Laboratory 4

Objectives

- To ligate both digested
  - plasmid (vector) and
  - insert (PCR product)
- To transform *E. coli* DH5 alpha cells

Safety measures

- You will work with a **genetically modified organism** (GMO). Adopt safe working practices to protect yourself and others.
  - Wear a lab coat, gloves and safety goggles
  - Clean bench before and after use with 70% EtOH
  - Dispose all waste in yellow biohazard bag.
  - Clean any spillage with 70% EtOH
  - Don’t touch your face, door knobs, screen etc with gloves while handling bacteria.
  - After removal of gloves, wash your hands.

Summary of experimental steps

Steps performed by us

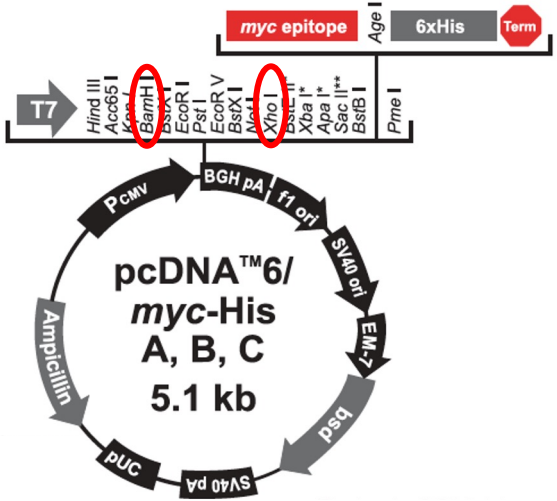
1. Digestion of PCR product (insert) and plasmid (vector)
2. Dephosphorylation of vector
3. Extraction / Purification of digested products from agarose gel

Laboratory 4

- DNA Ligation of purified vector and insert
- Transformation of competent bacteria

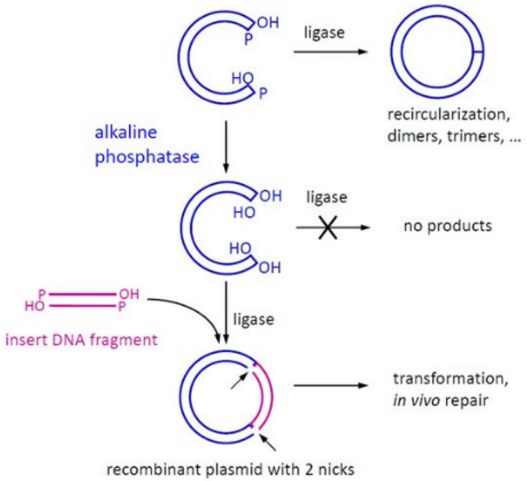
1. Restriction enzyme digestion of vector and insert

- Digestion of PCR product (Amy2 amplicon = insert) and pcDNA myc-His plasmid (vector) with *Bam*HI and *Xho*I restriction enzymes



2. Dephosphorylation of plasmid vector

- Alkaline Phosphatase catalyzes the removal of 5´ phosphate from DNA and RNA.
- Phosphatase-treated fragments lack the 5´ phosphoryl termini required by ligases and cannot self-ligate.
- This property is used to avoid recircularization of the plasmid (without insert).



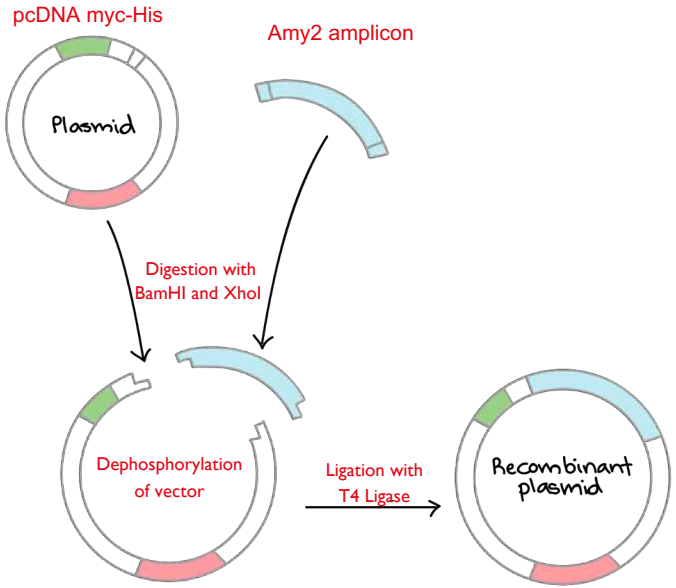
3. Extraction of digested DNA from agarose gel

https://www.addgene.org/protocols/gel-purification/

Procedure

- Run agarose gel
- Cut band of interest under UV
- Dissolve gel containing DNA
- Purify with spin column

