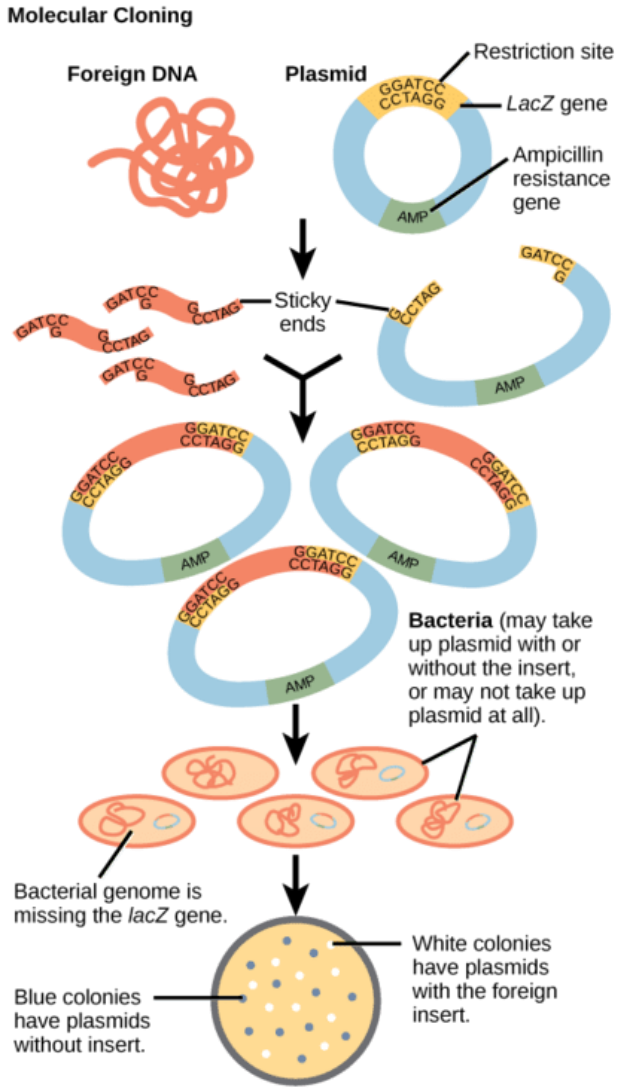
DNA cloning



Matsapume Detcharoen
2/2564

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

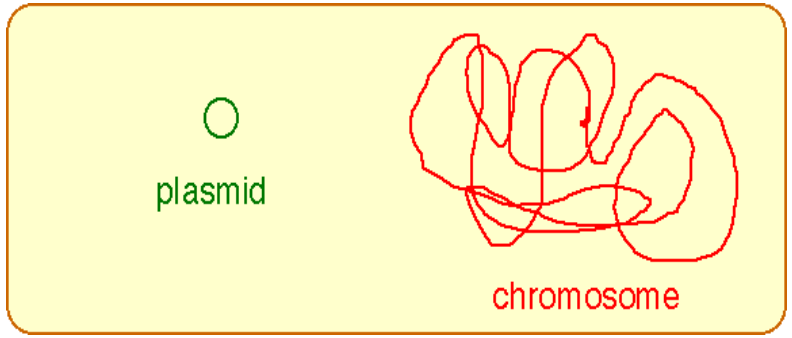
- Massive amplification of DNA sequences
- Stable propagation of DNA sequences
- A single DNA molecule can be amplified allowing it to be:
  - Studied - sequenced
  - Manipulated - mutagenized or engineered
  - Expressed - generation of protein

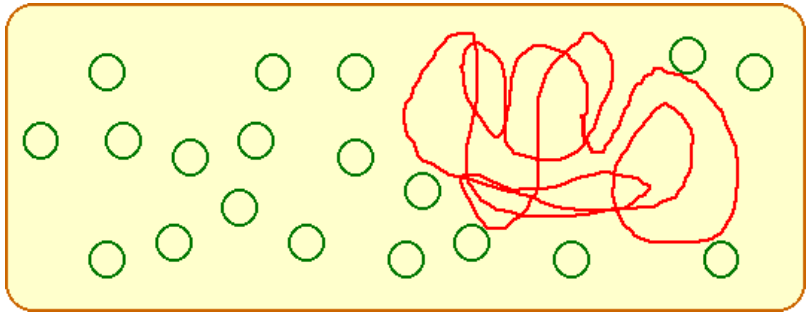
Cloning vectors

- Cloning **vectors** are DNA molecules that are used to "transport" cloned sequences between biological hosts and the test tube.
- Cloning 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.

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





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.

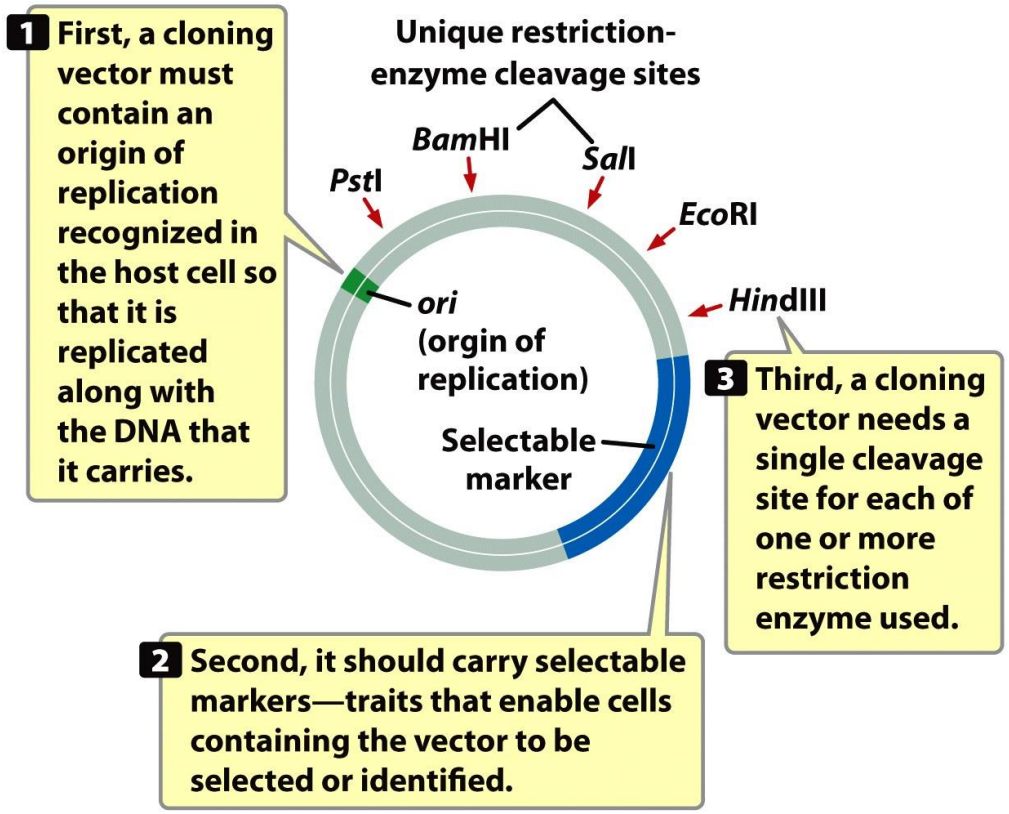
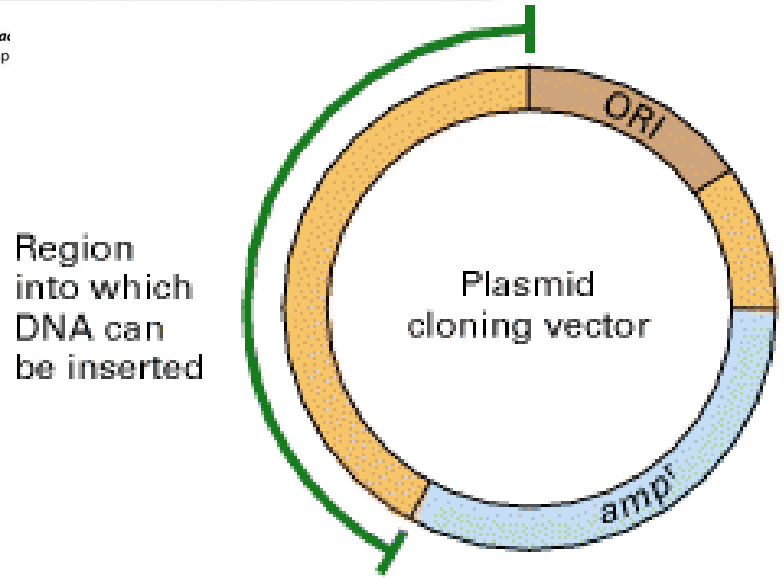
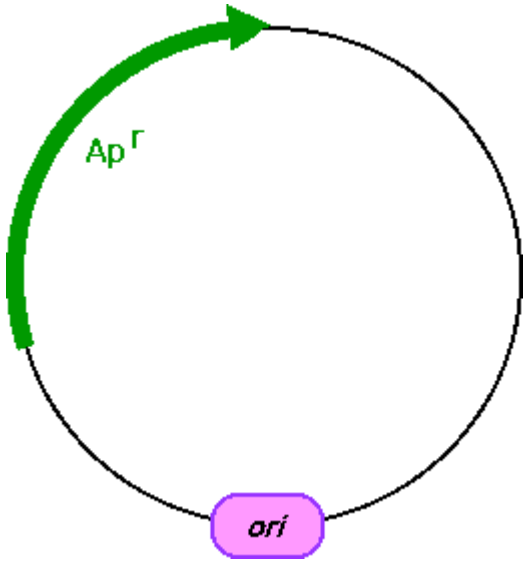


Figure 19-5
Genetics: A Conceptual Approac
© 2009 W. H. Freeman and Comp



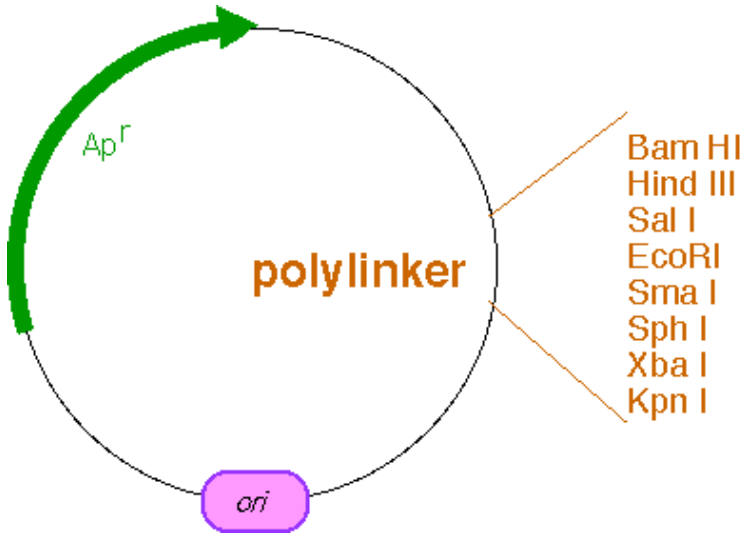
Selective marker

- **Selective marker** is required for maintenance of plasmid in the cell.
- Because of the presence of the selective marker the plasmid becomes useful for the cell.
- Under the selective conditions, only cells that contain plasmids with selectable marker can survive
- Genes that confer resistance to various antibiotics are used.
- Genes that make cells resistant to ampicillin, neomycin, or chloramphenicol are used



