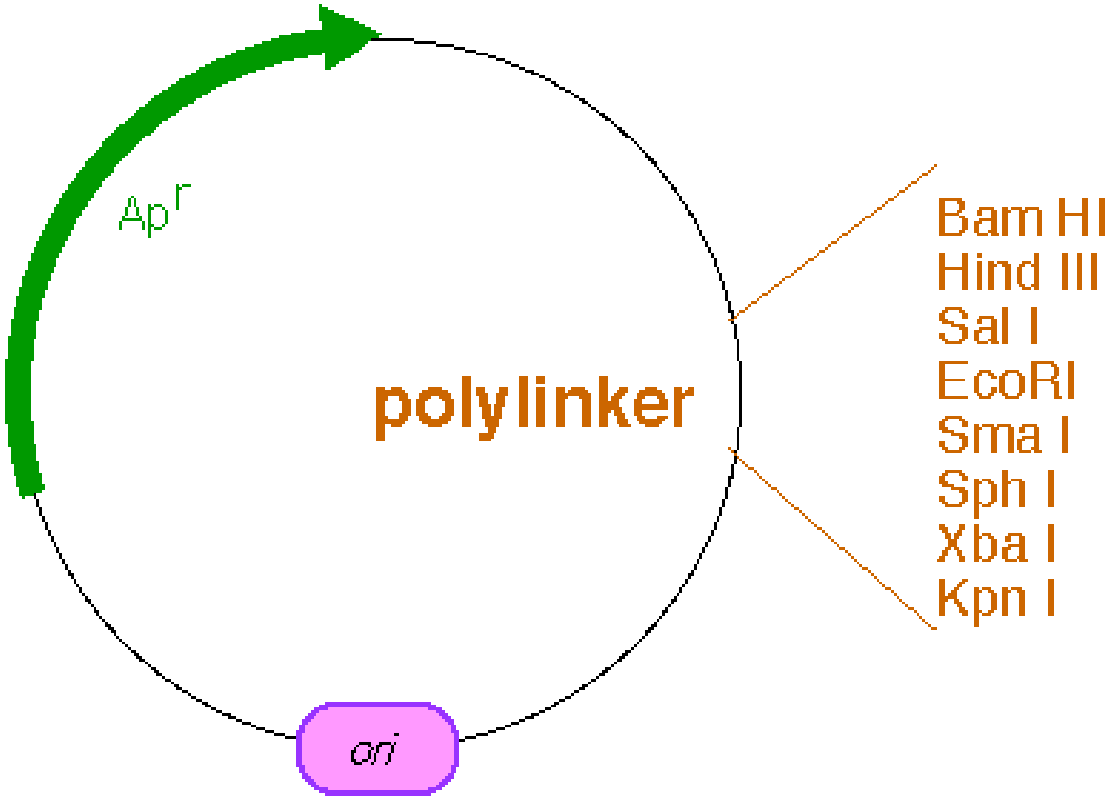
Selective marker

- **Selective marker** is required for maintenance of plasmid in the cell.
- Under the selective conditions, only cells that contain plasmids with selectable marker can survive.
- Genes that confer resistance to various antibiotics (e.g. ampicillin, neomycin, or chloramphenicol) are used.



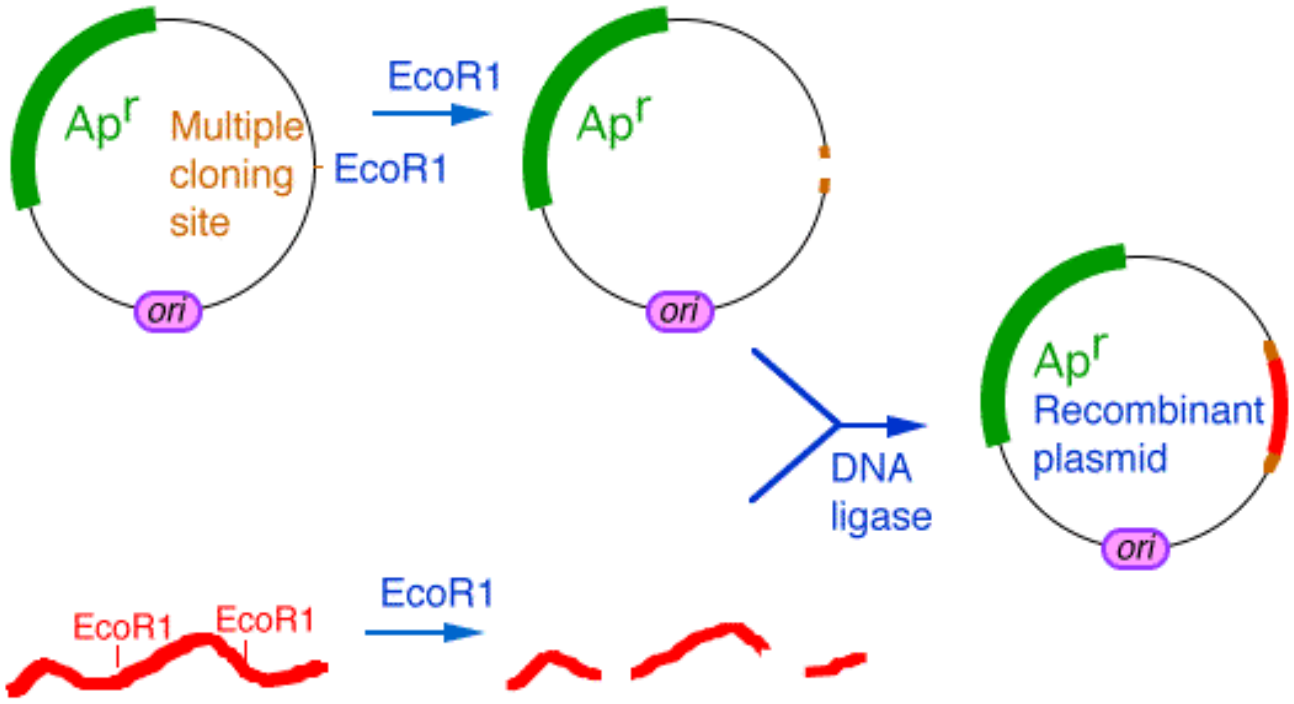
Multiple cloning site

- Cloning vectors contain a **multiple cloning site** or **polylinker,** a DNA segment with several unique sites for restriction endonucleases located next to each other.

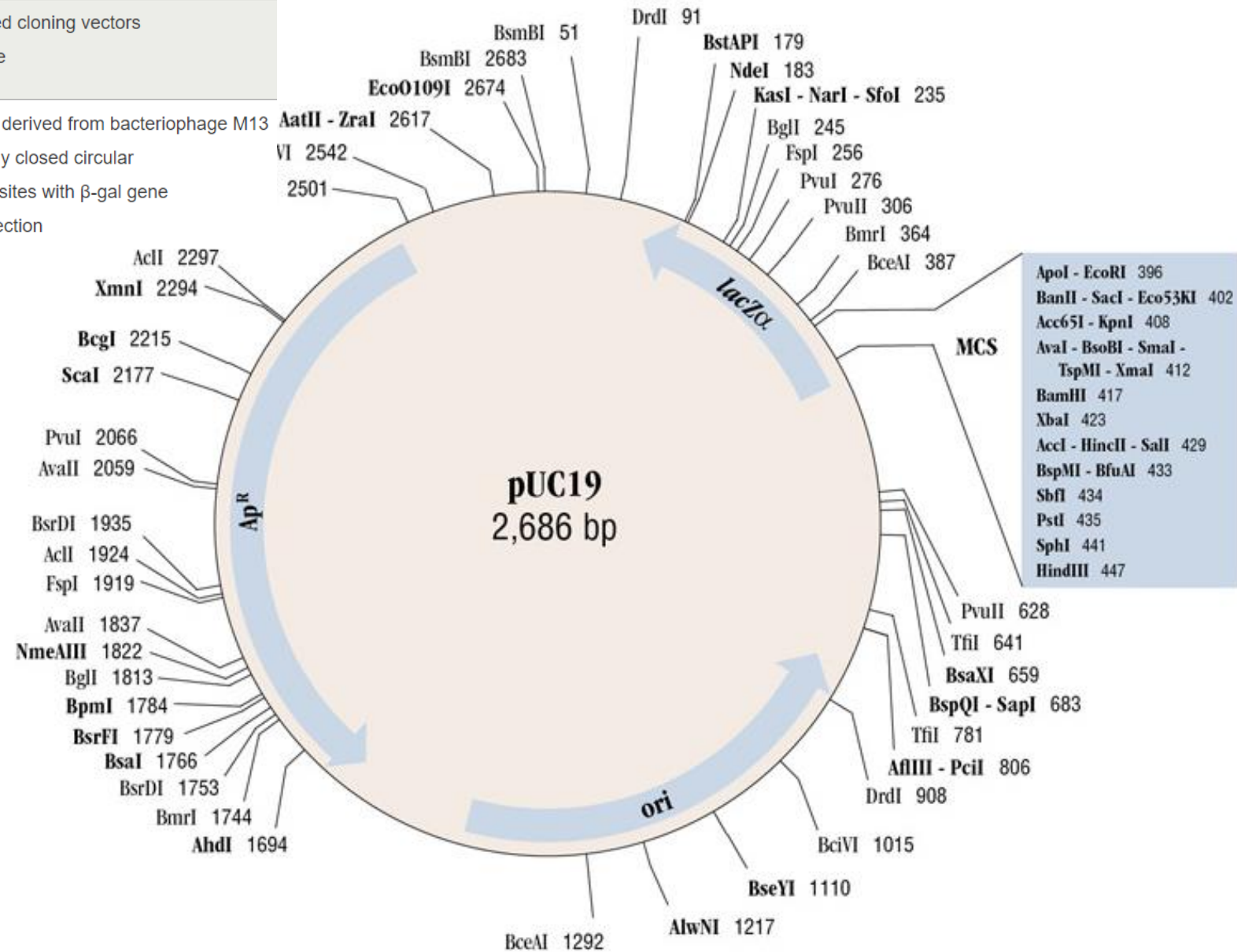


Multiple cloning site

- Gene to be cloned can be introduced into the cloning vector at one of the restriction sites present in the polylinker.



Cloning Plasmid/DNA	NEB #	Features
pBR322 Vector	N3033S/L	• Commonly used cloning vectors • Tet, Amp resistance
pUC19 Vector	N3041S/L	• Commonly used cloning vectors • Amp resistance
M13mp18 RF I DNA	N4018S	• Phage vectors derived from bacteriophage M13 • DNA, covalently closed circular • 13 Unique RE sites with β-gal gene • Blue-white selection



Cloning and Expression Vectors

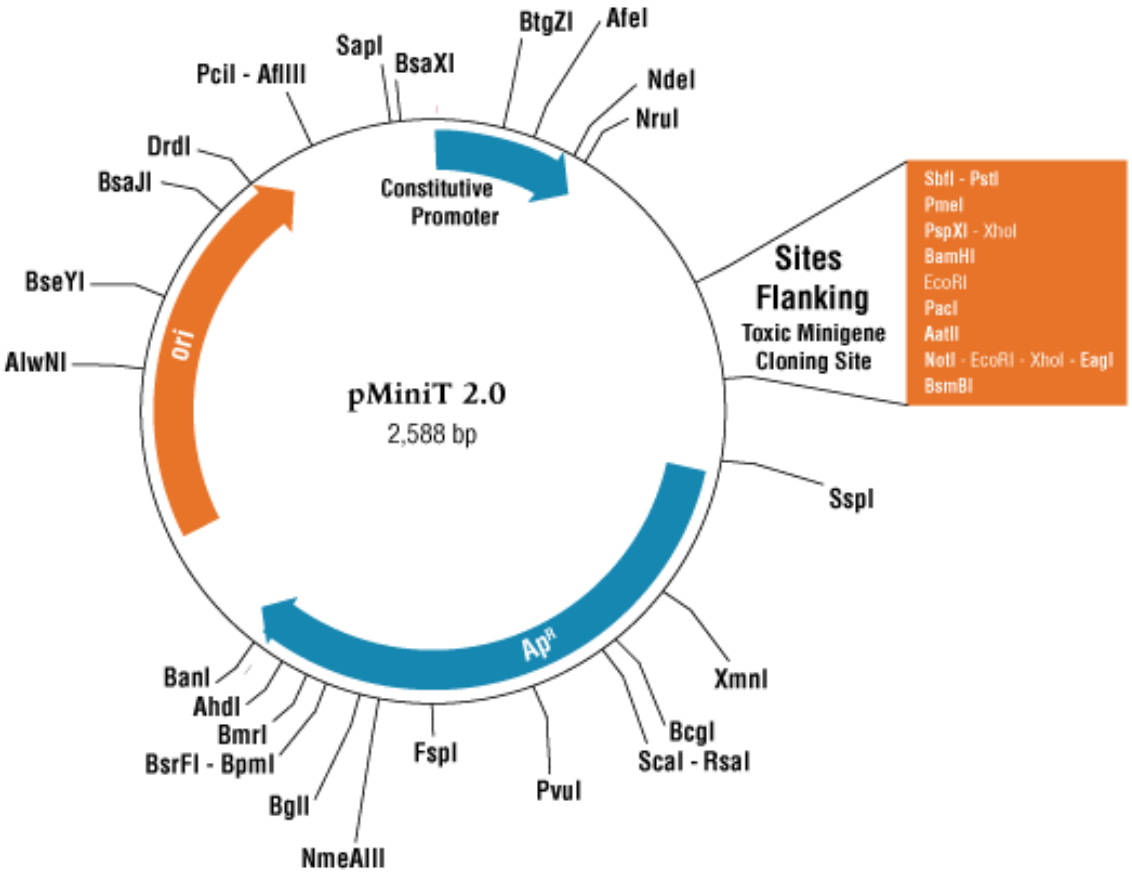
- Vectors are DNA molecules used to carry and deliver foreign DNA into host organisms.
- There are 2 main types of vectors used in molecular biology:
  - Cloning vectors
  - Expression vectors

Cloning Vectors:

Purpose: to facilitate the insertion and propagation of DNA fragments, within host cells.