DNA Ligation



Example

$$\frac{\text{Insert size (kb)}}{\text{Vector size (kb)}} \text{ (mass ratio)} \quad \text{x} \quad \frac{\text{Insert}}{\text{Vector}} \text{ (desired mole ratio)} = \frac{\text{ng Insert}}{\text{ng Vector } (e.g., 50 \text{ ng})}$$

For example, you have **50 ng** of a **4 kb vector** and you want to ligate your **insert (2 kb)** at a **1:10 vector to insert ratio**. How much insert must you add to your reaction?

$$\frac{2 \text{ kb}}{4 \text{ kb}} \quad \text{x} \quad \frac{10}{1} \quad = \quad \frac{\text{X ng}}{50 \text{ ng}} \qquad \text{X= 250}$$

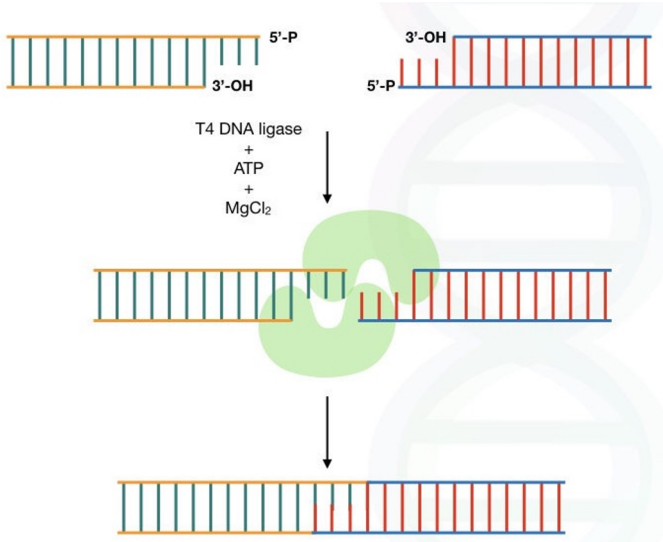
So…..what volume would you add to your reaction if your stock concentration of insert was 25 ng/µL?

$$\text{Volume need} = \frac{\text{Amount desired}}{\text{Concentration}} = \frac{250\text{ng}}{25\text{ng/µl}} = 10\text{µl}$$

For optimal ligation we will use a molar ratio of **1:3 vector to insert** and **50 ng vector**.
Based on vector and insert length, calculate the amount of insert corresponding to 3-fold molar excess
(help: NEB biocalculator)

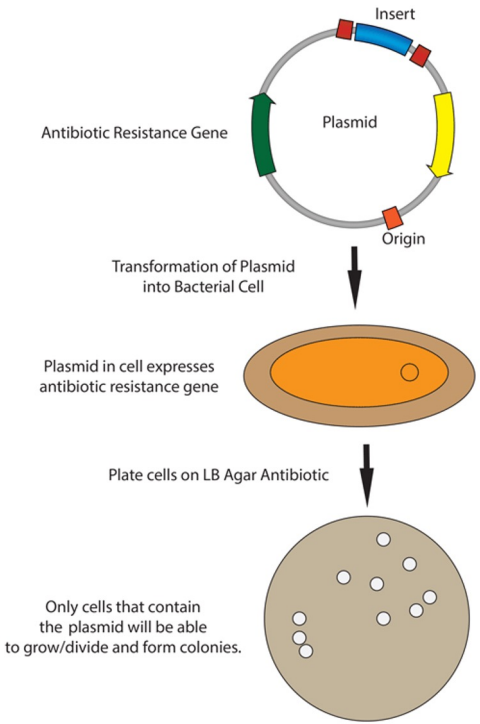
T4 Ligase

- Catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA.
- Ligates DNA restriction fragments (vector and insert) with overhanging, cohesive ends.