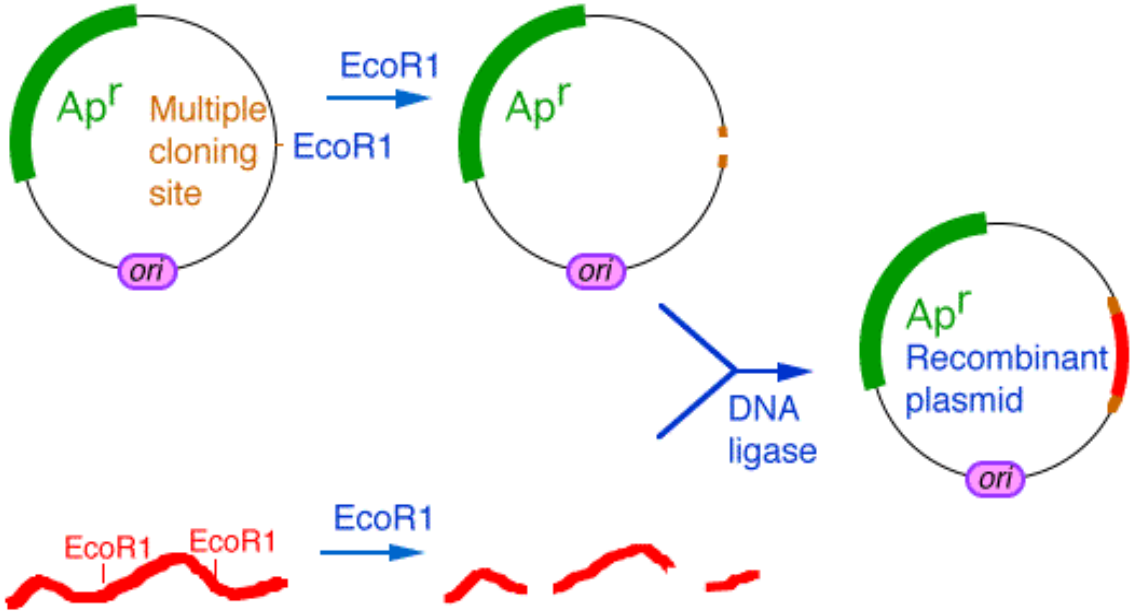
Multiple cloning site

- Many cloning vectors contain a **multiple cloning site** or **polylinker**: a DNA segment with several unique sites for restriction endo- nucleases located next to each other
- Restriction sites of the polylinker are not present anywhere else in the plasmid.
- Cutting plasmids with one of the restriction enzymes that recognize a site in the polylinker does not disrupt any of the essential features of the vector

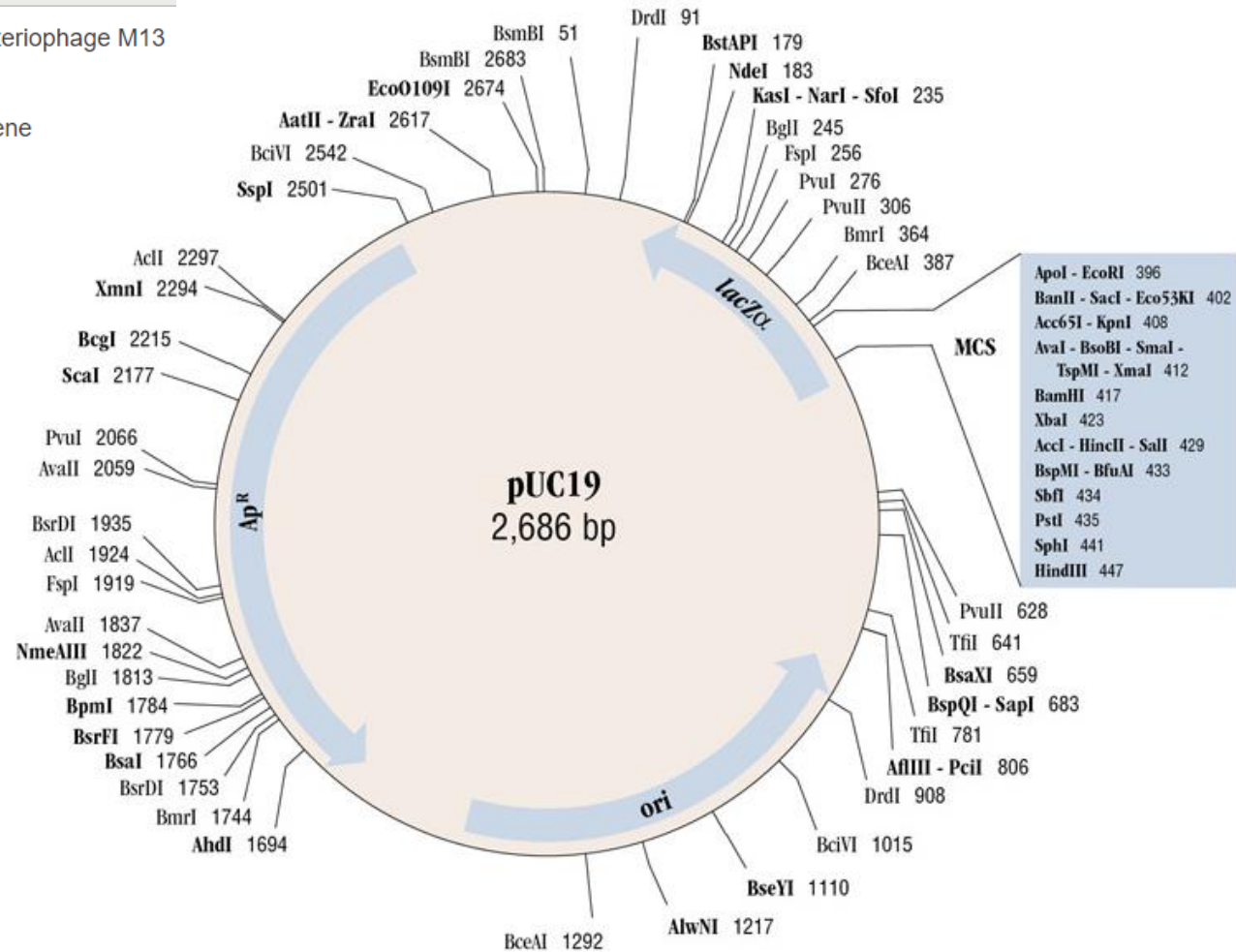


Multiple cloning site

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

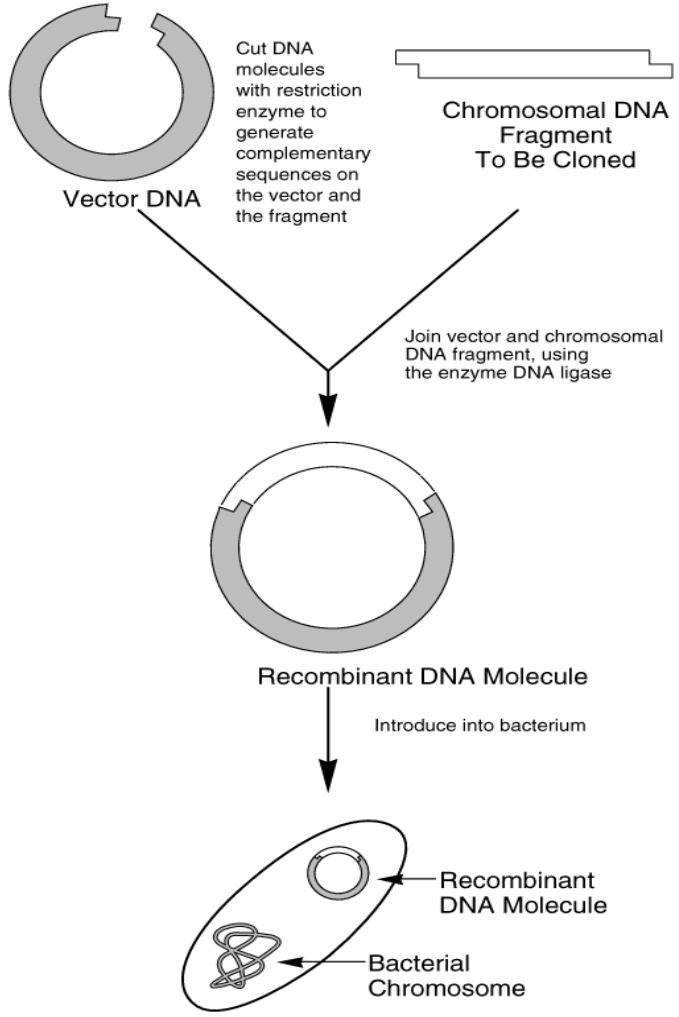


Cloning Plasmid/DNA	NEB #	Features
pBR322 Vector	N3033S/L	• Commonly used cloning vectors • Tet, Amp resistance
pUC19 Vector	N3041S/L	• Commonly used cloning vectors • Amp resistance
M13mp18 RF I DNA	N4018S	• Phage vectors derived from bacteriophage M13 • DNA, covalently closed circular • 13 Unique RE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



Cloning 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)



PCR cloning considerations

- **Nature of the Insert:** not all PCR fragments will clone with the same efficiency into the same vector.
- **Insert size:** The size of the fragment being cloned is a primary contributor to the overall cloning efficiency. 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.