Features: contain elements such as origin of replication (ori), selectable markers, and multiple cloning sites (MCS).

Advantages: versatile tools for generating libraries of DNA fragments, studying gene function, and preparing DNA samples for further analysis.

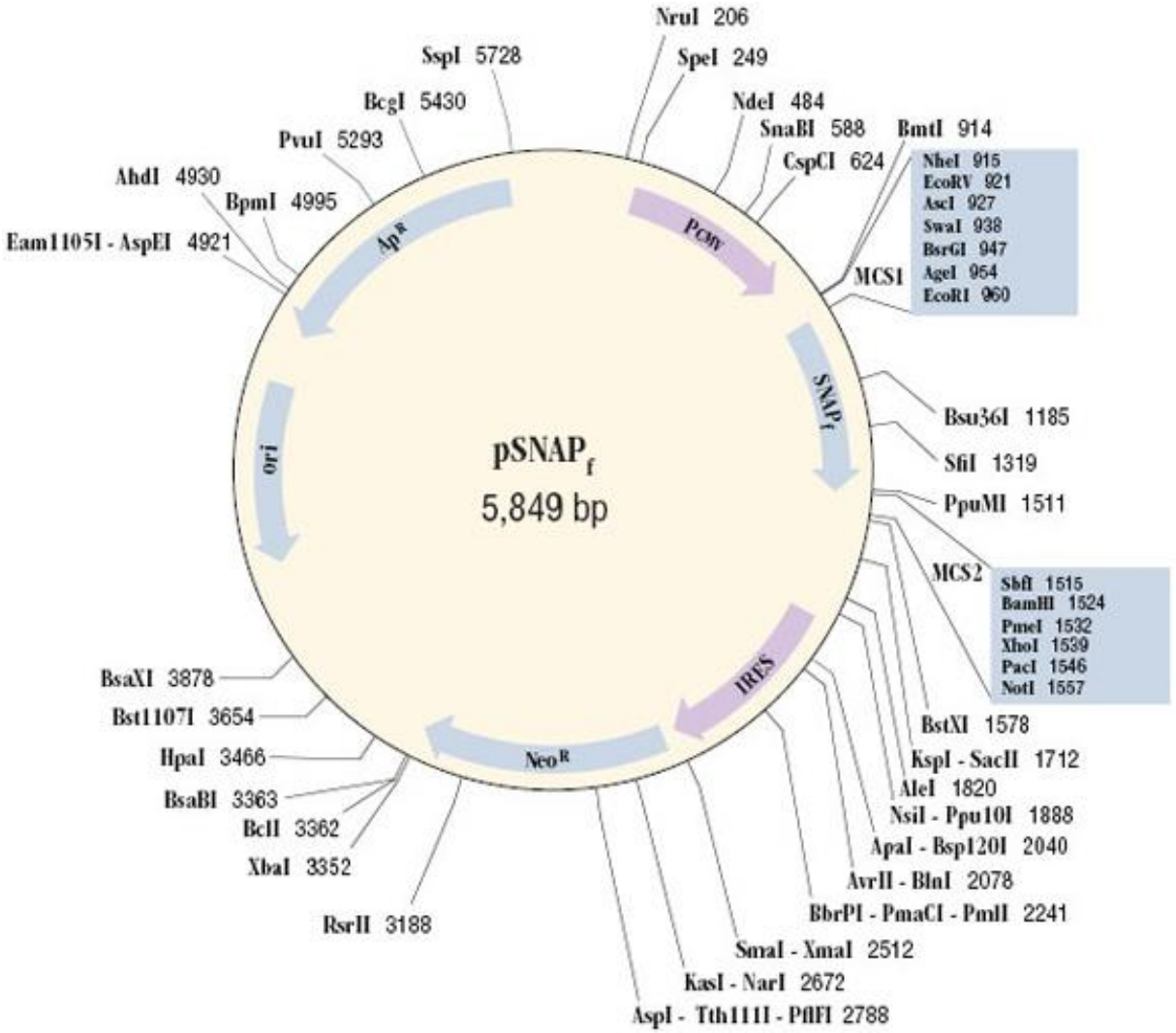


Expression Vectors:

Purpose: a subset of cloning vectors designed to clone DNA fragments and expression of the cloned gene in host cells.

Features: strong promoters, enhancers, transcription terminators, and translation initiation sites.

Advantages: producing proteins of interest in large quantities for research, pharmaceuticals, and biotechnology applications.



Overview of the steps

1. **Selection of DNA source**
   DNA fragment or gene of interest. Genomic DNA, cDNA, or synthetic DNA fragments.

2. **DNA fragmentation**
   The selected DNA may require to be fragmented into smaller pieces using restriction enzymes. Usually, these enzymes generate DNA fragments with sticky ends that are compatible with other fragments cut by the same enzyme.

3. **DNA vector selection**
   Vectors have features like origin of replication, selectable markers, and cloning sites.

Overview of the steps

4. **Ligation**
   The DNA fragment and vector are joined using DNA ligase, creating recombinant DNA molecules.

5. **Transformation**
   The recombinant DNA molecules are introduced into host cells.

6. **Selection and screening**
   Selectable markers within the vector, such as antibiotic resistance genes, are used to identify cells that have successfully incorporated the DNA of interest.

Overview of the steps

7. **Amplification**
   The transformed cells are allowed to grow and multiply. As the host cells divide, the recombinant DNA also replicates, leading to the production of multiple copies of the cloned DNA fragment.

8. **DNA extraction**
   Once transformed cells have been grown, the cloned DNA can be extracted from the cells. Plasmid extraction.

PCR cloning considerations

- **Nature of the Insert:** not all PCR fragments will clone with the same efficiency into the same vector.

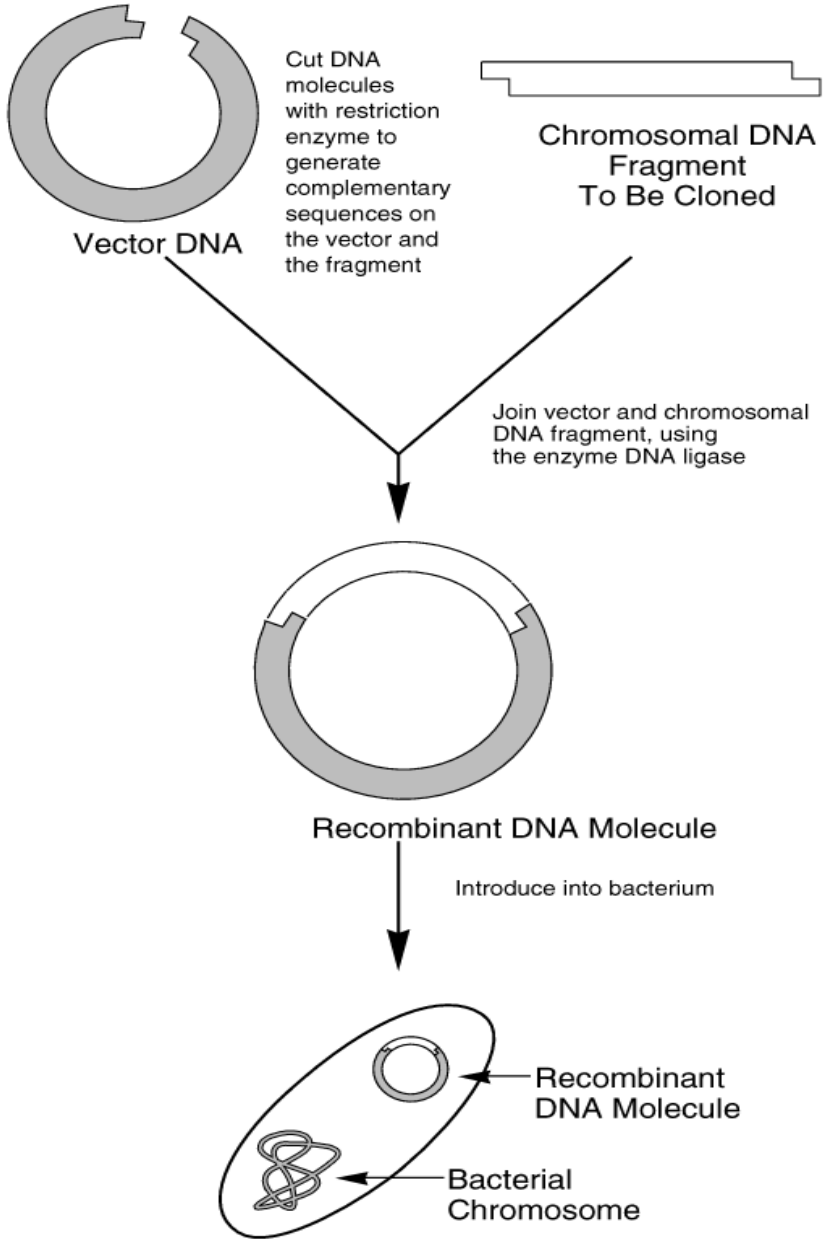
- **Insert size:** Large fragments of DNA (≥ 5 kb) are amenable to cloning in high-copy number vectors, yet at a much lower efficiency.

- **Vector-to-insert ratio:** Optimization of molar concentration ratios of the vector to insert is critical to ensure efficient cloning. Vector to insert ratio of 1 to 3 is common.

https://nebiocalculator.neb.com/#!/ligation

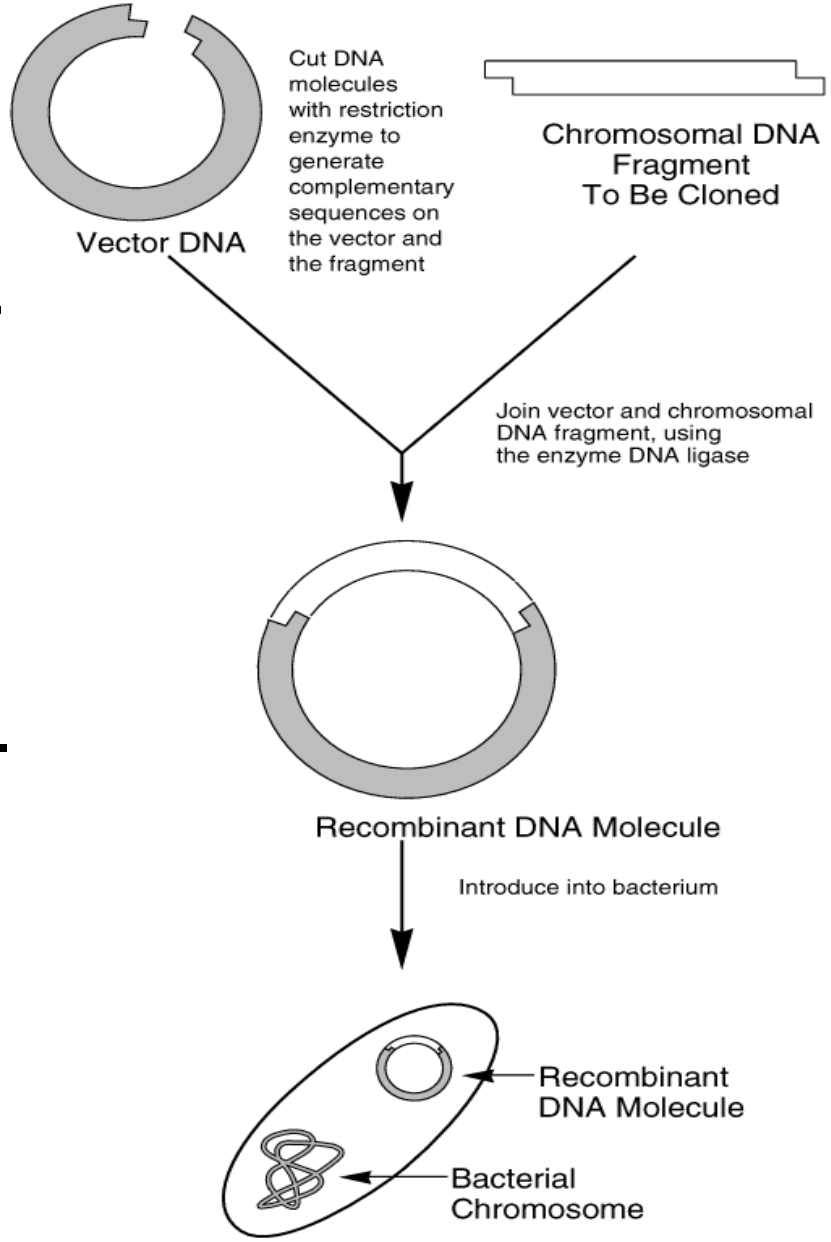
A brief cloning process

- Gene of interest is cut out with RE.
- Host plasmid is cut with same RE.
- Gene is inserted into plasmid and ligated with ligase.
- New plasmid inserted into bacterium (transform).