Sticky end DNA ligation

Bacterial Transformation



https://www.addgene.org/protocols/bacterial-transformation/

Selection by Antibiotic Resistance

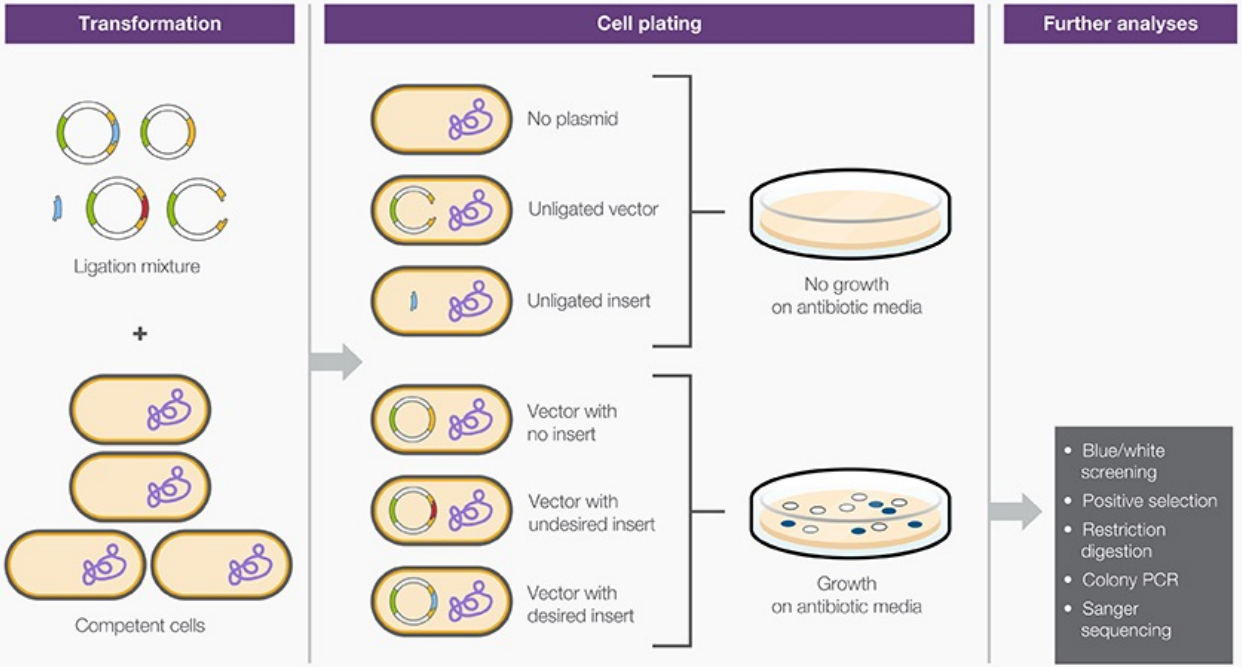


image: https://www.thermofisher.com

Controls

- **Negative control** (vector self-ligation)
  - Allows to determine background colonies (trace of undigested vector DNA)
  - Linearized plasmid (vector) cannot replicate in bacteria.
  - Dephosphorylated vector cannot “self-ligate”.
- **Transformation control** (undigested plasmid)
  - Allows to determine **transformation efficiency** of competent cells (known amount of plasmid DNA).

Heat-Shock Transformation

- **DH5α competent cells** should be handled carefully (do not vortex)
- Add ligation mix to competent cells and incubate on ice for 30 min (gently mix by pipetting up and down or tapping the tube)
- **Heat-shock** the cells at **42°C** for **exactly 20 sec** (temperature and timing are critical for this step)
- Incubate cells on ice for 2 min
- Carefully add 250 µl SOC medium to cells
- Allow cells to recover for 1h at 37°C

Label LB-Plates

- Label your plates around the edge (not the lid) with the following information:
  - Competent cells
  - Sample condition
  - Group number
  - Date

- Plates should be **incubated 'upside down'** to prevent condensation formed on the inside of the lid to fall on top of the bacteria.

Plating transformed cells

- Plate 200 µl of transformed bacteria for each condition
  - Ligation (vector + insert)
  - Negative control (vector only)
  - Transformation control (undigested plasmid)

- LB agar plates with **ampicillin** are used to selectively grow transformed bacteria (plasmid encodes an ampicillin resistance gene)
- Different techniques can be used to spread the bacteria on LB agar plates
  - We will use a disposable sterile **spreader**
  - Sterile **glass beads** can also be used to spread the bacteria on the plate

Plating transformed cells

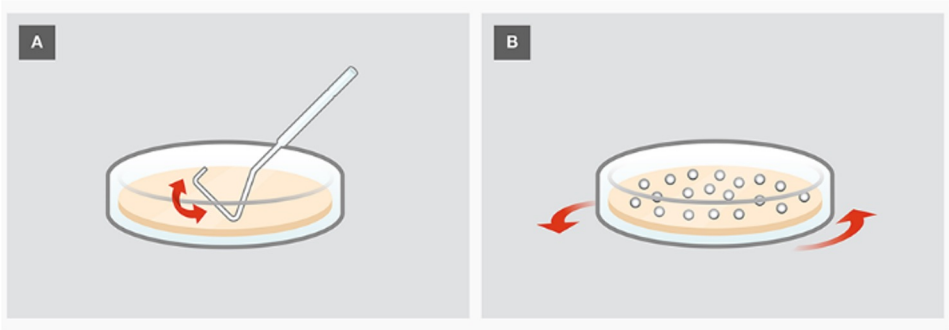


Figure 7. Two common plating methods. (A) Spreading with a sterile hockey stick spreader. (B) Spreading with sterile 4 mm glass beads and gentle swirling of the plate.