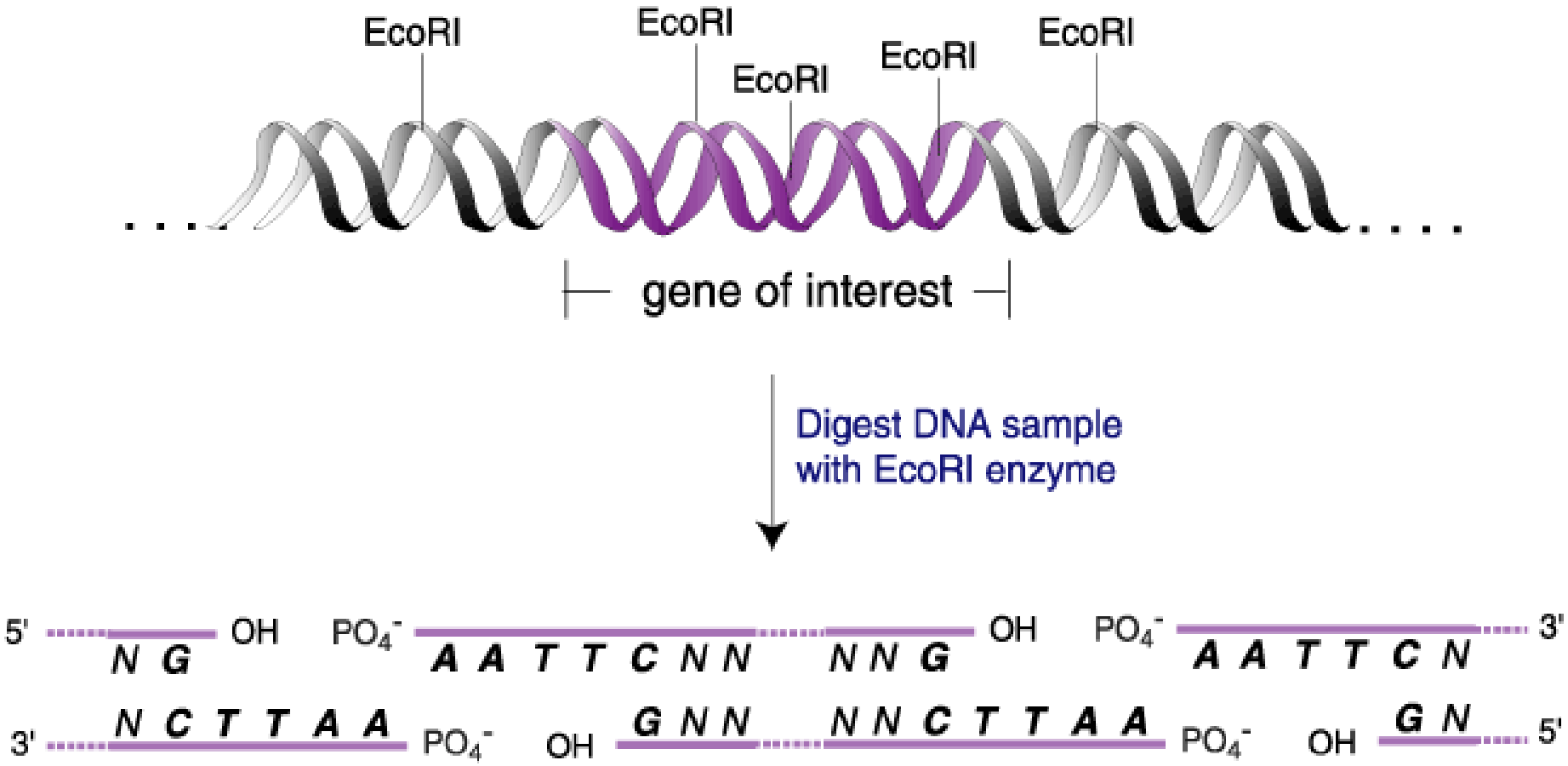
http://www.insilico.uni-duesseldorf.de/Lig_Input.html

Plasmid cloning strategy

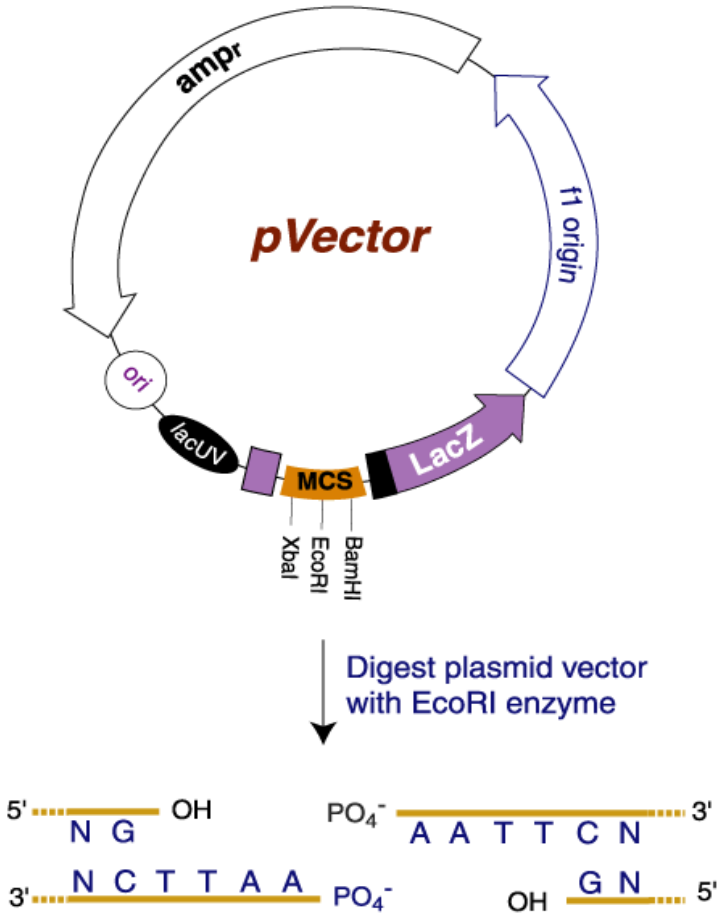
5 steps

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.