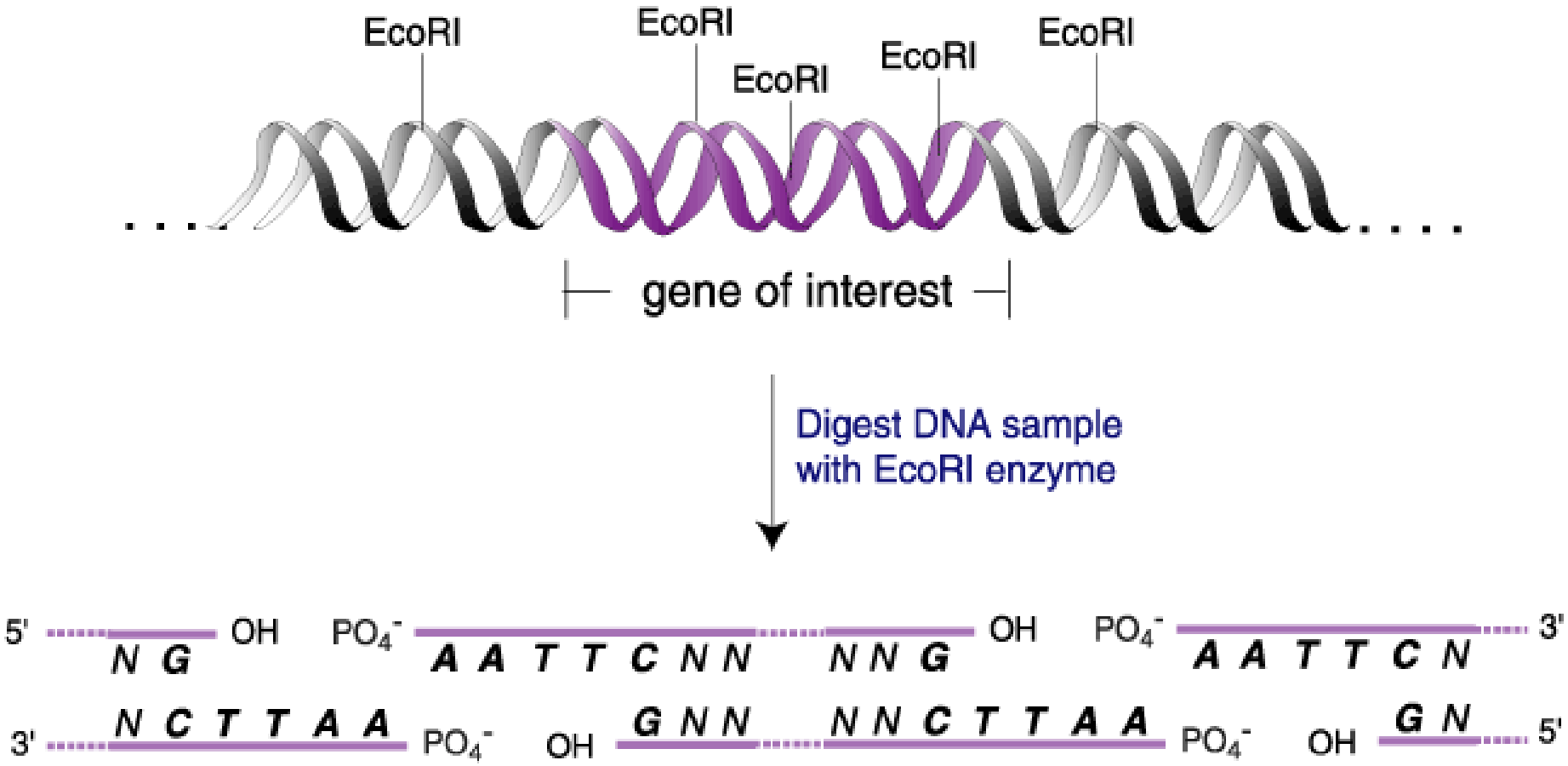


A brief cloning process

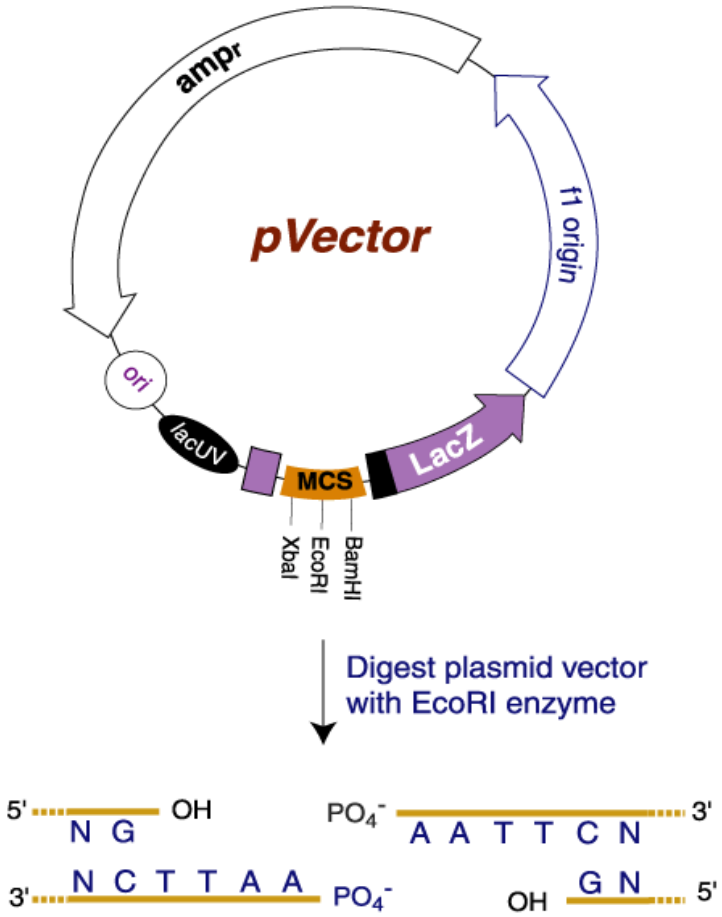
1. Restriction Enzyme digest of DNA sample.
2. Restriction Enzyme digest of DNA plasmid vector.
3. **Ligation** of DNA sample products and plasmid vector.
4. **Transformation** with the ligation products.
5. **Growth** on agar plates with selection for antibiotic resistance.



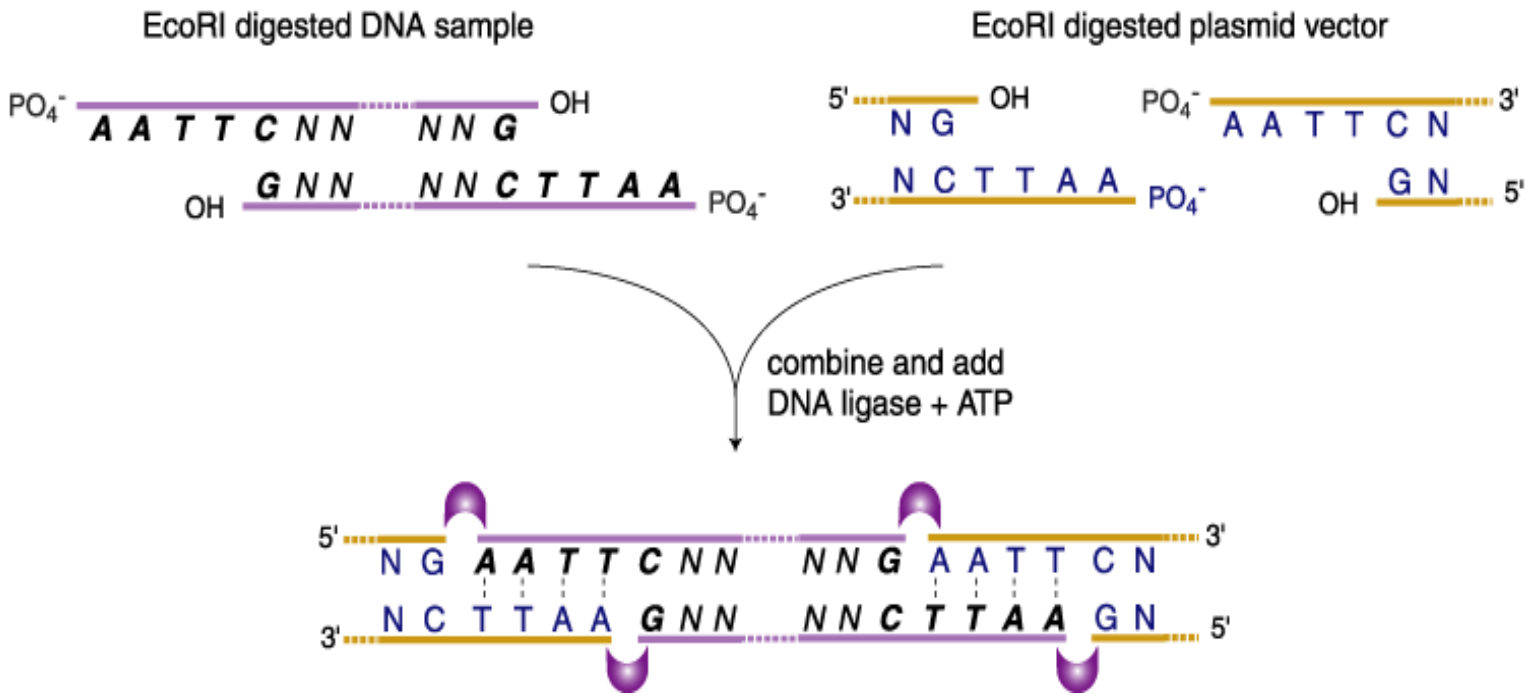
Step 1
RE digestion of DNA sample



Step 2
RE digestion of plasmid DNA



Step 3
Ligation of DNA sample and plasmid DNA

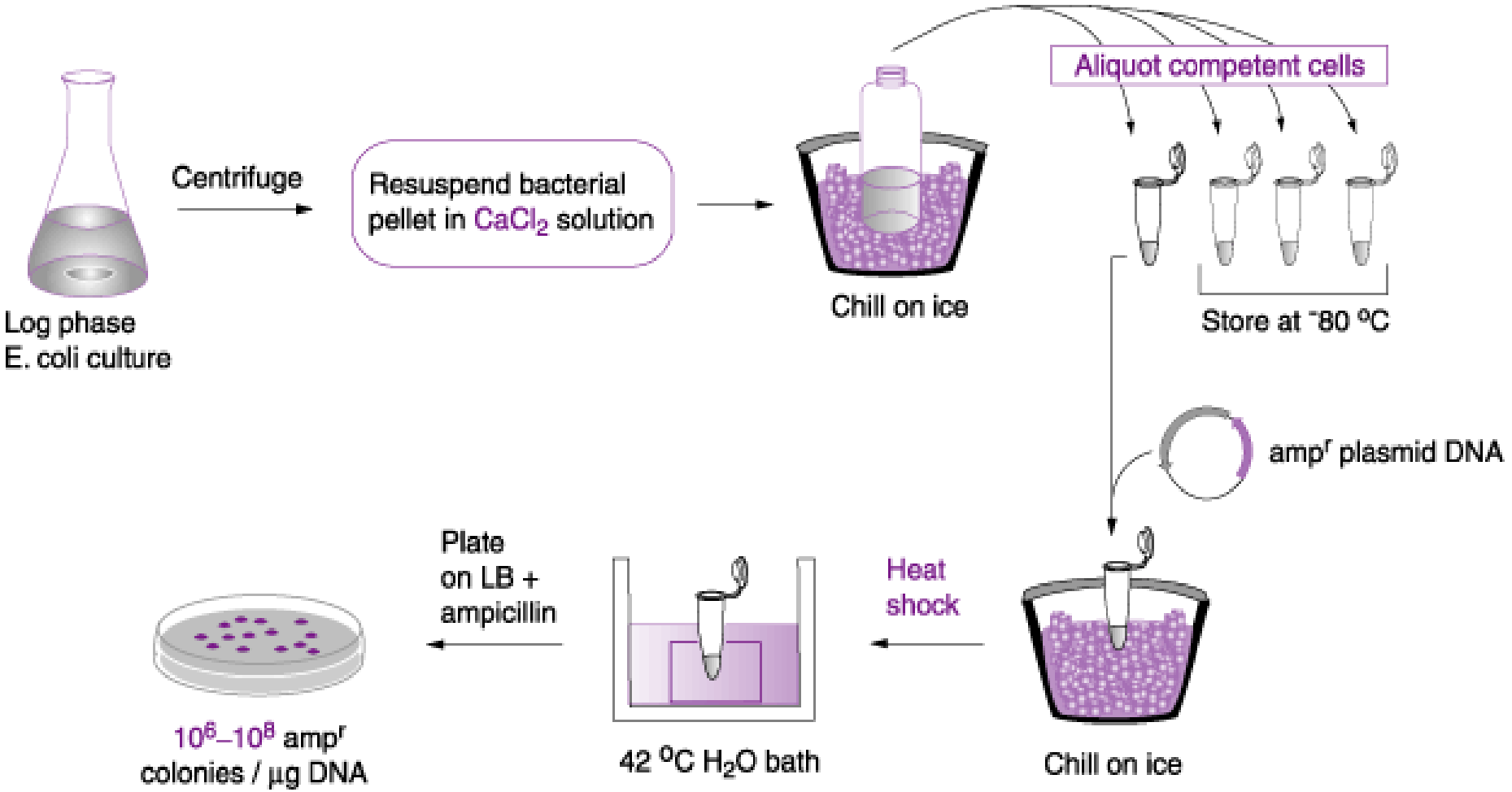


Step 4
Transformation of ligation products

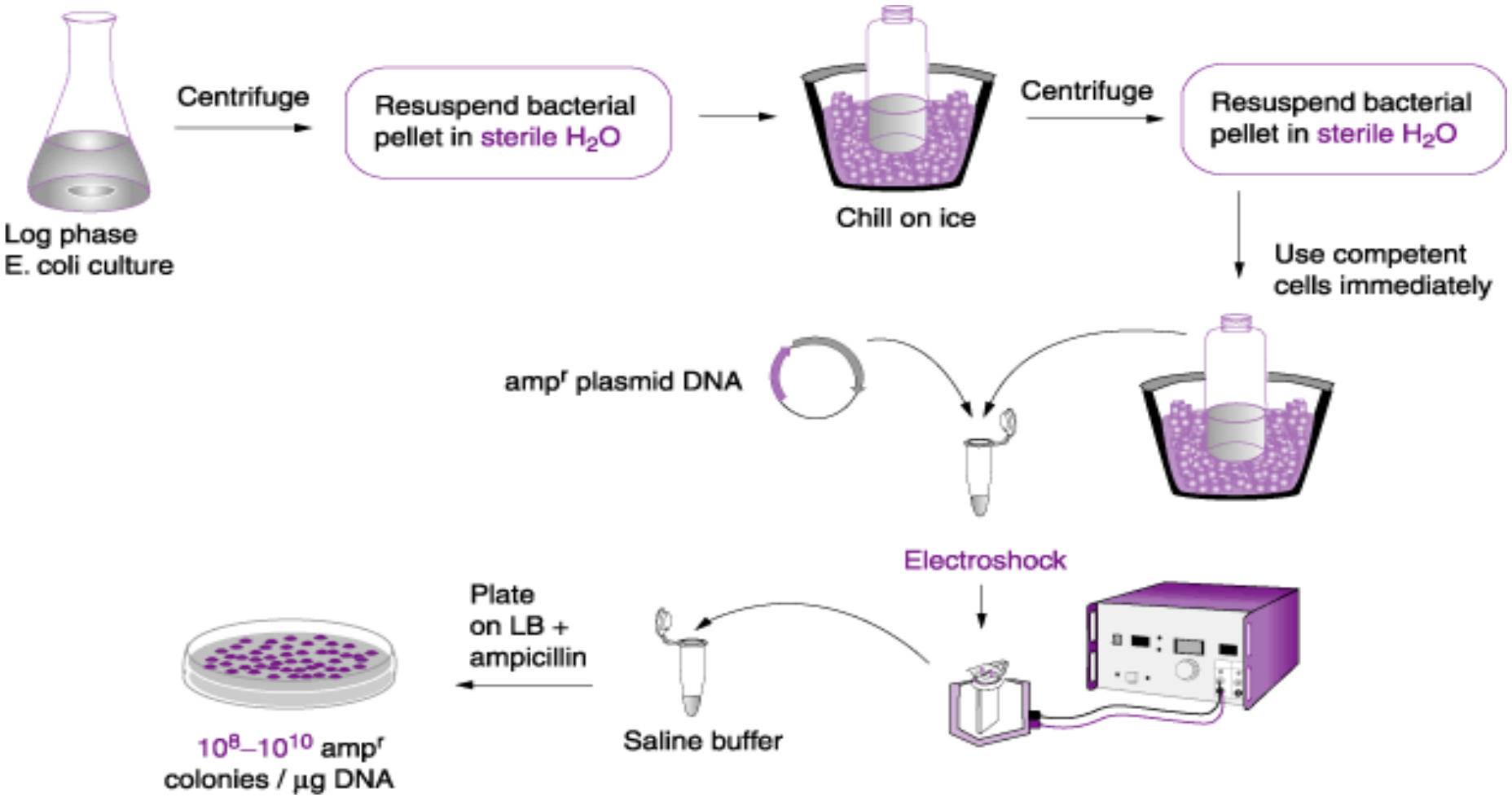
- The process of transferring exogenous DNA into cells is call “**transformation**”.