bacterial-transformation-workflow-plating

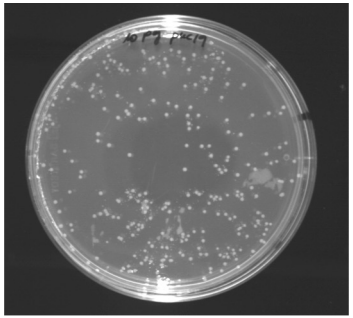
Transformation Efficiency of Competent Cells

To calculate the transformation efficiency, divide the number of colonies (plate: transformation control) by the amount of DNA added, and factor in cell dilution (if performed), using the following formula:

$$\text{Transformation efficiency (CFU/µg)} = \frac{\text{Number of transformants (CFU)}}{\text{DNA added to the cells (µg)}} \times \frac{\text{Volume of transformation (µL)}}{\text{Volume of cells plated (µL)}} \times \text{Cell dilution factor (in plating)}$$

Example:

- 10 pg plasmid DNA (transformation control)
- number of colonies: 200
- µl plated: 200
- Transformation efficiency = 3×10^7 cfu per µg plasmid DNA



Single colony streaks

Use a NEW sterile tip for each streak!
Do not make holes.

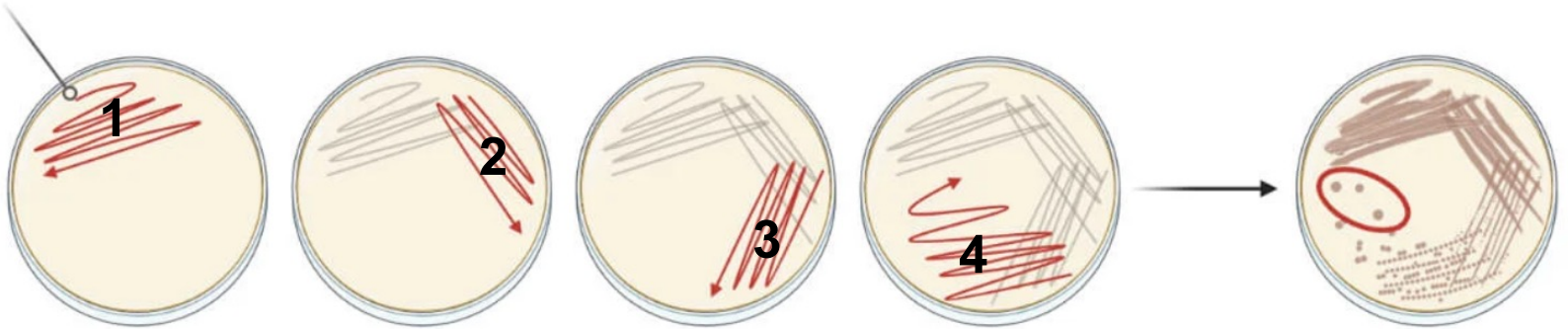
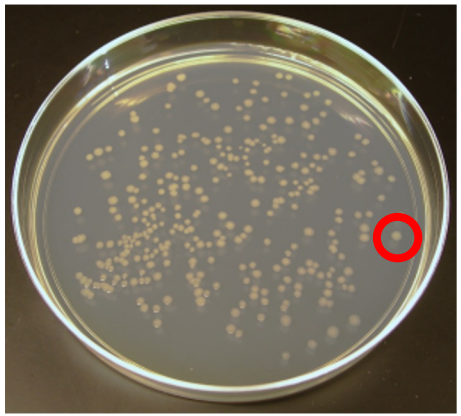


image: https://microbenotes.com/streak-plate-method-principle-methods-significance-limitations/

Four “dilution streaks”(2 to 4; direction is indicated by arrows) were made from the original inoculum.

Drawing the desired pattern at the bottom of the plate may assist with the streaking.

Inoculating single colonies



Transformants on LB-Amp plate A
(vector + insert ligation)

pick single colony using sterile tip

Snap cap tube containing
LB-Amp medium
incubate at 37°C
in bacterial shaker

- Growth in the presence of oxygen, carbon and nitrogen sources.
- Turbidity of the culture is proportional to bacterial cell number.