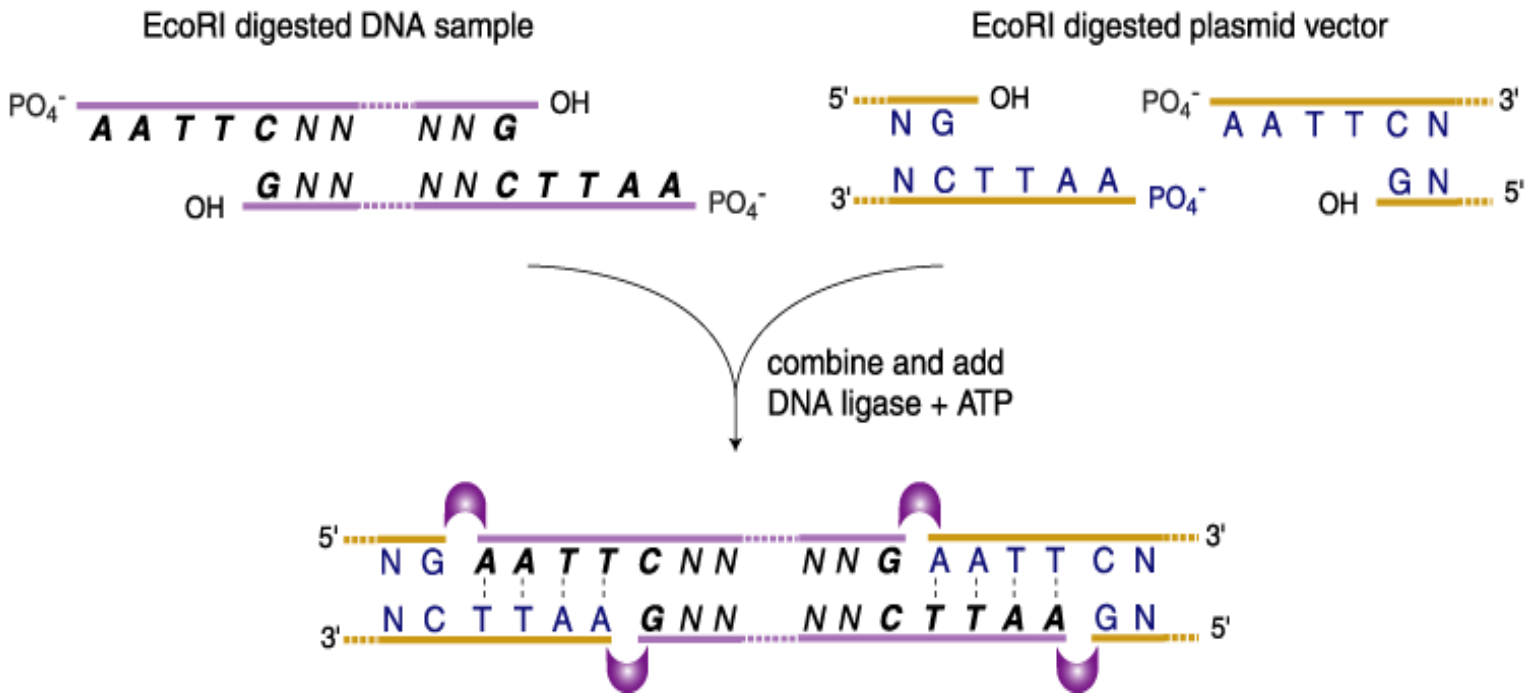
Step 1
RE digestion of DNA sample



Step 2
RE digestion of plasmid DNA



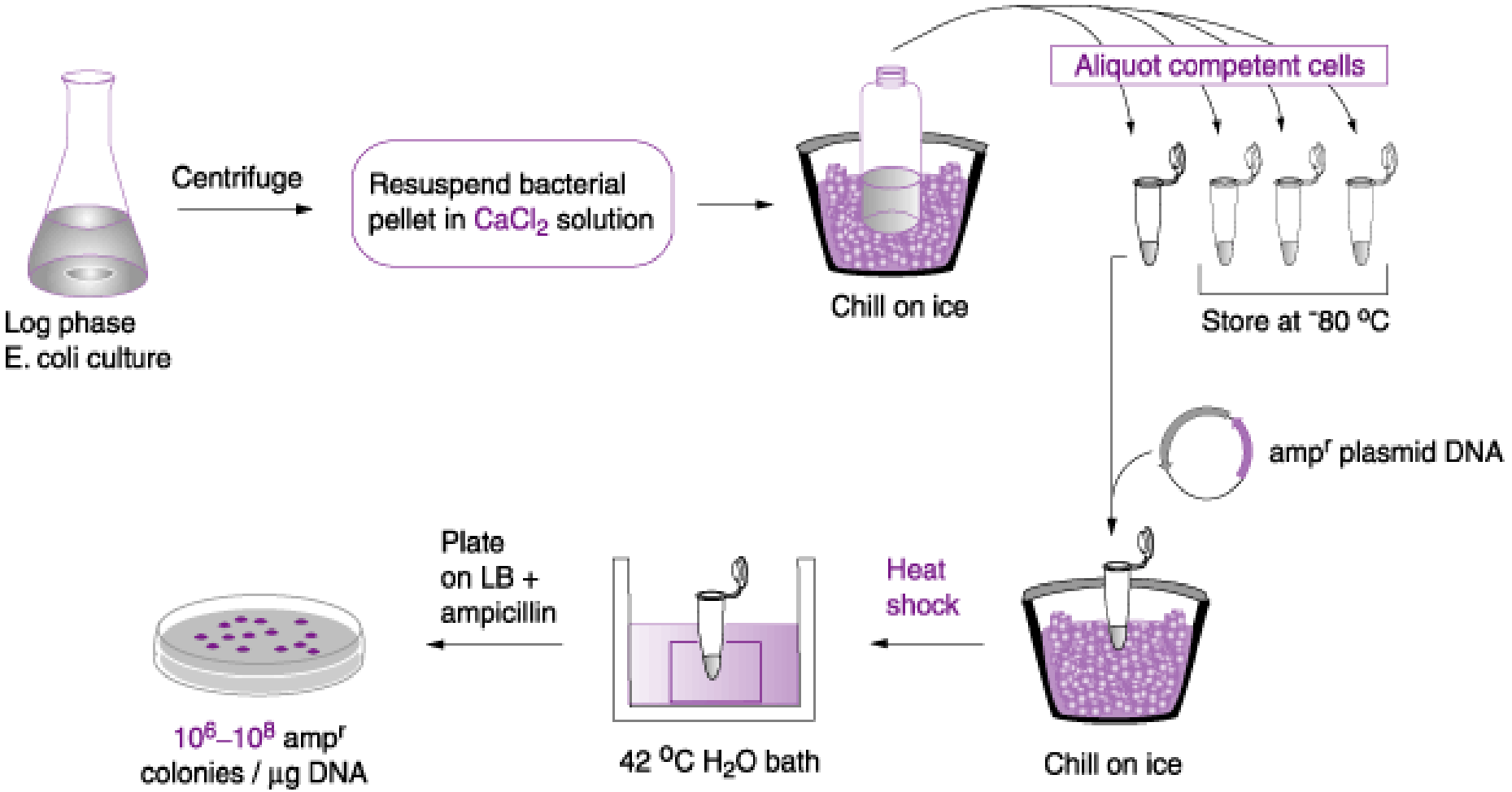
Step 3
Ligation of DNA sample and plasmid DNA



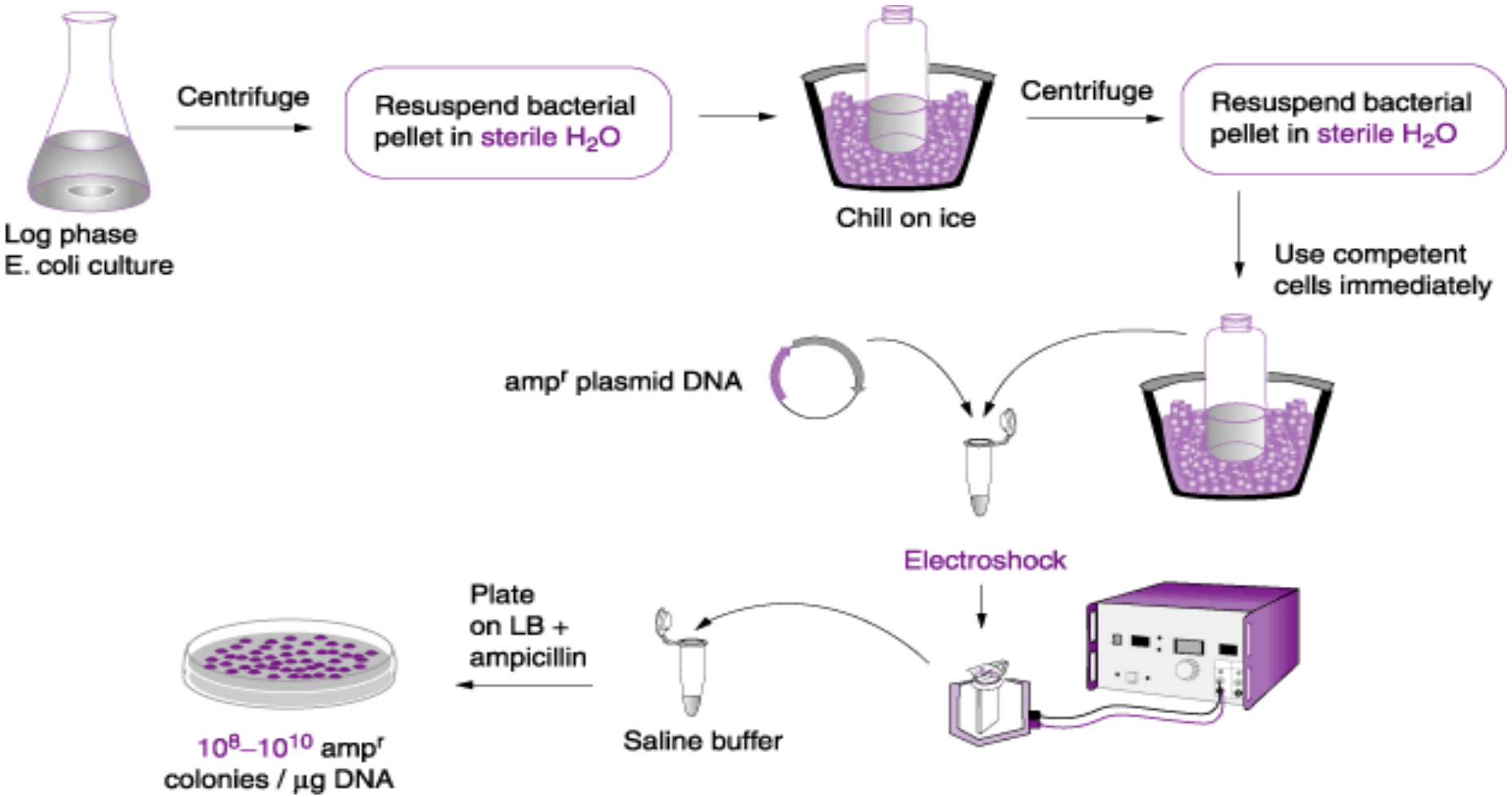
Step 4
Transformation of ligation products

- The process of transferring exogenous DNA into cells is call **“transformation”**
- There are basically two general methods for transforming bacteria. The first is a **chemical** method utilizing $CaCl_2$ and heat shock to promote DNA entry into cells.
- A second method is called **electroporation** based on a short pulse of electric charge to facilitate DNA uptake.

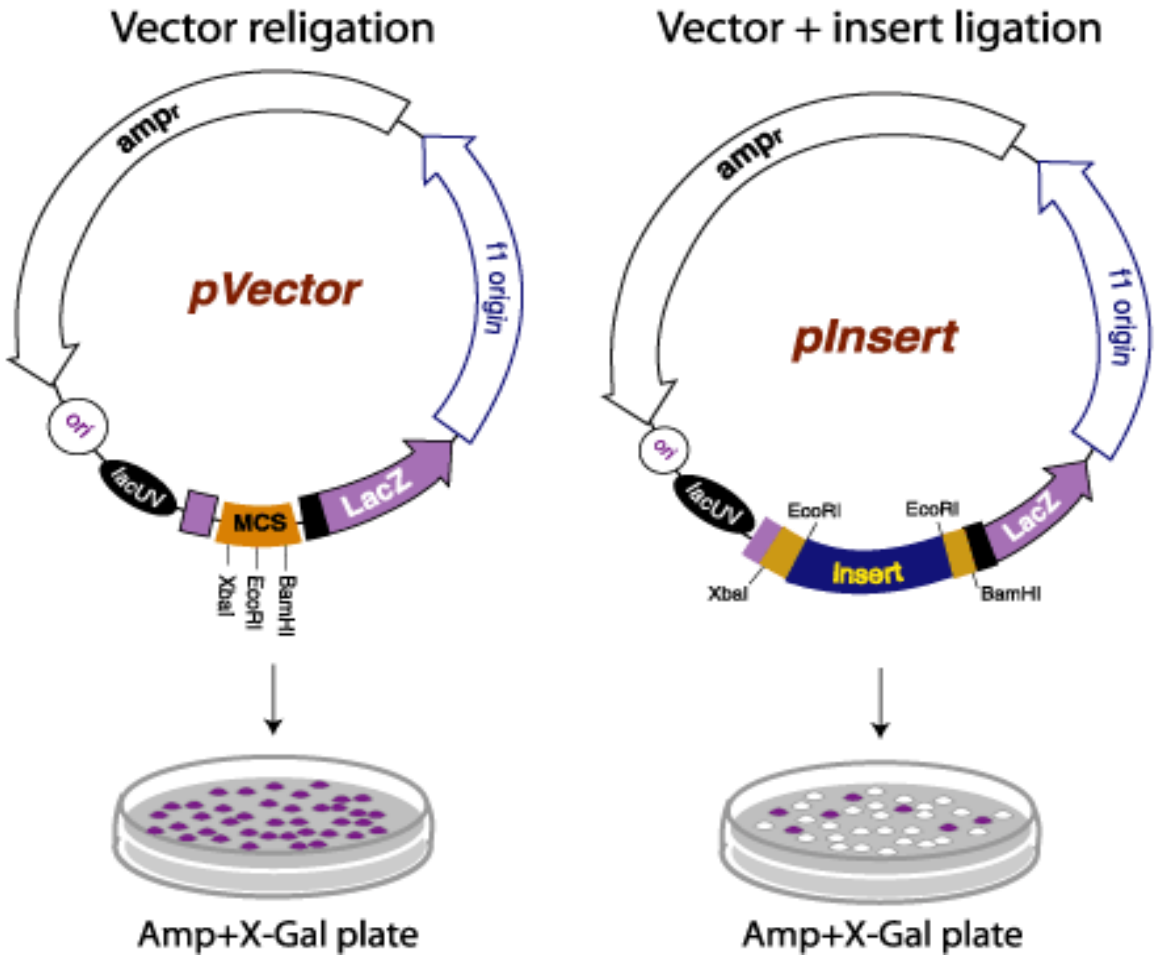
Chemical transformation with calcium chloride