- There are basically two general methods for transforming bacteria.
  - **Chemical** method utilizing $CaCl_2$ and heat shock to promote DNA entry into cells.
  - Electroporation, based on a short pulse of electric charge to facilitate DNA uptake.

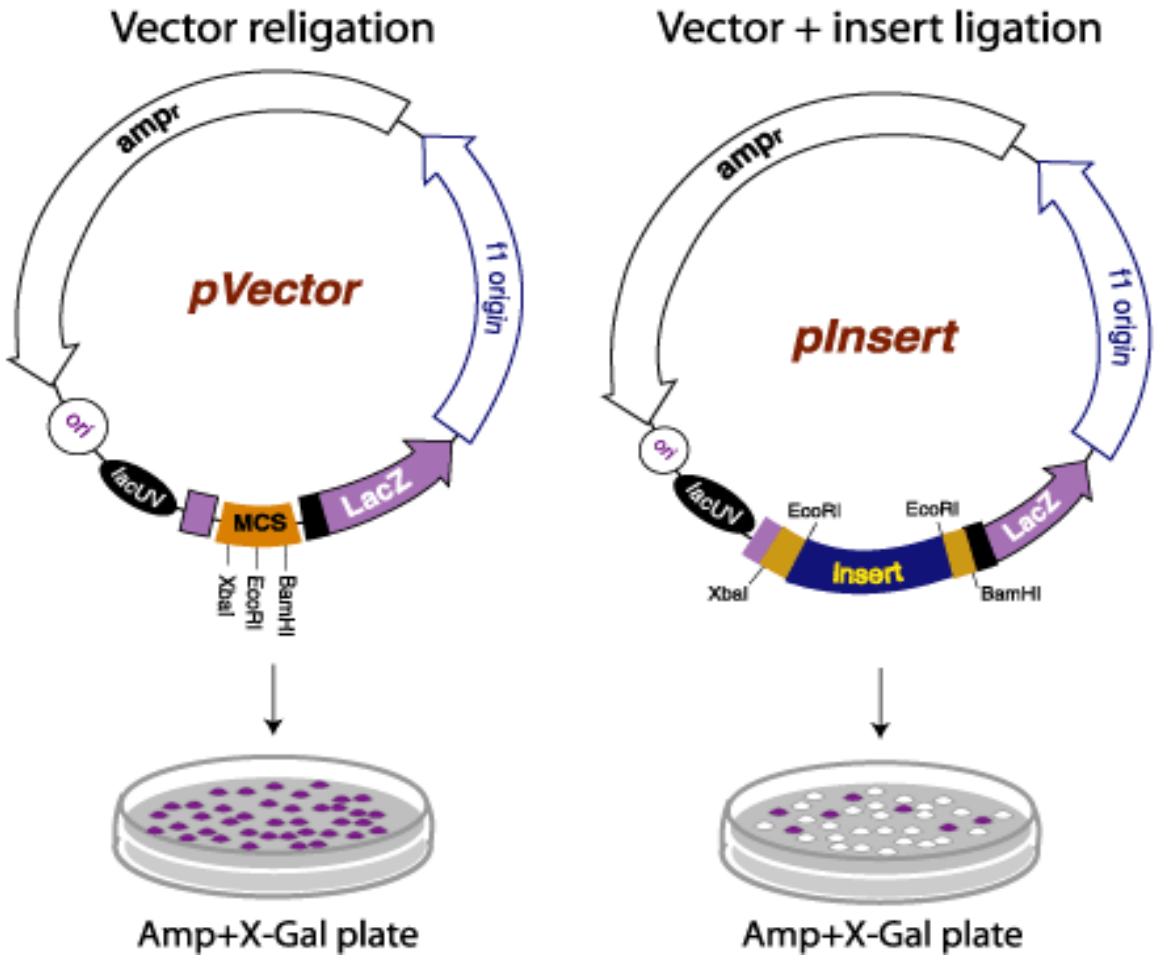
Chemical transformation with calcium chloride

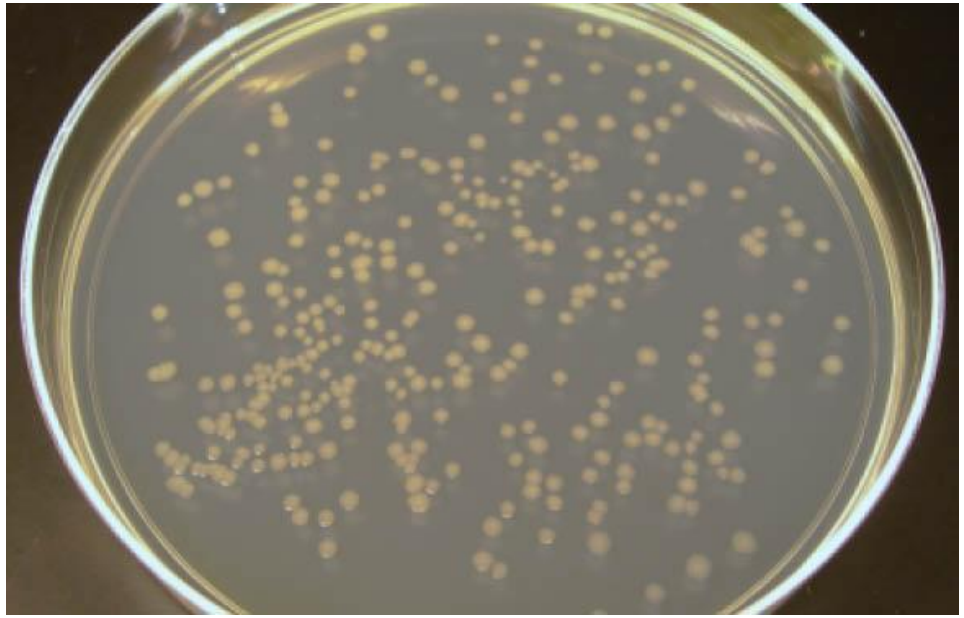


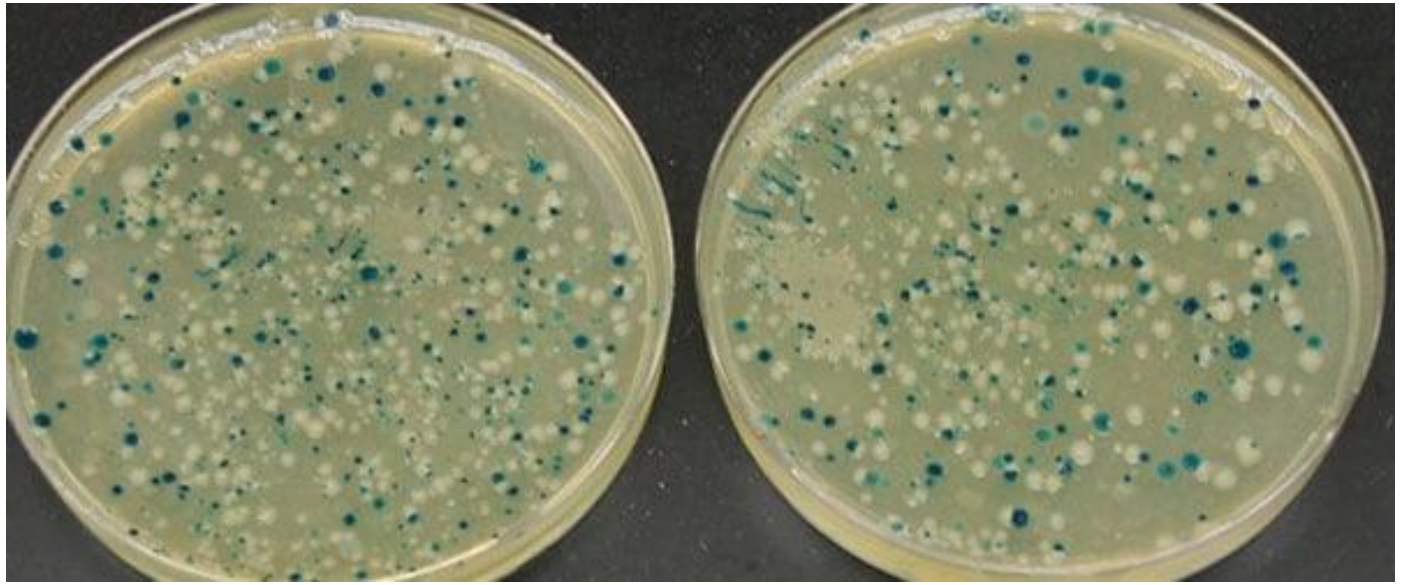
Transformation by electroporation



Step 5
Growth on agar plates







Blue-white screening

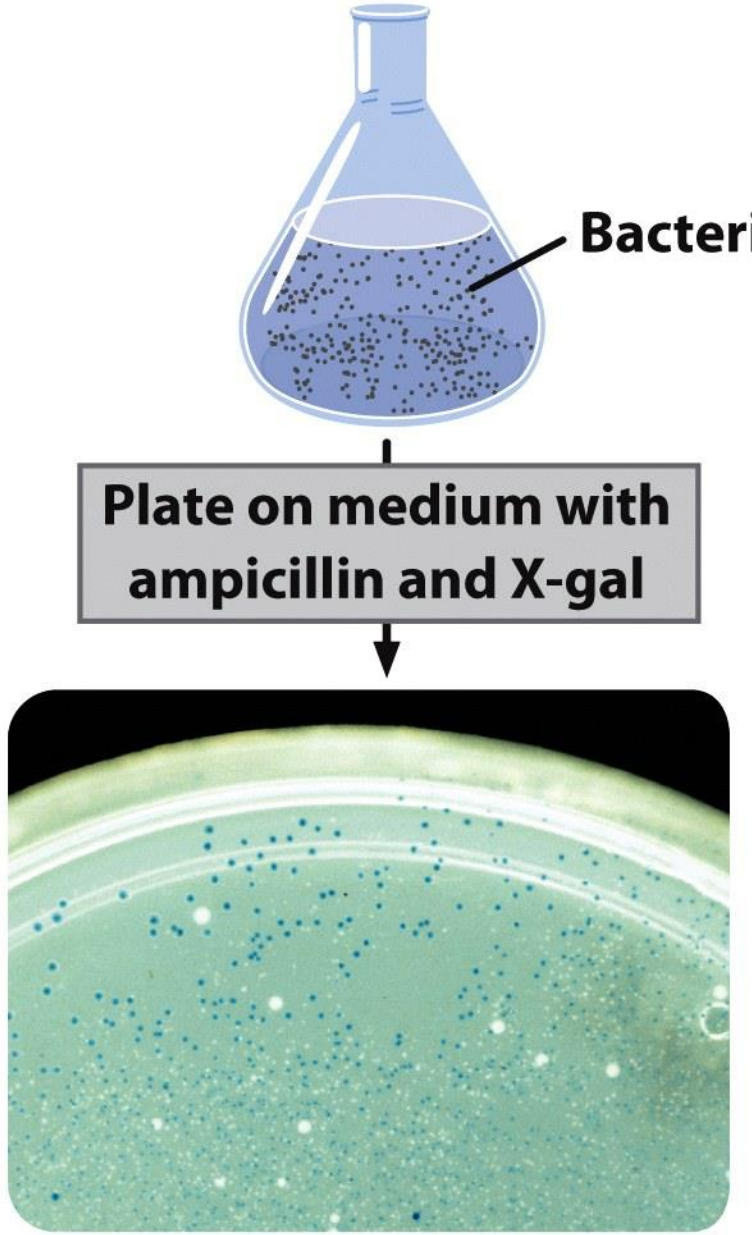


Figure 19-8 part 2
Genetics: A Conceptual Approach, Third Edition

Conclusion: A white colony consists of bacteria carrying a recombinant plasmid.