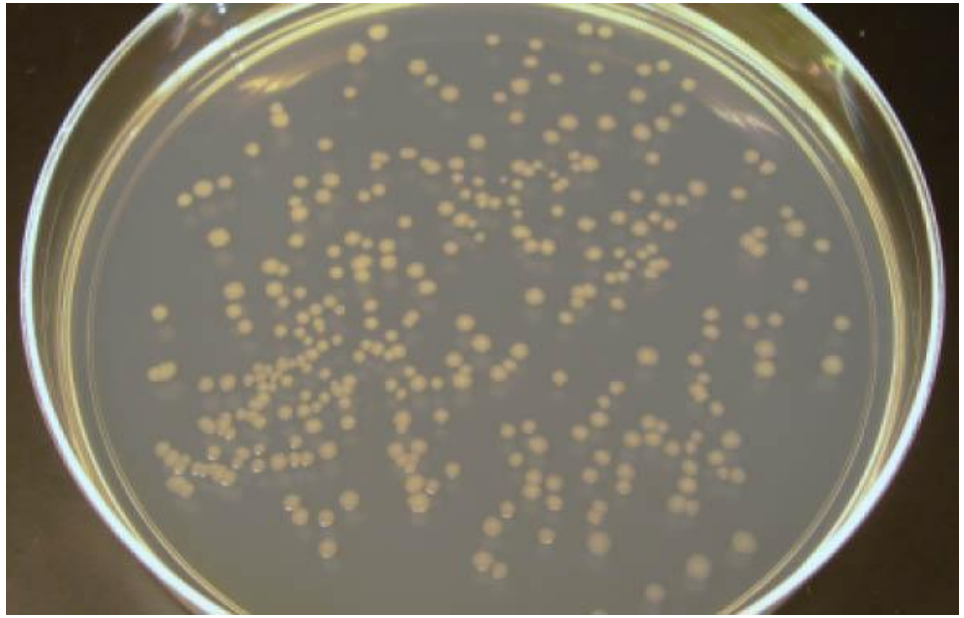


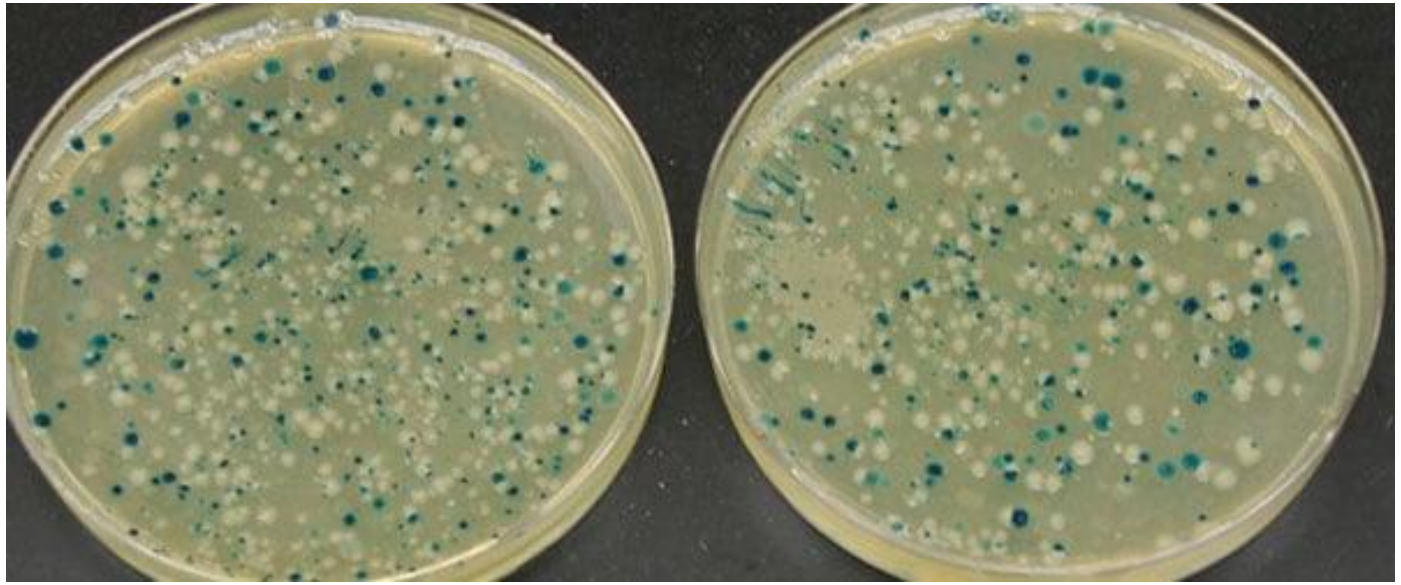
Transformation by electroporation

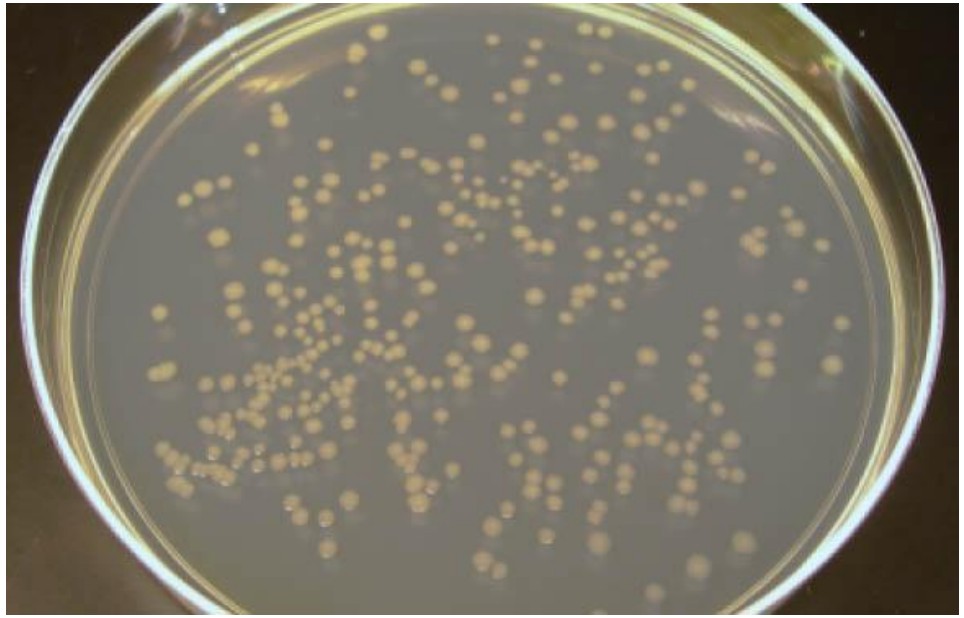


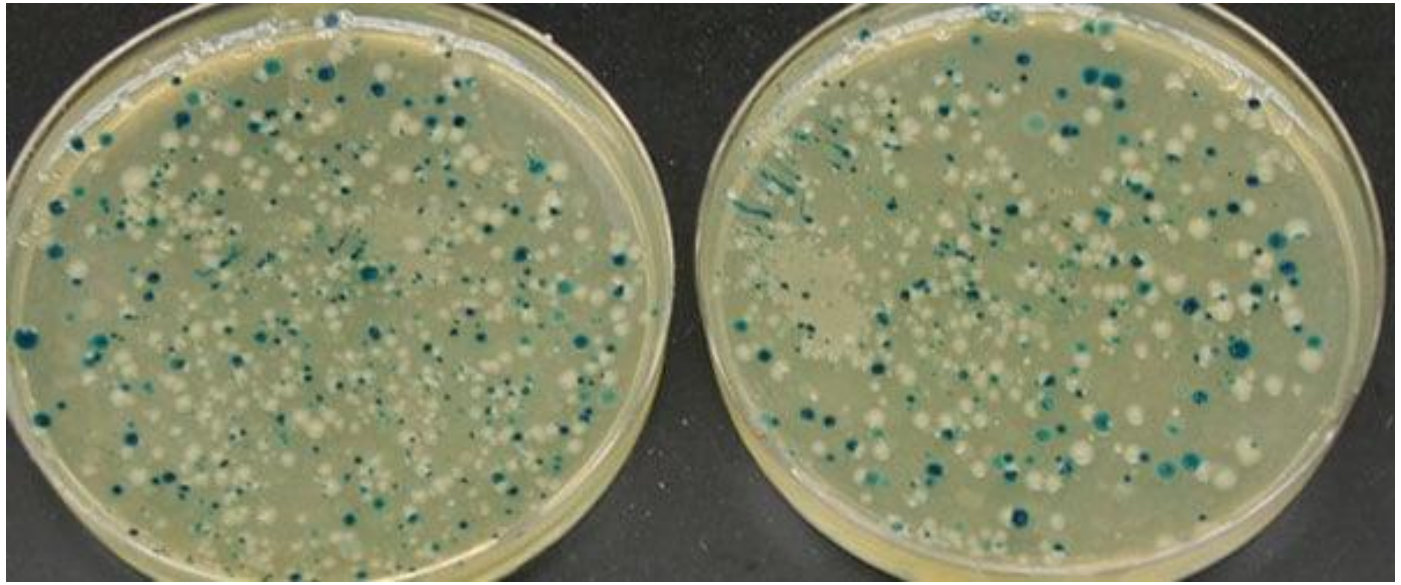
Step 5
Growth on agar plates



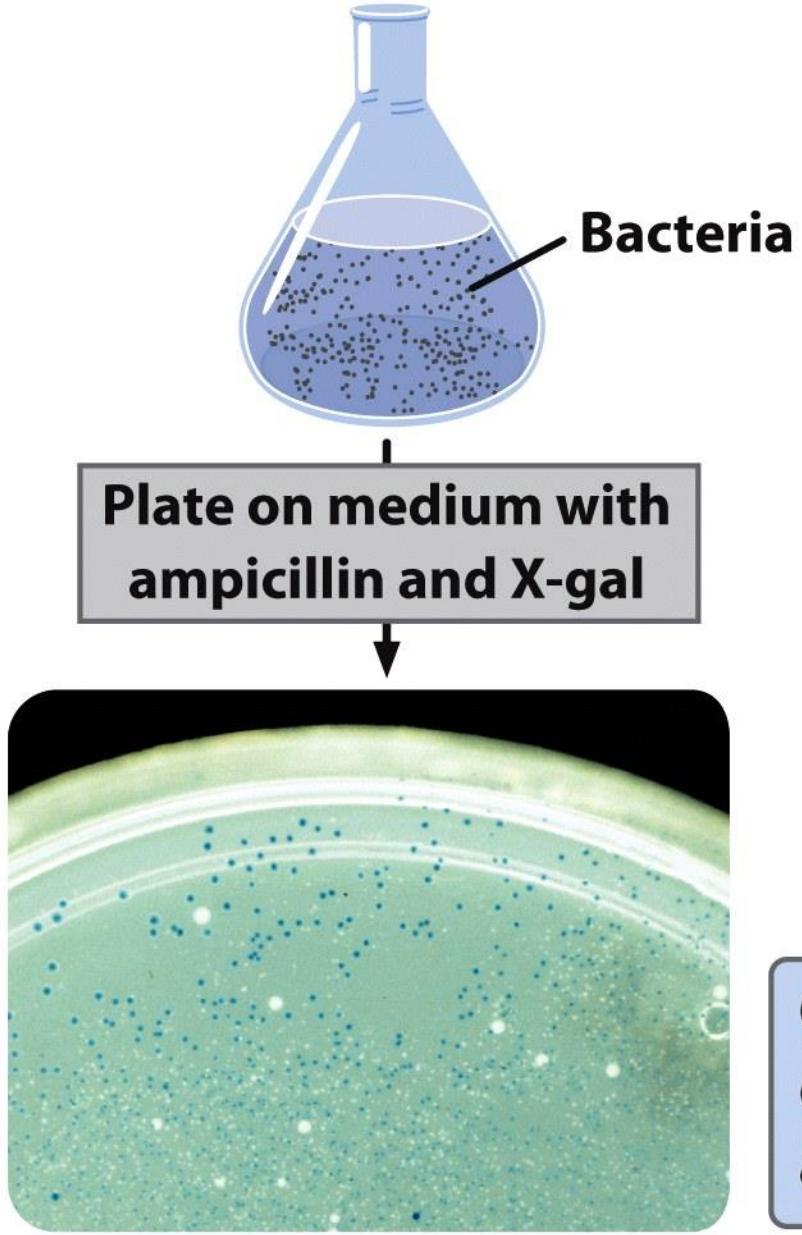








Blue-white screening



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

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

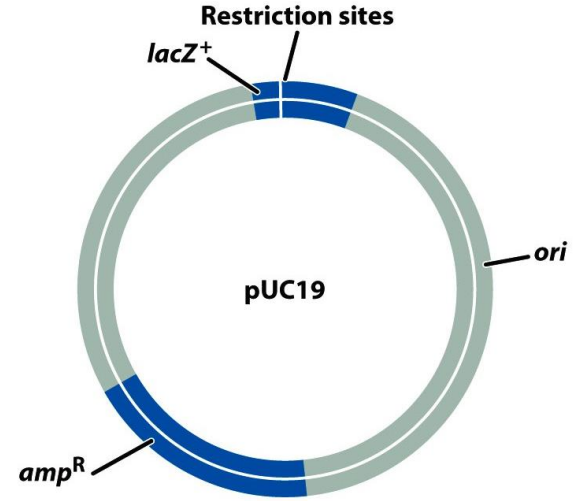
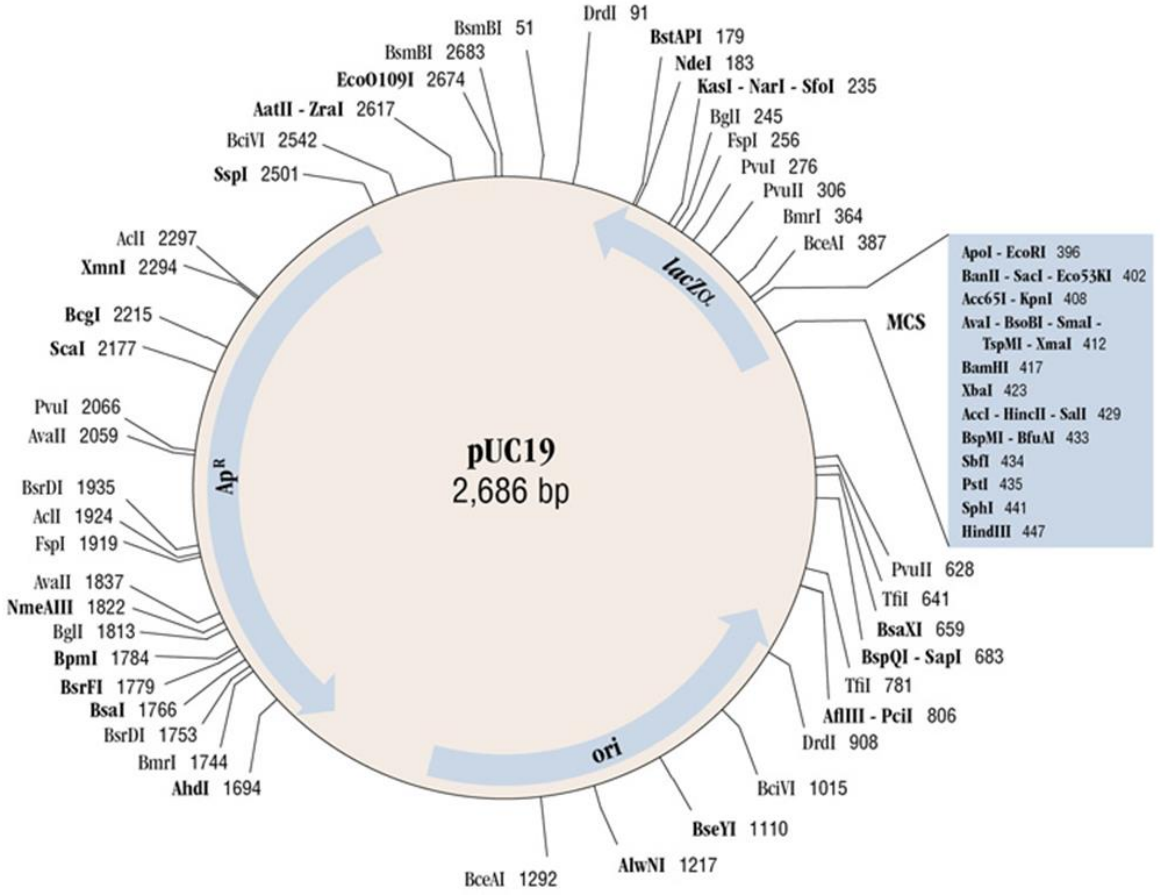


Figure 19-6
Genetics: A Conceptual Approach, Third Edition

- **X-gal** is used to test activity of the enzyme β-galactosidase.
- X-gal is an analogue of lactose and may be hydrolyzed by the β-galactosidase which cleaves the β-glycosidic bond in D-lactose.

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

Autoclaved LB agar
+ X-Gal
+ IPTG



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

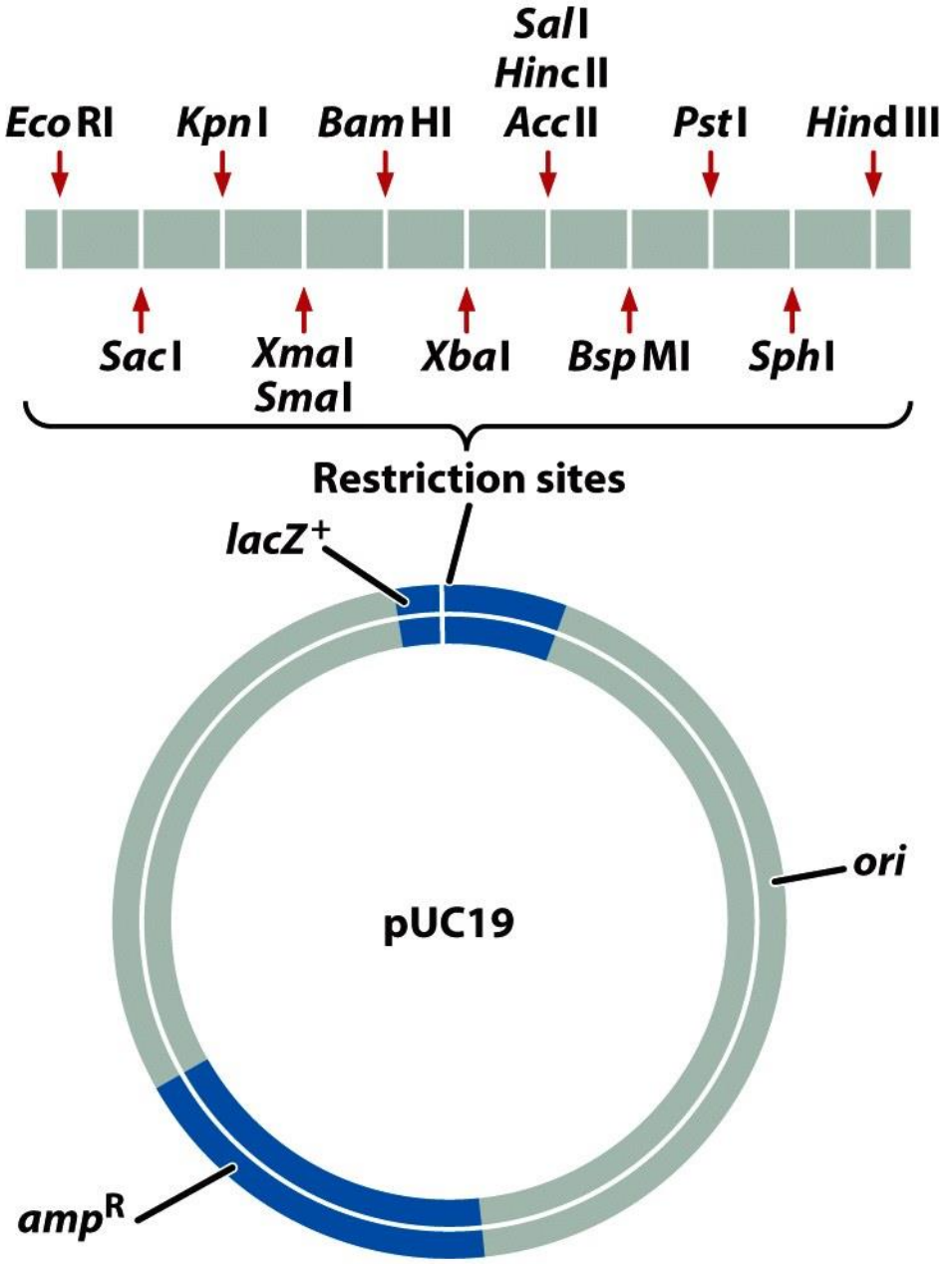
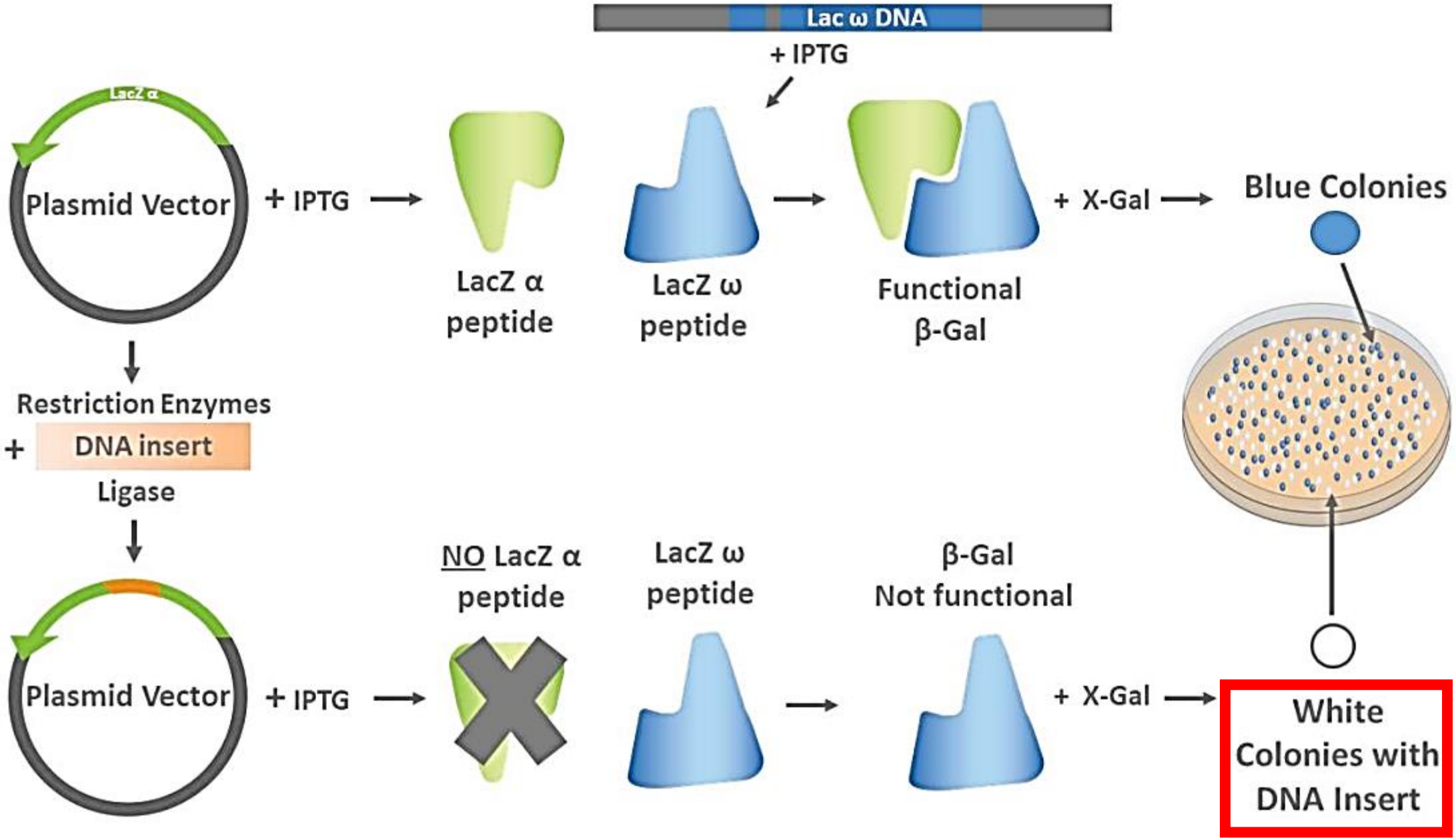