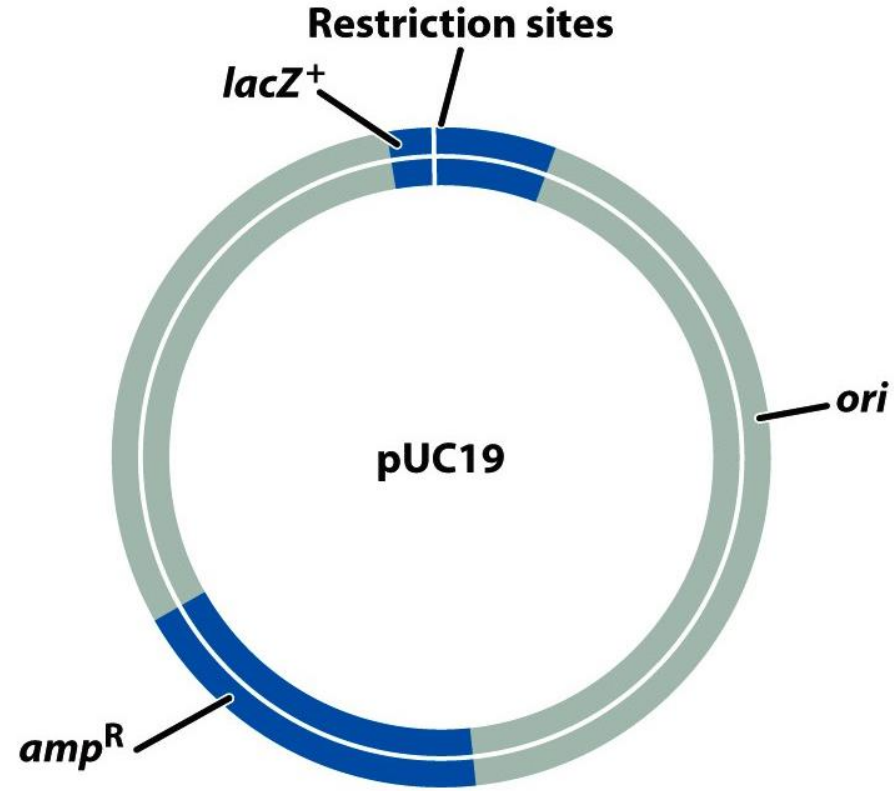
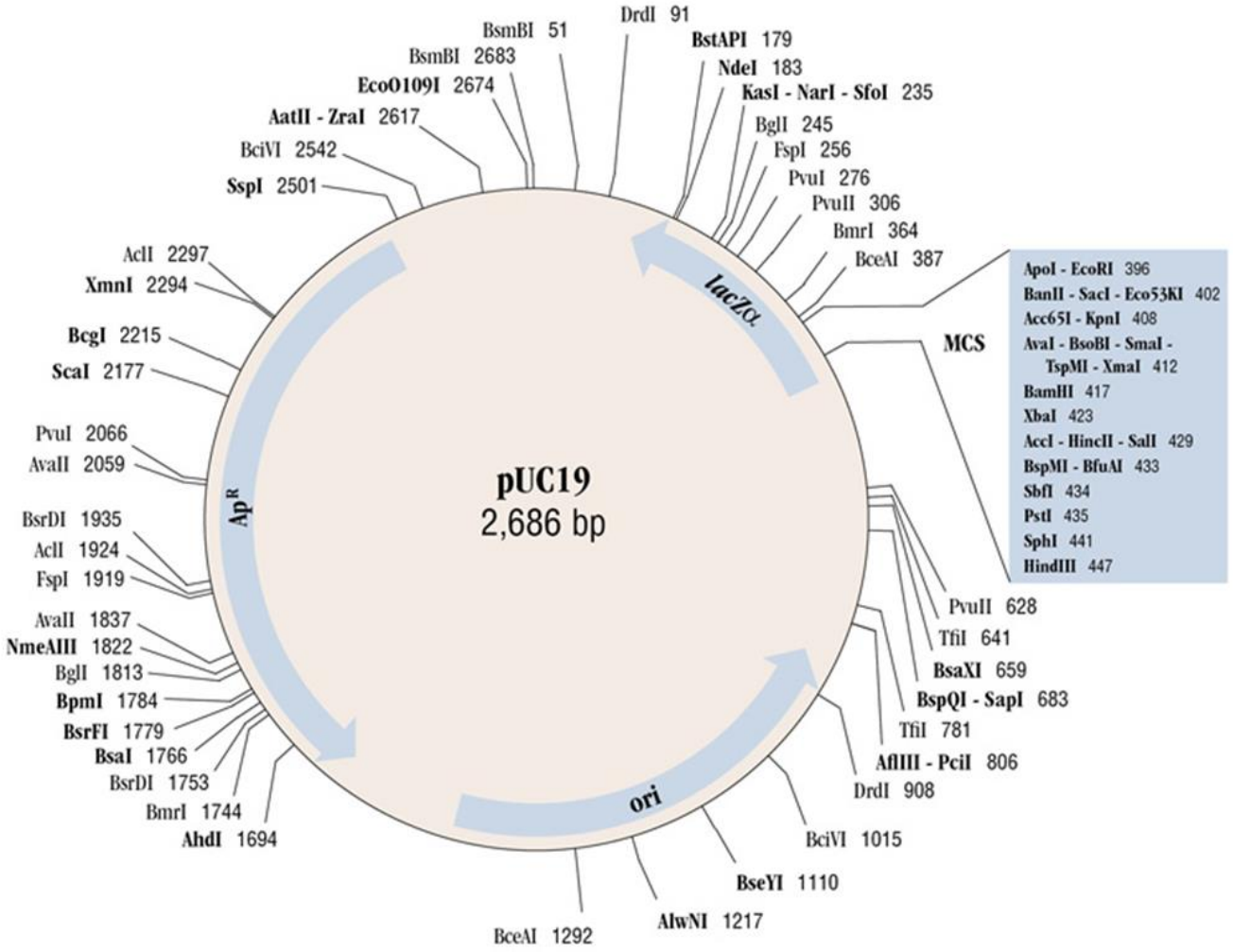


Figure 19-6
Genetics: A Conceptual Approach, Third Edition

- **X-gal** is used to test activity of the enzyme β-galactosidase.
  - an analogue of lactose.

- **IPTG** is an inducer enzyme for X-Gal

Autoclaved LB agar
+ X-Gal
+ IPTG



No insert > *lacZ* expressed > enzyme > blue colony

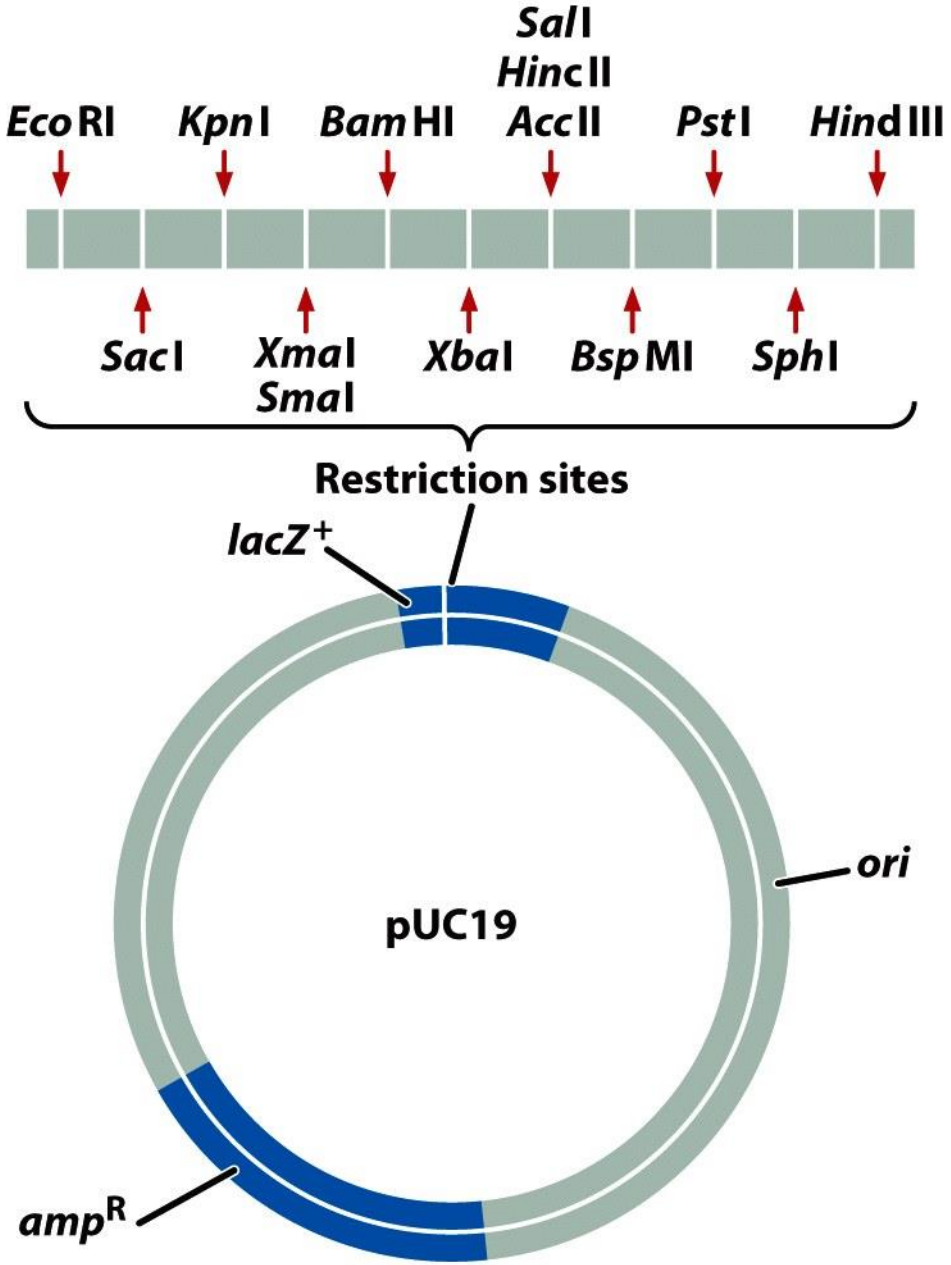
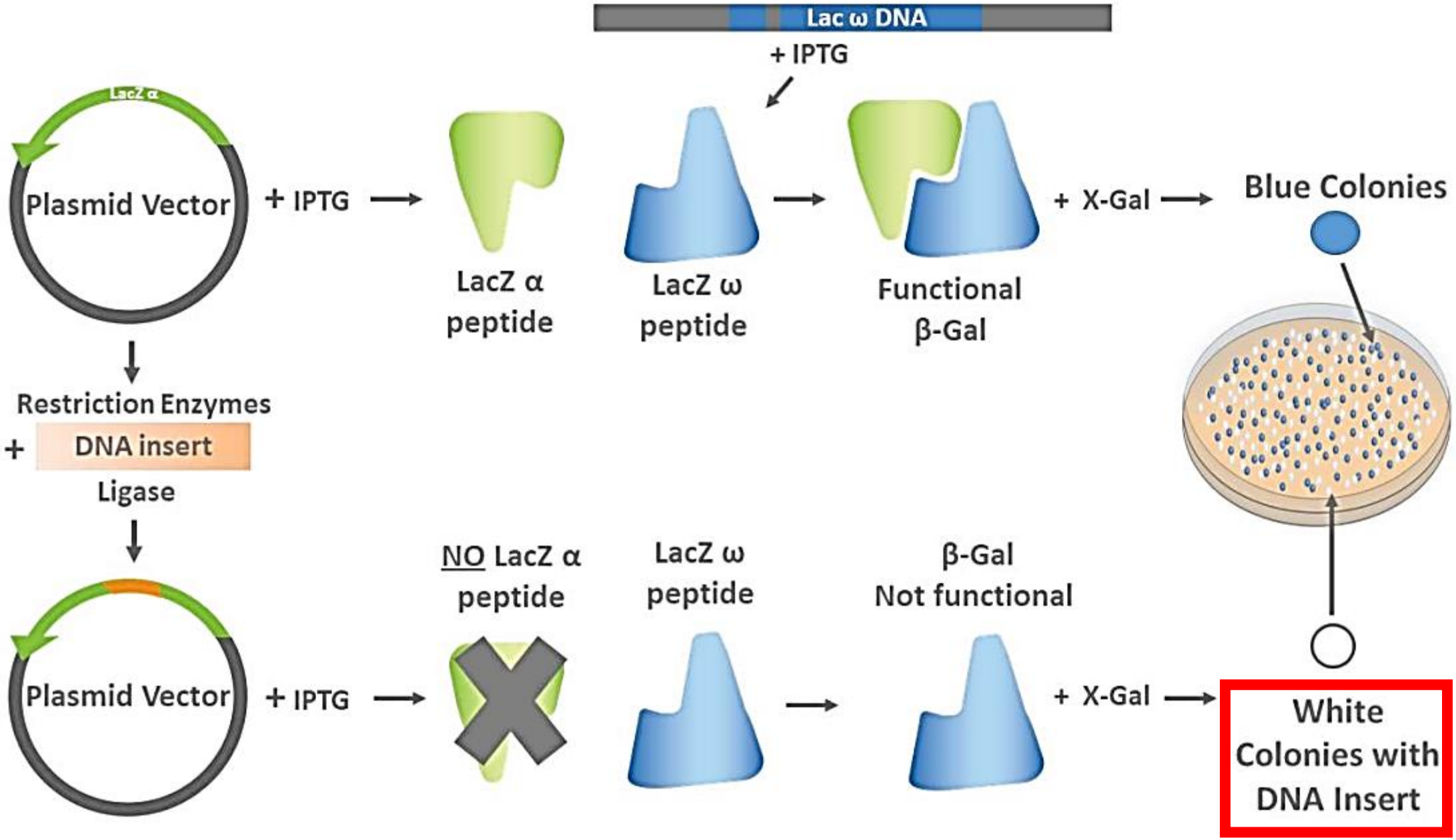


Figure 19-6
Genetics: A Conceptual Approach, Third Edition


Lac ω DNA
+ IPTG
LacZ α
Plasmid Vector
+ IPTG
LacZ α peptide
LacZ ω peptide
Functional β-Gal
+ X-Gal
Blue Colonies
Restriction Enzymes
+ DNA insert
Ligase
NO LacZ α peptide
LacZ ω peptide
β-Gal Not functional
Plasmid Vector
+ IPTG
+ X-Gal
White Colonies with DNA Insert