Figure 19-6
Genetics: A Conceptual Approach, Third Edition


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

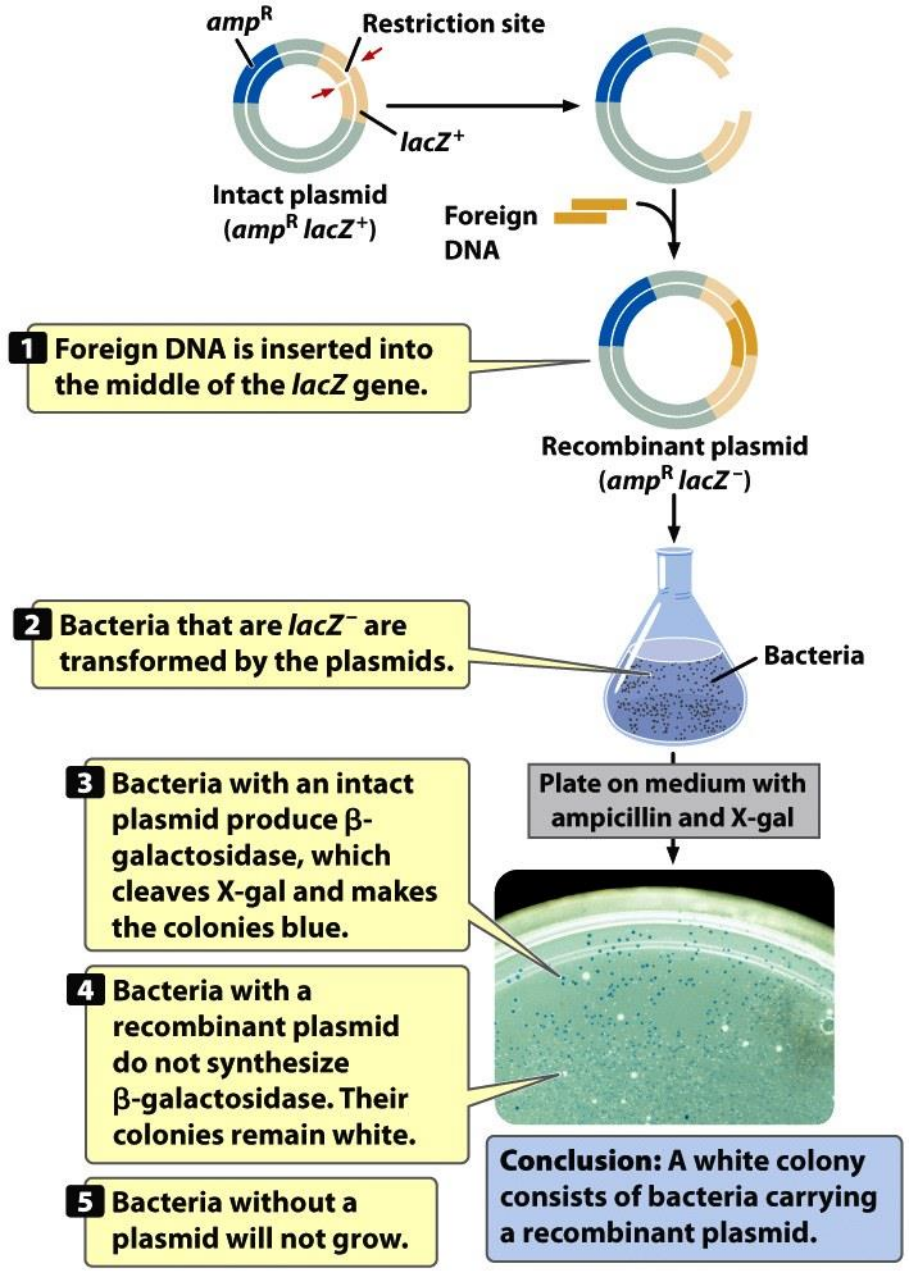


Figure 19-8
Genetics: A Conceptual Approach, Third Edition

- **Blue colonies** represent Ampicillin-resistant bacteria that contain pVector and express a functional **alpha fragment** from an intact *LacZ* alpha coding sequence.

- **White colonies** represent Ampicillin-resistant bacteria that contain insert and do **not** produce *LacZ* alpha fragment

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**

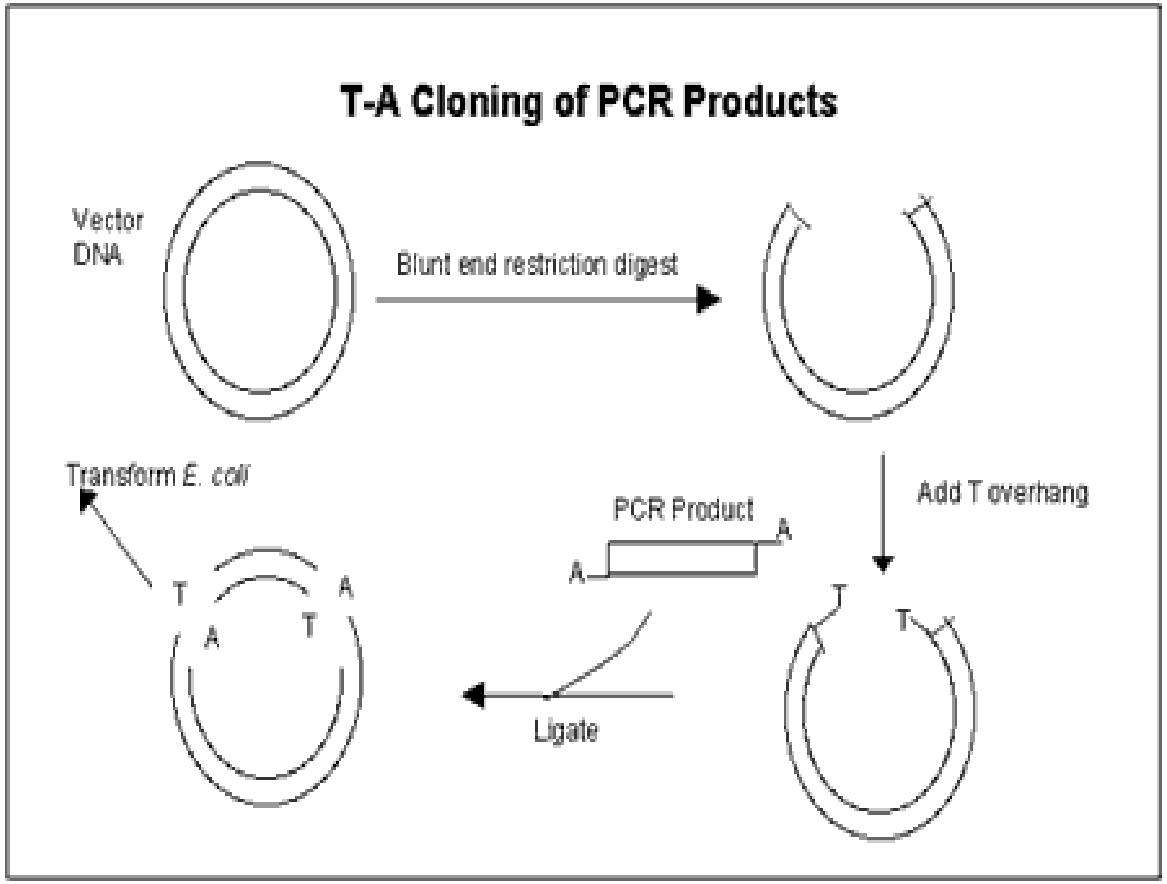
PCR cloning strategies

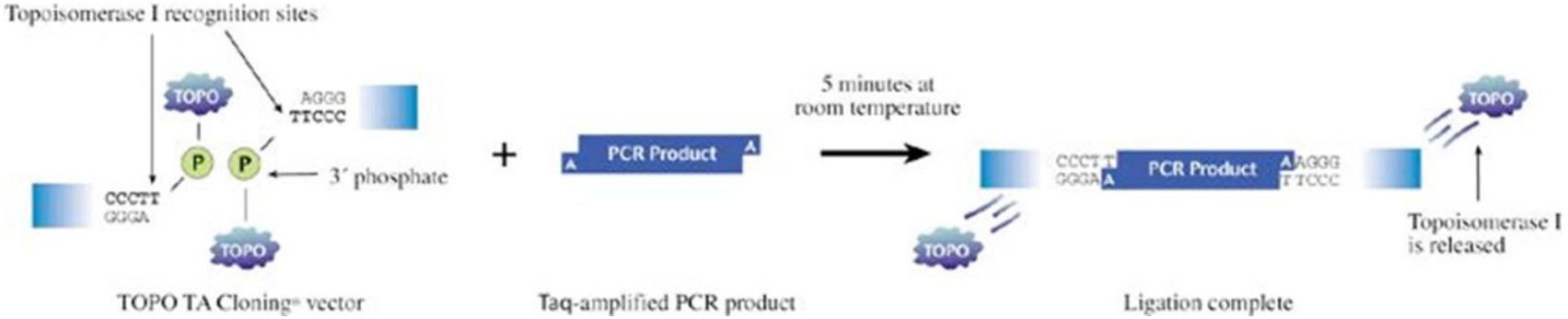
Cloning methods for PCR products are divided into three types:

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

TA Cloning

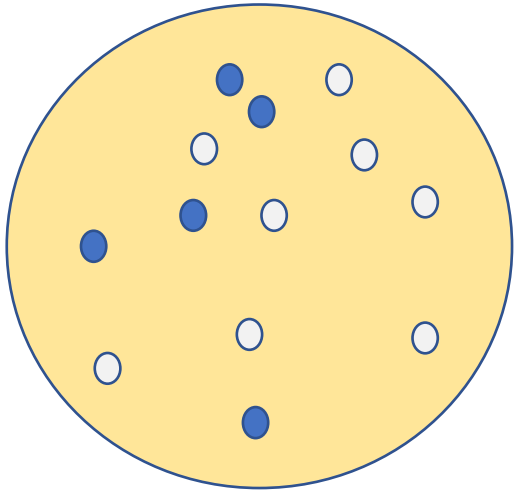
- When DNA fragments are generated *Taq* polymerase adds 1 or 2 extra adenines onto the end of 3’ end of blunt ds DNA
- Several commercially available kits take advantage of this ability
- Use a plasmid vector with thymidine residues linked onto the 3’ ends of linearized plasmid DNA





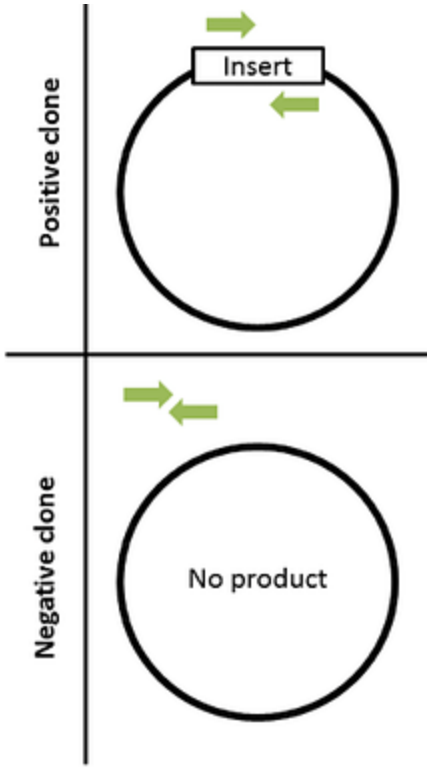
Analysis of cloned DNA

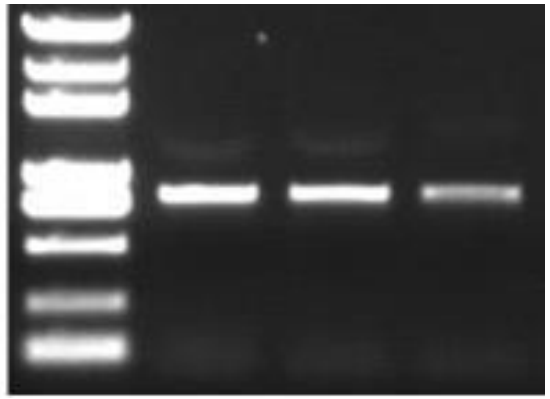
- Is it the one you wanted?
- What are its molecular characteristics?
- **Gel electrophoresis:** separates DNA fragments by molecular weight
- **DNA sequencing:** provides *complete order of bases* in a DNA fragment
- **Colony PCR**



H2O + cells
95 ˚C 5 min

Use the solution as template for PCR with gene specific primers



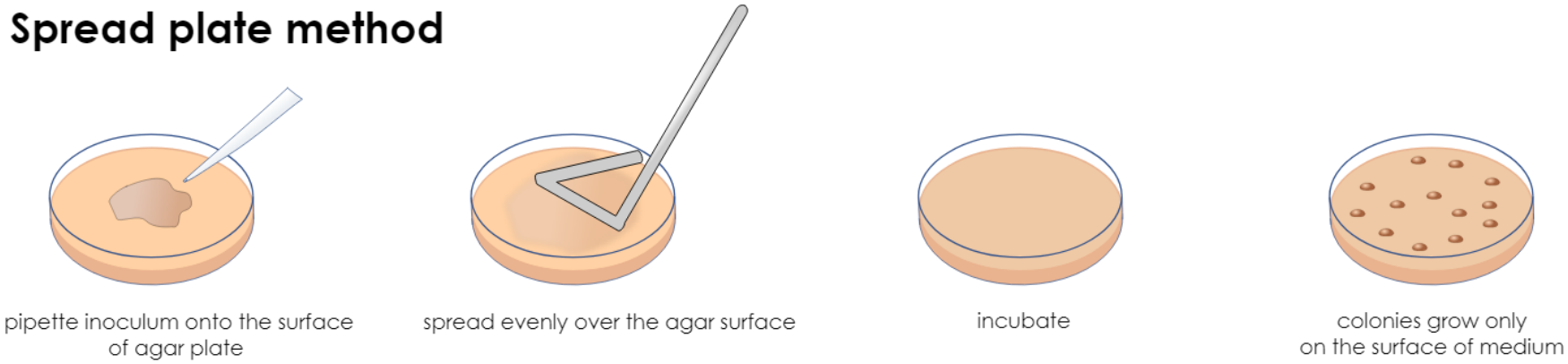


Aseptic technique
Good microbiology techniques

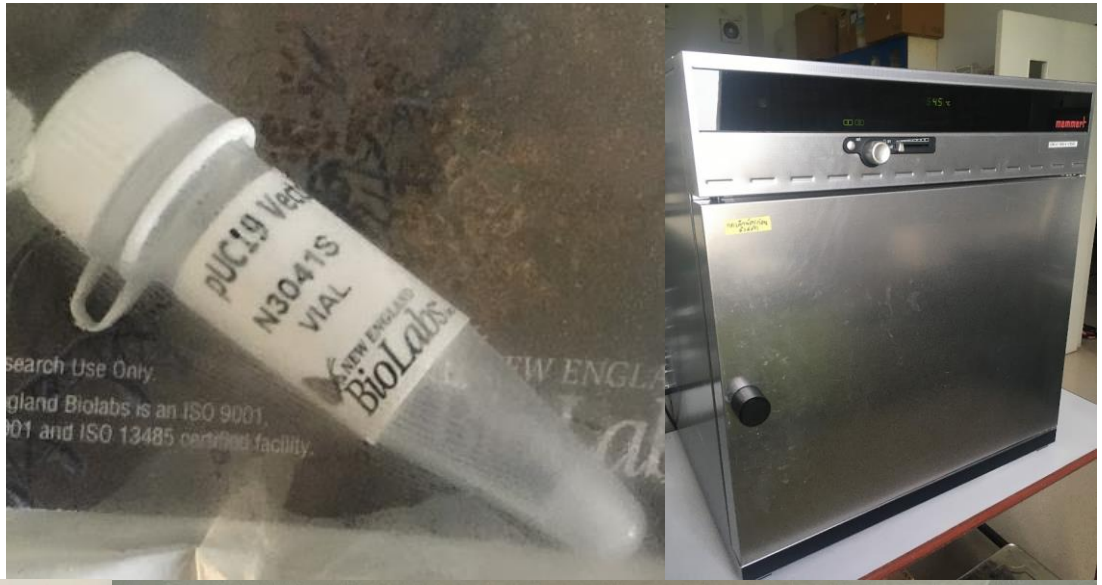
Pour plates

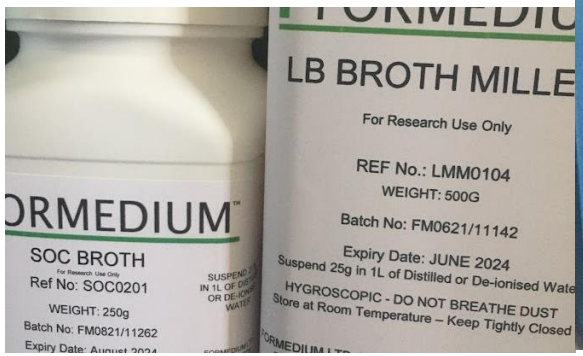
Spread plate

…



- 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**

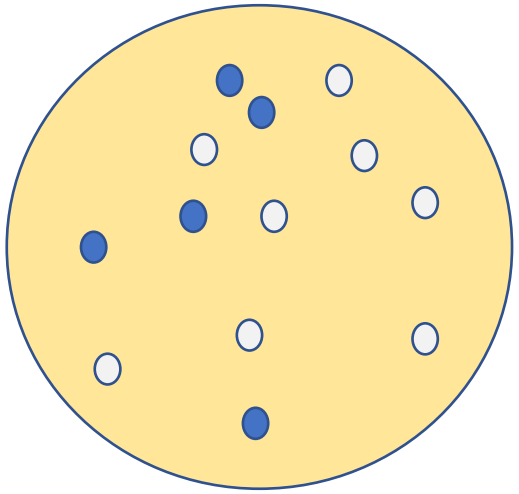
Protocol

3. Ligate cloning vector with your PCR product
    - **Vector:insert = 1:3**
        - PCR product 524 ng/µl, 498 bp
        - pTZ57R/T vector 2886 bp
        - Use 165 ng vector (3 µl)
        - Use calculator

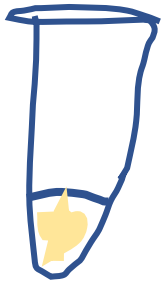
          http://www.insilico.uni-duesseldorf.de/Lig_Input.html
        - Use 85.4 ng PCR product, = 0.16 µl
        - 1 µl vector + 0.16 µl product + 2 µl 5x buffer + 6.5 µl water + 0.3 µl T4 ligase
    - Ligate overnight (16-18 hours)

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