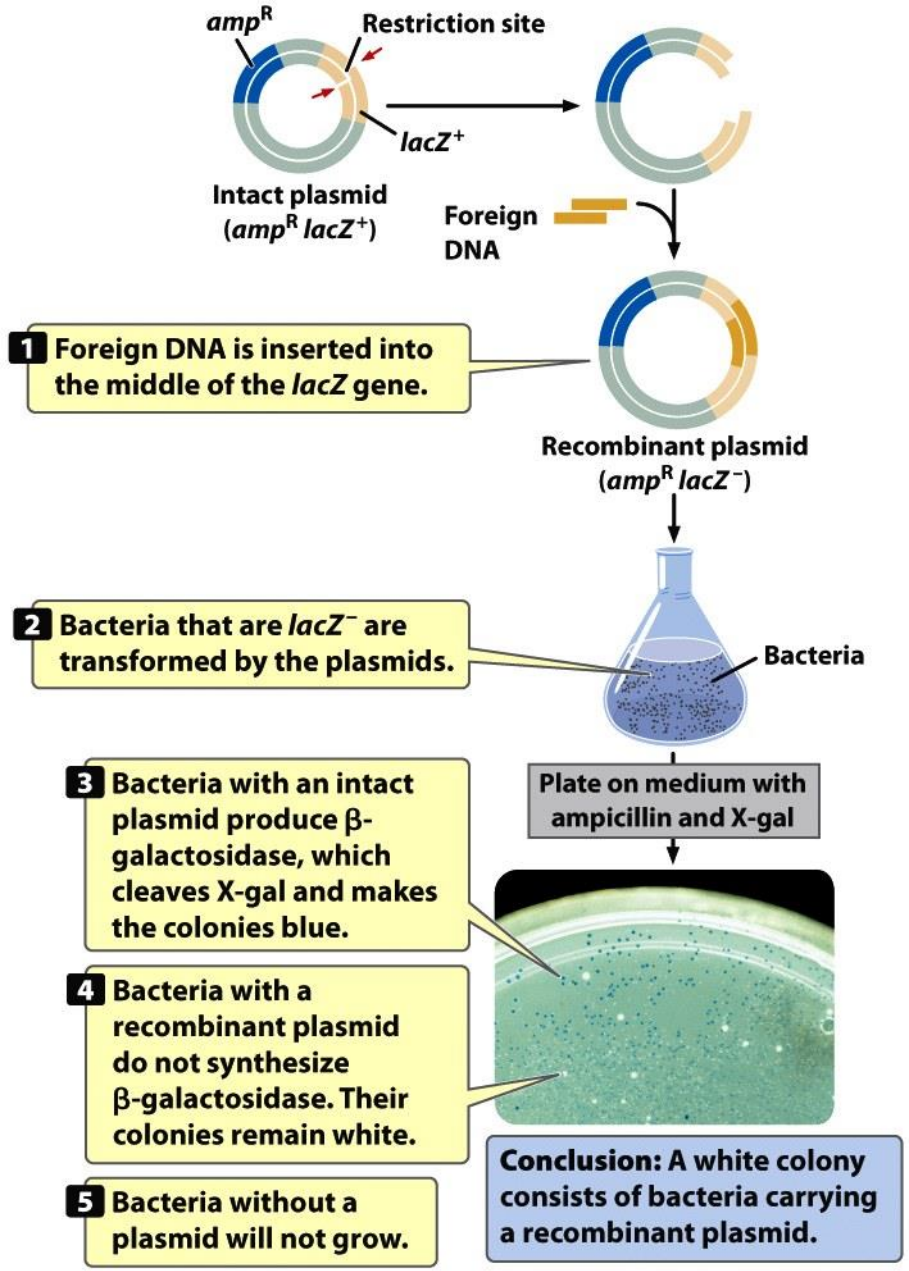


Figure 19-8
Genetics: A Conceptual Approach, Third Edition

Terms used in cloning

- **DNA recombination:** The DNA fragment to be cloned is inserted into a vector.
- **Transformation:** The recombinant DNA enters into the host cell and proliferates.
- **Selective amplification**
- **Isolation of desired DNA clones**

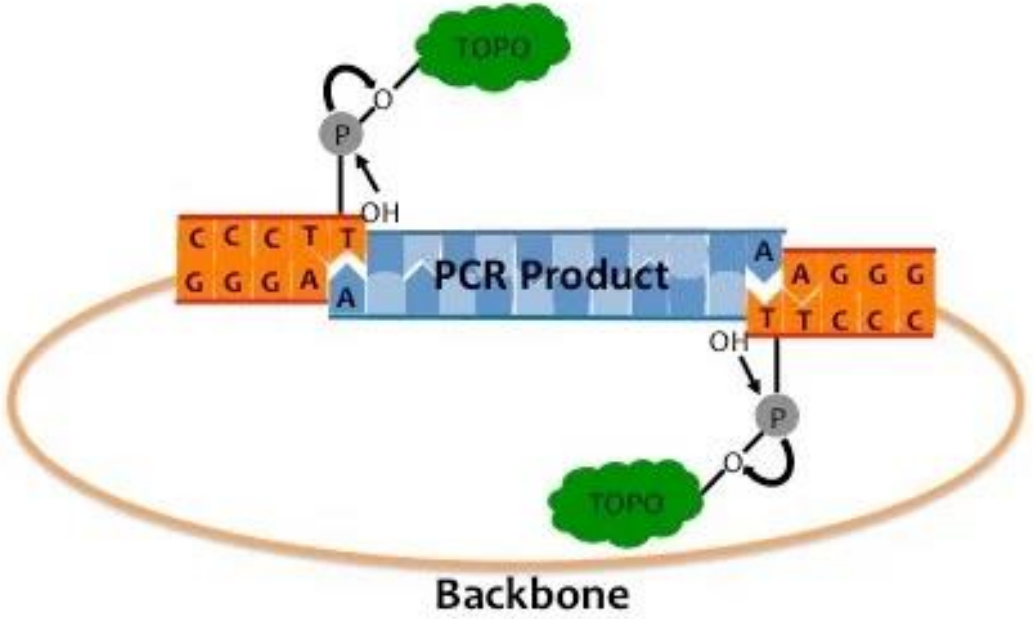
PCR cloning strategies

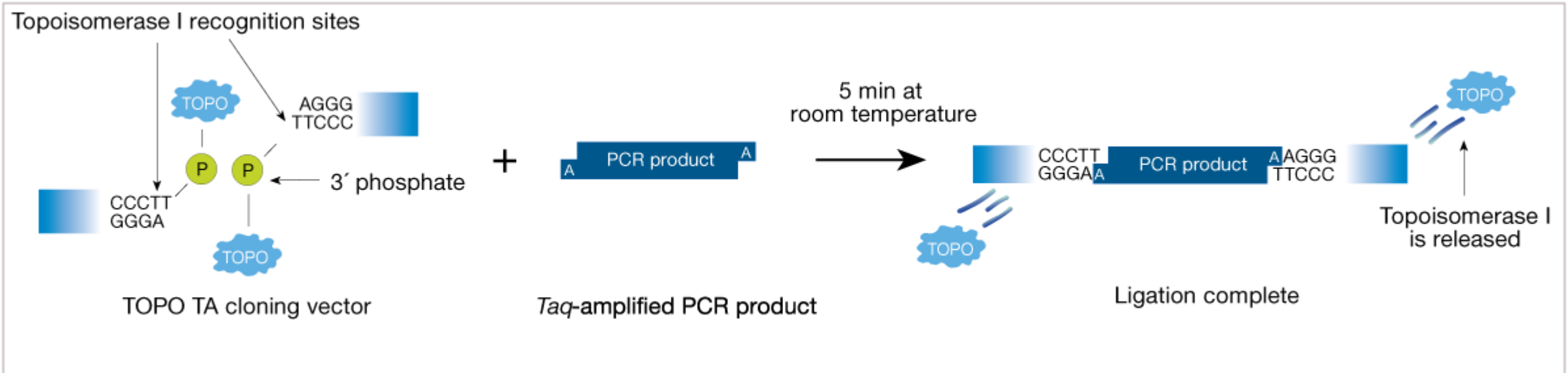
Cloning methods for PCR products are divided into three types:

- Blunt-end cloning
- Sticky-end cloning
- TA cloning

TA Cloning

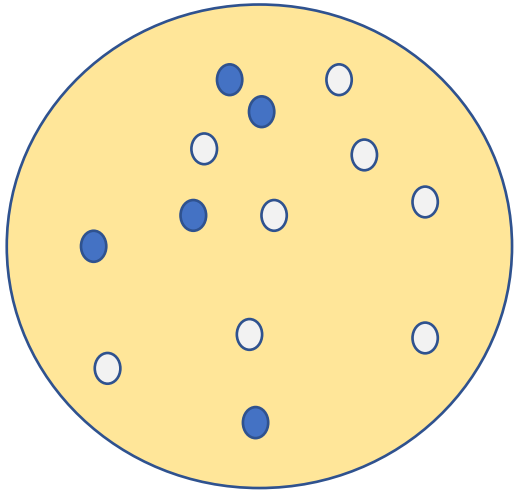
- When DNA fragments are generated, *Taq* polymerase adds 1 or 2 extra adenines onto the end of 3’ end of blunt dsDNA.
- Use a plasmid vector with thymidine residues linked onto the 3’ ends of linearized plasmid DNA.





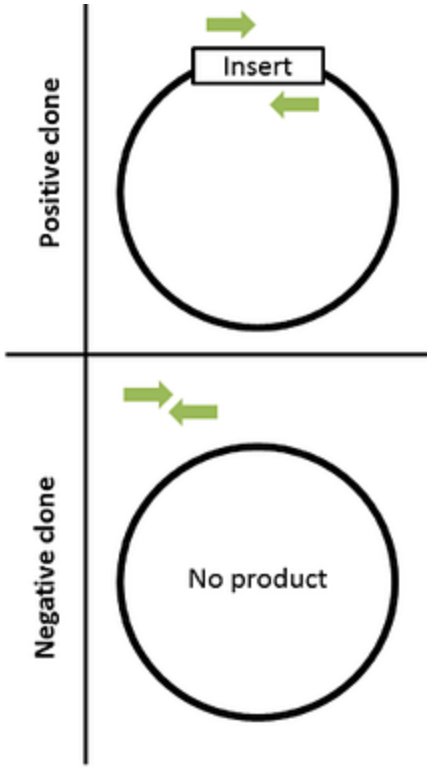
Analysis of cloned DNA

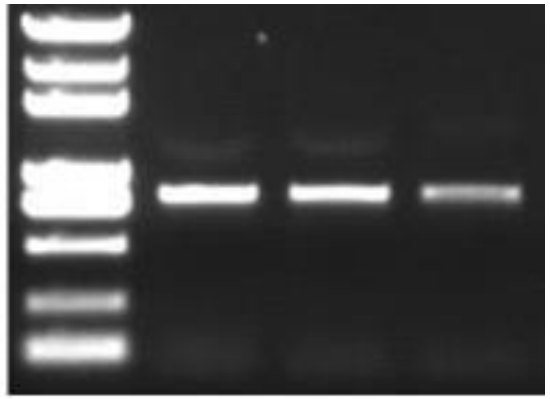
- Is it the one you wanted?
- What are its molecular characteristics?
- **Gel electrophoresis:** separates DNA fragments by molecular weight
- **DNA sequencing:** provides *complete order of bases* in a DNA fragment
- **Colony PCR**



H2O + cells
95 ˚C 5 min

Use the solution as template for PCR with gene specific primers



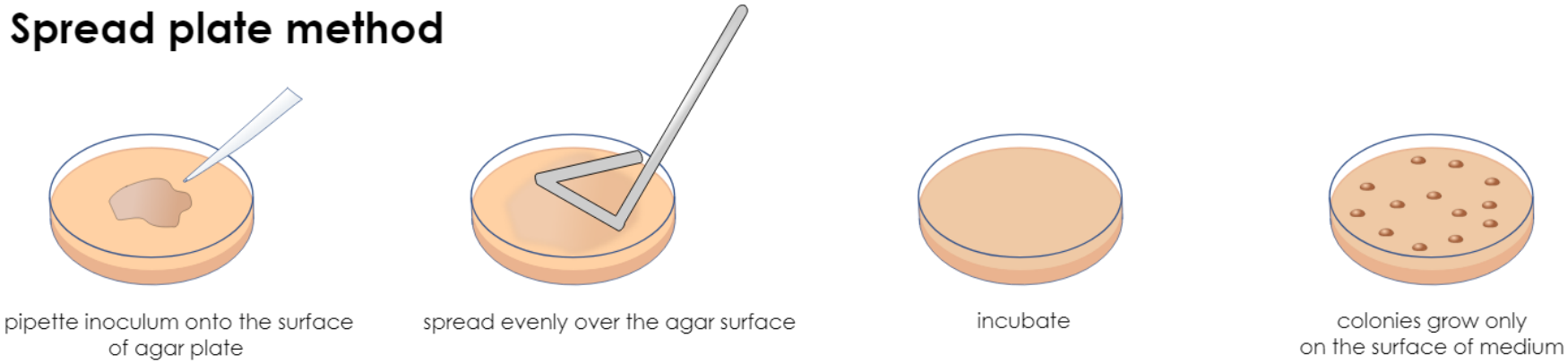


Aseptic technique
Good microbiology techniques

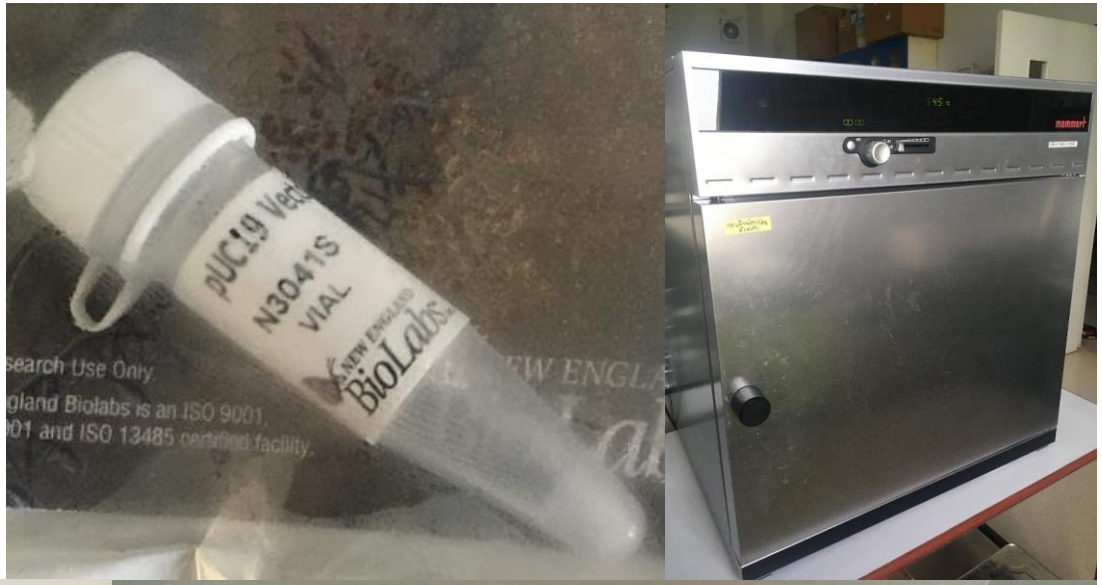
Pour plates

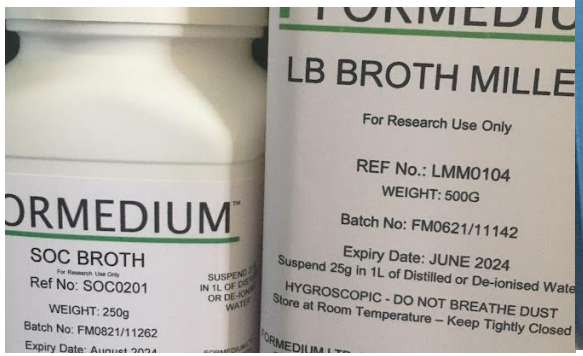
Spread plate

…



- PCR products
- Cloning vector
- DNA ligase (T4)
- Competent cells *E. coli*
- LB agar plates + X-Gal + IPTG
- SOC broth/LB broth
- Shaker
- Incubator
- Water bath





LB agar plates

- Autoclave **LB agar**, cool down
- For 1 ml LB: Add 0.2 mg **X-Gal** + 1 mM **IPTG** + antibiotic, mix
- Pour plate
- Plate can be stored at 4 °C, remember to **warm** the plate at 37 °C before use.
- Spread the transformed competent cells (better to split into 2 different volume, e.g. 100 and 50 µl)

Protocol

1. Clean your PCR products (from gel, clean with column, do not use enzymes)
2. Thor competent cells **on ice**

Protocol

3. Ligate cloning vector with your PCR product
    - **Vector:insert = 1:3**

      PCR product 524 ng/µl, 498 bp

      pTZ57R/T vector size = 2886 bp

      • Want to use 165 ng vector (=3 µl)

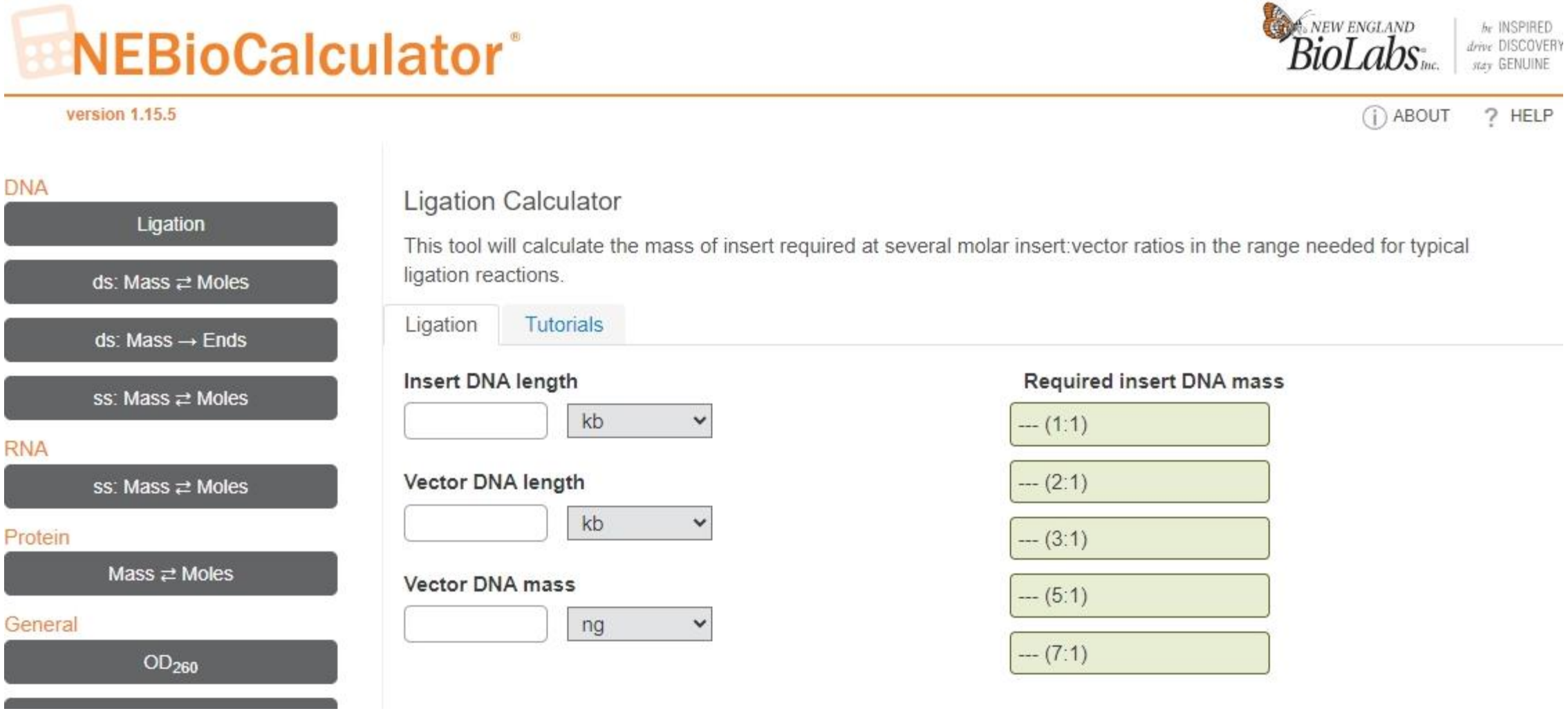
      • Use calculator

      https://nebiocalculator.neb.com/#!/ligation

      Use 85.4 ng PCR product, = 0.16 µl

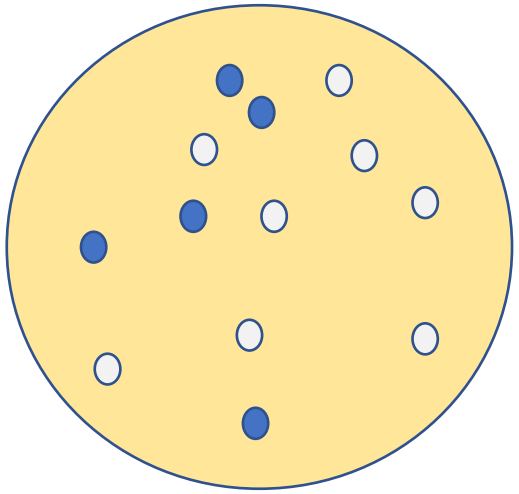
    - Ligate overnight (16-18 hours)

Protocol

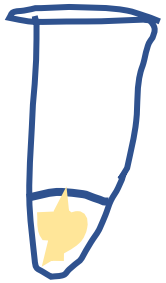

NEBioCalculator®
version 1.15.5
NEW ENGLAND BioLabs Inc.
be INSPIRED drive DISCOVERY stay GENUINE
ABOUT
HELP
DNA
Ligation
ds: Mass ⇄ Moles
ds: Mass → Ends
ss: Mass ⇄ Moles
RNA
ss: Mass ⇄ Moles
Protein
Mass ⇄ Moles
General
OD260
Ligation Calculator
This tool will calculate the mass of insert required at several molar insert:vector ratios in the range needed for typical ligation reactions.
Ligation
Tutorials
Insert DNA length
kb
Vector DNA length
kb
Vector DNA mass
ng
Required insert DNA mass
--- (1:1)
--- (2:1)
--- (3:1)
--- (5:1)
--- (7:1)

Protocol

4. 50 µl competent cells + 2.5 µl ligation mixture
5. On ice for 20 min
6. 42 °C 50 sec
7. On ice for 5 min
8. Add 900 µl SOC medium
9. Incubate at 37 °C for an hour, shake
10. Spread plates (split into 2 different volumes e.g. 50 and 100 µl)
11. Grow overnight
12. Collect white colonies, **colony PCR**

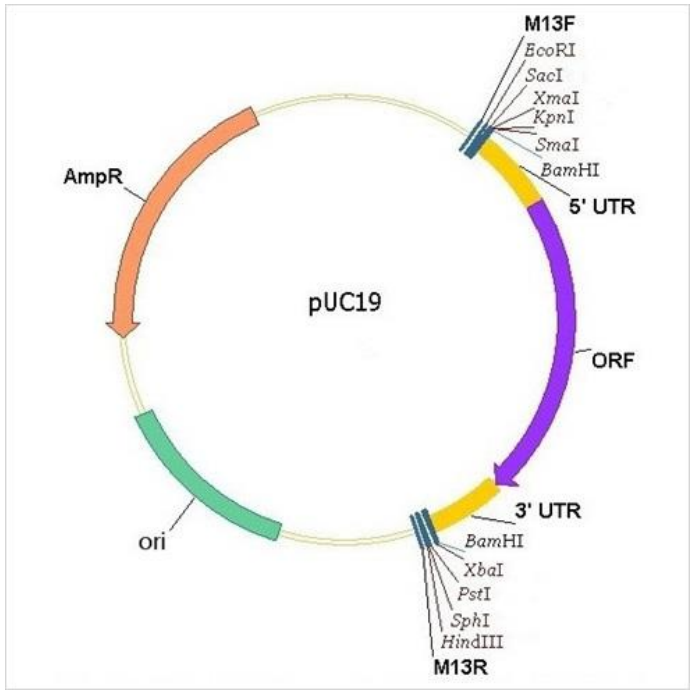


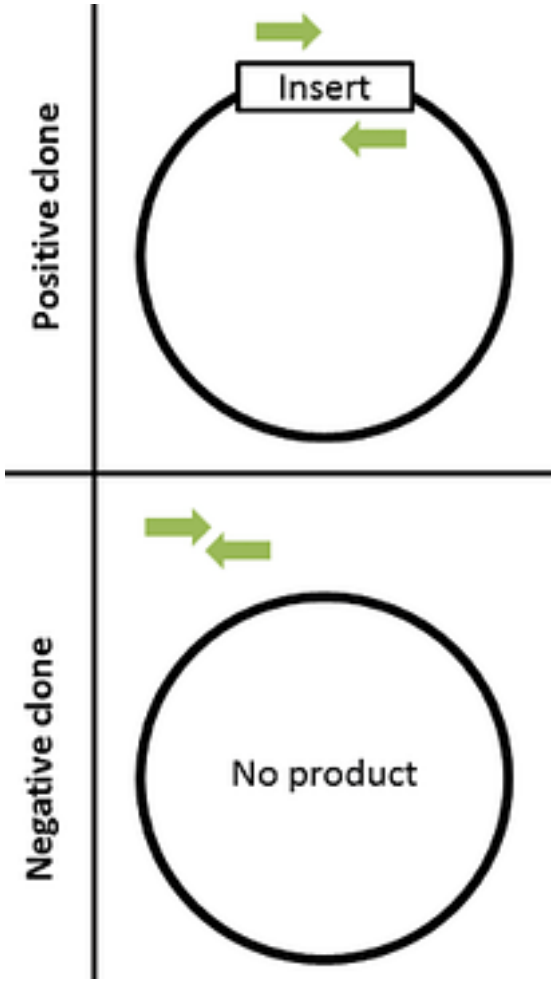
Grow the rest of the cells in new LB broth, grow O/N, for plasmid extraction

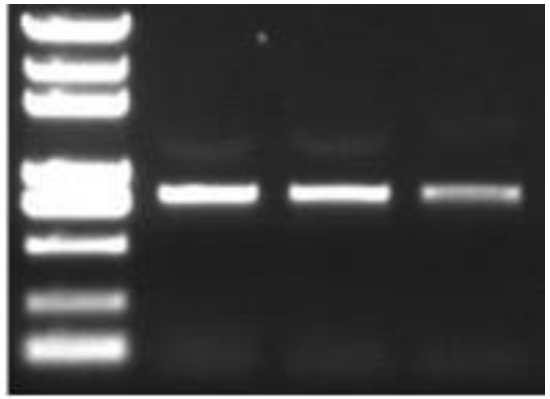


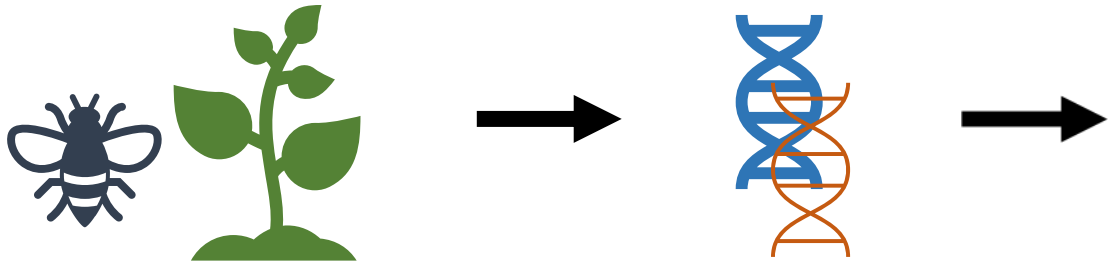
H_2O + cells
95 ˚C, 5 min

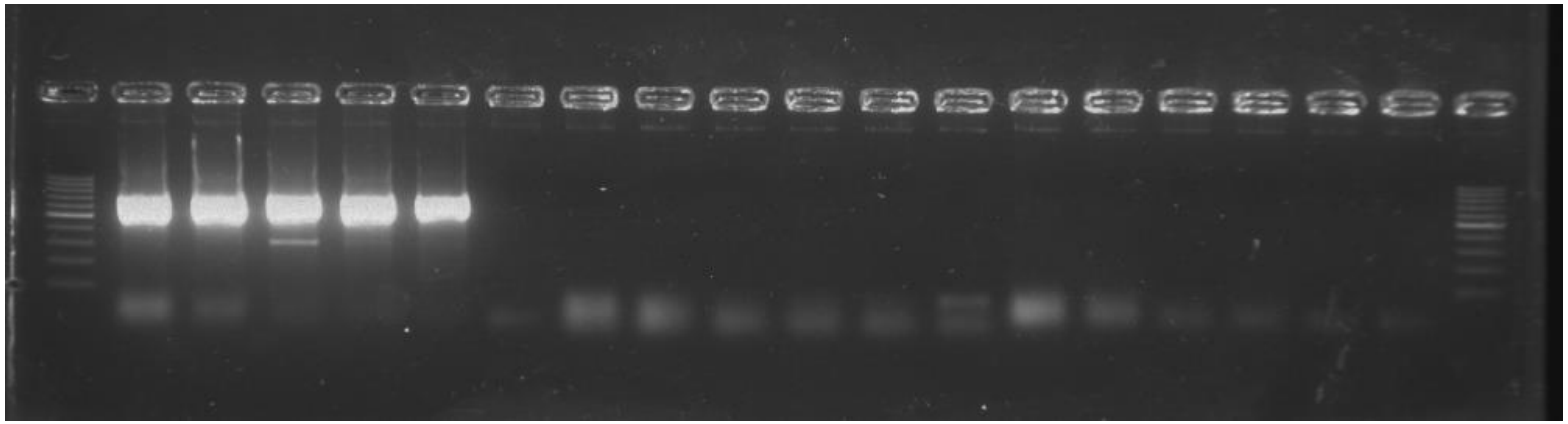
Use the solution as template for PCR with gene specific primers





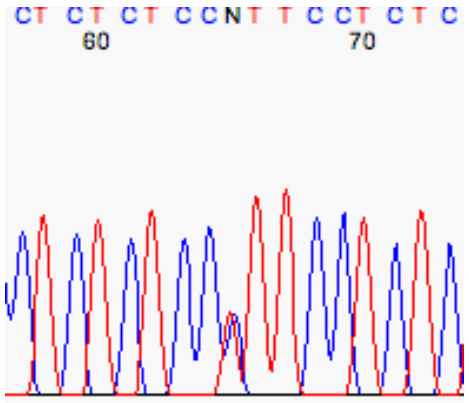


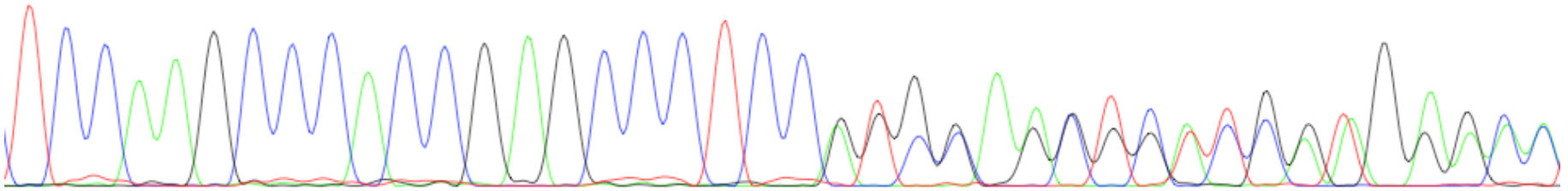




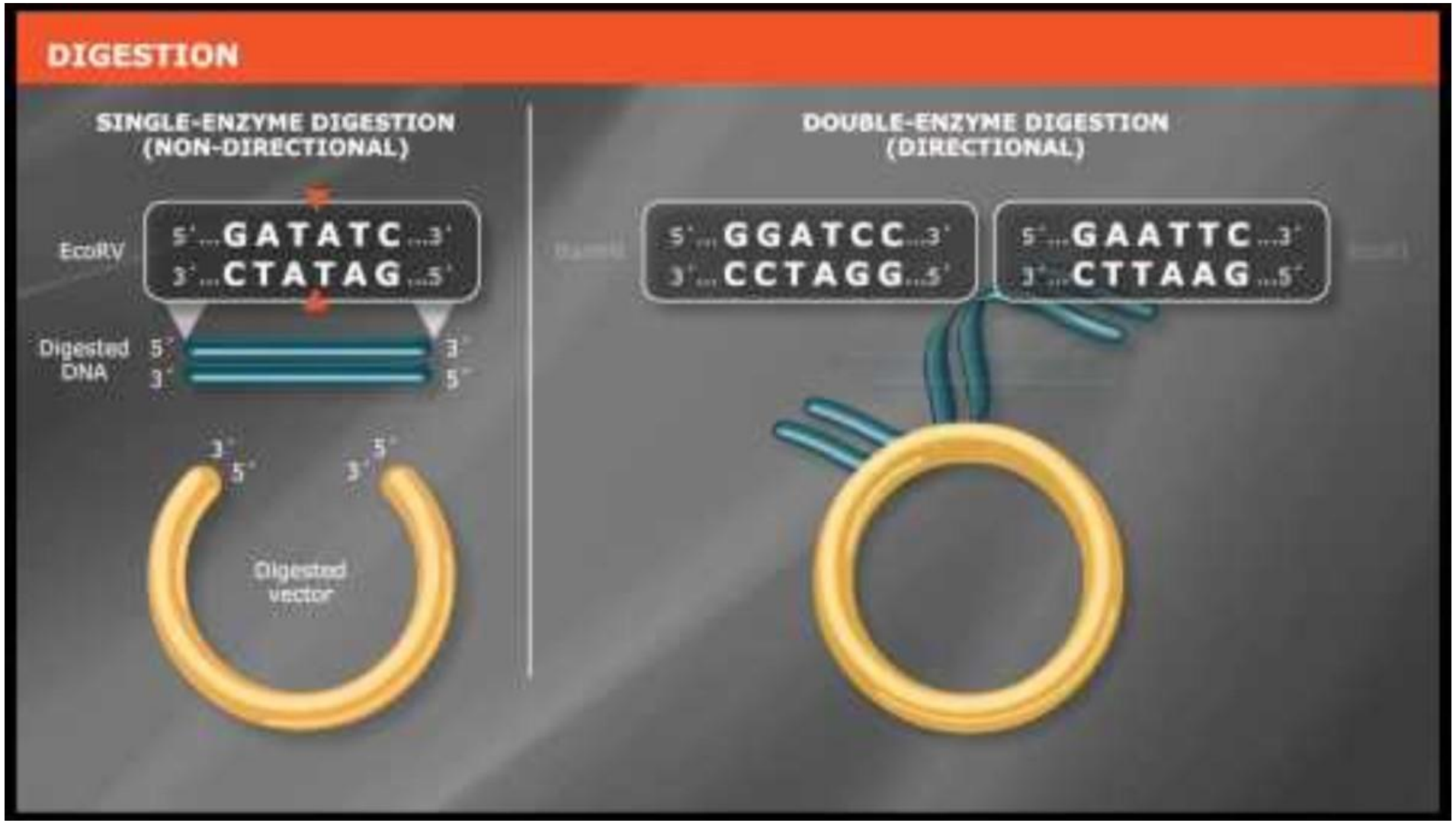
Nice. Thick bands 😇 😇

but



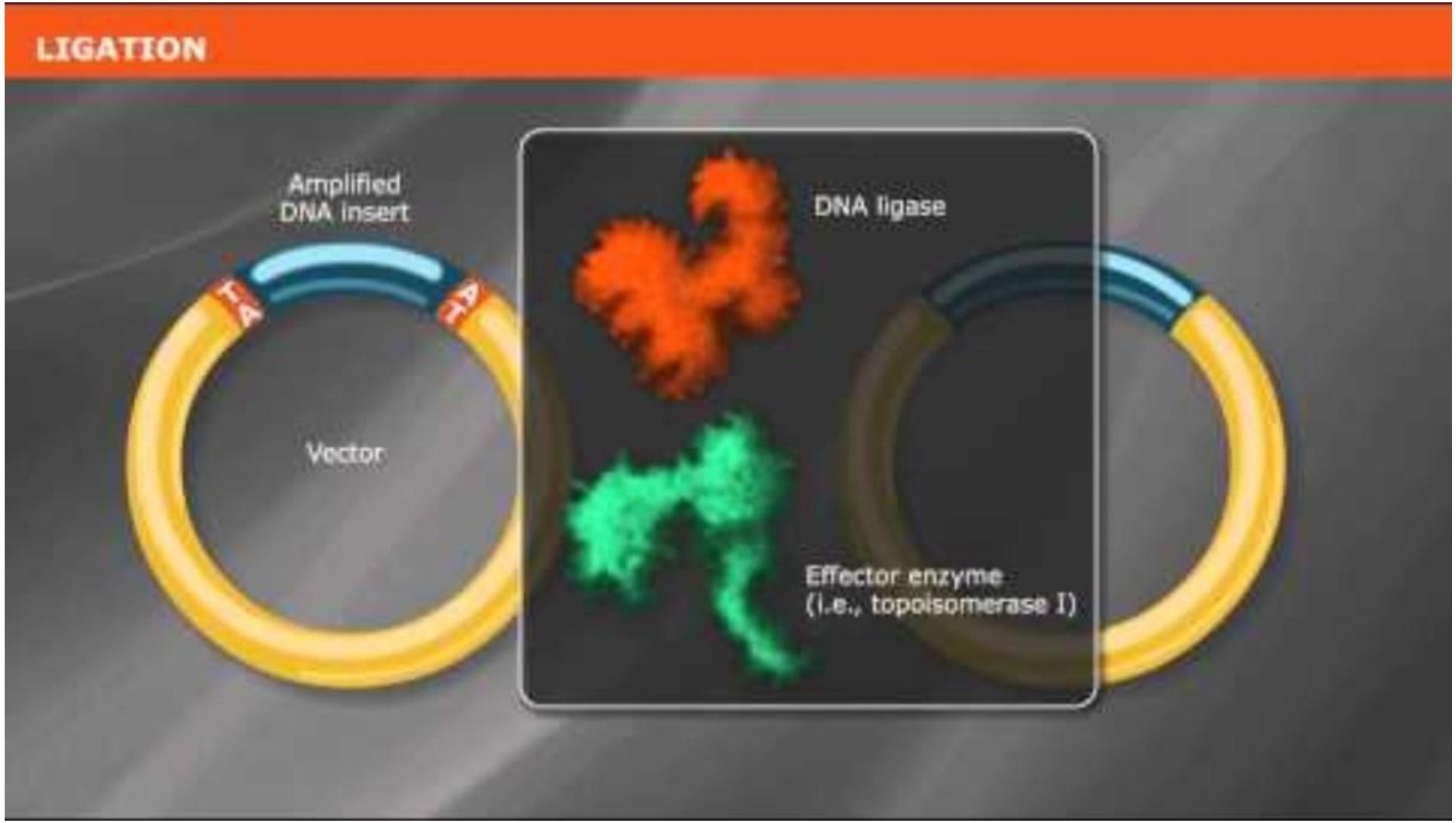


Overview of Traditional Cloning



https://www.youtube.com/watch?v=b9Lm2TUDN2M

Overview of PCR Cloning



https://www.youtube.com/watch?v=E7eqc4r2iwI

How do I calculate how much DNA to add to a ligation reaction?

https://www.neb.com/en/tools-and-resources/video-library/quick-tips-how-do-i-calculate-how-much-dna-to-add-to-a-ligation-reaction

Ligation Protocol with T4 DNA Ligase (M0202)

Protocol

1. Set up the following reaction in a microcentrifuge tube on ice.
   (T4 DNA Ligase should be added last. Note that the table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes.) Use NEBioCalculator to calculate molar ratios.

COMPONENT	20 µl REACTION
T4 DNA Ligase Buffer (10X)*	2 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

   ** The T4 DNA Ligase Buffer should be thawed and resuspended at room temperature.*
2. Gently mix the reaction by pipetting up and down and microfuge briefly.
3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
5. Heat inactivate at 65°C for 10 minutes.
6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

https://www.neb.com/en/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

Ligation Protocol for Cloning with Blunt/TA Ligase Master Mix (M0367)

Protocol

1. Transfer master mix to ice prior to reaction set up. Mix tube by finger flicking before use.
2. Combine 20–100 ng of vector* with a 3-fold molar excess of insert and adjust volume to 5 µl with dH_2O.
3. Add 5 µl of Blunt/TA Ligase Master Mix and mix thoroughly by pipetting up and down 7-10 times or by finger-flicking.
4. Incubate at room temperature (25°C) for 15 min, place on ice.
5. Use for transformation or store at -20°C.
6. **Do not heat inactivate.**

https://www.neb.com/en/protocols/2012/08/27/transfer-master-mix-to-ice-prior-to-reaction-set-up-mix-tube-by-finger-flicking-before-use-combin

Transformation Protocol (M0367)

Protocol

1. Thaw competent cells on ice.
2. Aliquot 50 µl of cells into a 1.5 ml microcentrifuge tube.
3. Add 2 µl of the ligation reaction to the cells and mix by finger-flicking. Do not vortex the tube.
4. Incubate the tube on ice for 30 minutes. Do not mix.
5. Heat shock at 42°C for 30 seconds, then place on ice for 2 minutes.
6. Add 950 µl recovery media (e.g. SOC) to the tube and incubate for one hour at 37°C with rotation or shaking (200–250 rpm).
7. Spread 100 µl of the outgrowth (undiluted or diluted 1:5 with recovery media) onto appropriate antibiotic selection plates and incubate overnight at 37°C.

https://www.neb.com/en/protocols/2012/08/27/chemically-competent-strains-of-e-coli-commercially-available-or-prepared-by-user-can-be-transfor