

Session 6

Ligation and Transformation

Learning Objective:

In this lab you will learn what components go into a ligation reaction and the conditions under which T4 DNA ligase is able to join DNA pieces. Understand transformation of a plasmid into a host strain to complete the cloning process. Gain practical experience with the importance of selection and antibiotic resistance.

Introduction

In previous sessions we have seen how we can get pieces of DNA by isolating it from cells, cutting it with restriction digestion, and amplifying with PCR. Today we will put the DNA pieces together with a process called ligation. Ligation permanently attaches strands of DNA so that they can be put into a cell and maintained. The process of putting DNA into a cell is called transformation. After transformation, the cells containing the plasmid are selected with antibiotic completing the cloning process.

Background:

Ligation: Joining DNA Fragments

In earlier weeks you learned how DNA can be cleaved in specific locations by restriction endonucleases. The opposite process, **ligation** joins DNA strands together. Enzymes called ligases can join two double-stranded pieces of DNA together or a single double-stranded piece of DNA to itself. There are two general varieties of ligation, **blunt-end** ligation and **sticky-end** ligation, which refer to the absence or presence of overhangs on the ends of the DNA being ligated. Blunt-end ligation has the benefit of being very generally applicable, while sticky-end has the benefit of high specificity and efficiency.

In this lab session you will be doing a sticky-end ligation. The parts that we are going to put together are BBa_J45200, a protein generator for alcohol acetyltransferase I, and pSB4A5. Two sessions ago, using EcoRI and PstI, you digested the backbone and cut out the “purple”-producing enzymes. You will now insert ATF1, the “banana smell”-producing enzyme. These pieces represent everything required to express the gene in *E. coli* and produce a functional protein. The EcoRI overhang from the vector matches the EcoRI overhang in the gene, the PstI overhang in the gene matches the PstI overhang in the vector.

Transformation: Putting Foreign DNA into a Cell

Transformation is a process by which cells pick up foreign DNA from their surroundings and incorporate it into the cytoplasm. Transformation is a common method for introducing plasmids into a host cell. For a transformation to be successful, the cells must be **competent**. There are two ways in which cells can be competent, **electrocompetent** and

chemically competent. To make the cells competent, they are treated using cold salt solutions and glycerol. They are cryogenically frozen in the very delicate competent state and defrosted just prior to use. Cells are transformed in different ways depending on the type of competency. For electrocompetent cells, a high voltage is passed through the media in a process called electroporation. For chemically competent cells, the cells are rapidly heated for a short time and then quickly cooled. Each of these processes allows DNA to enter the cell.

Following transformation, the cells are still very fragile. They are grown in rich media to allow them to recover from damaged state. If they contain the target DNA, they begin to build up selective resistance against an antibiotic. Following this period, the cells are plated on selective media so that only successfully transformed cells can grow and survive. After incubation, single monoclonal colonies can be selected which contain and check to verify the presence of the target DNA.

Some microorganisms can be made competent easily, while other cannot be made competent with our current understanding of their physiology. *E. coli* is made competent relatively easily by the methods described above. Many different strains of *E. coli* can be used depending on the desired application or experiment. The selection of a host strain is a complicated topic that requires a significant amount of background in molecular biology and genetics. For the purpose of this course, you only need to know that the strain we are using was derived from a non-pathogenic strain of *E. coli*; it was made stable for cloning applications and does not contain the *LacZ α* .

Laboratory Protocol

The exercise: You will be doing a “three-way” ligation to put together all of your cloning parts in a bio-bricks style assembly, finalizing your direct manipulation of DNA. Then, you will transform the ligated plasmid into chemically competent cells. Only products which were properly ligated and successfully transformed will confer resistance to ampicillin. You will then plate these transformations to obtain clones. A positive and negative control will help to determine if the transformation and/or ligation was a success.

Materials: Vector, pSB4A5 (digested with EcoRI and PstI)
PCR Product, BBa_J45200 (digested with EcoI & PstI)
T4 DNA Ligase
T4 DNA Ligase Buffer (10X)
1.5 mL Microcentrifuge Tubes
Deionized water
Chemically Competent Cells
Ligation Product
Sterile Water
Ice Bucket

Equipment: Heat Block
Microcentrifuge

Protocol: Part I – Three-Way Ligation of Promoter, Gene and Vector

1. label 2 1.5 mL microcentrifuge tube, one as control and one as ligation as well as your initials.
2. Add 1ul of each: vector, Gene to the tube marked ligation. Add 1ul of vector to the tube marked control
3. To each tube add 1 uL of the 10X T4 DNA ligase buffer
4. Bring total volume in each tube up to 9 uL with sterile deionized water
5. Add 1 uL T4 DNA Ligase Enzyme to each reaction mixture
6. Mix by pipetting up and down and spin to collect the liquid drop at the bottom of the tube
7. Incubate the ligations at room temperature for 10min.

Part II – Transformation of Ligation Product

1. Keep cells on ice at **ALL** times, and allow to defrost. NEVER vortex or use the pipette to mix competent cells.
2. To another tube, at 5 uL of the pUC19 control vector and label as a positive control.
3. To each of the ligation mixtures and controls, VERY GENTLY add 25 uL of chemically competent cells. Do NOT pipette up and down to mix. Each tube contains exactly 50 uL, so be very careful with the volume.
4. Incubate on ice for 30 minutes.
5. Place tubes in 42C incubator for **exactly 30 seconds.**
6. Remove the tubes and place them on ice.
7. Add 1000 uL of SOC media to each tube. Be very careful to use proper sterile technique as there is no antibiotic selection at this point!
8. Incubate the tubes at 37C for 1 hour.
9. Plate all of the transformation mixture onto an LB-Agar plate with kanamycin. Grow overnight at 37C.

Session 6: Post-Laboratory Exercises

Name: _____

Date: _____

- 1) Indicate the recipe for a three-way ligation. Your plasmid is 3000 bp and the PCR product is 500 bp. Assume that all nucleotide basepairs are 1000 Da.

Plasmid (10 ng/ μ L) _____ μ L (25 ng final)

PCR Product (50 ng/ μ L) _____ μ L (1 nM final)

Promoter (100 nM) 2 μ L

Ligation Buffer (10X) _____ μ L

T4 DNA Ligase (Enzyme) 0.5 μ L

Water μ L

Total 20 μ L

- 2) For the ligation in Question 1:
- a. Calculate the number of plasmid, PCR product, and promoter segments (number of molecules) in the mix.

 - b. What is the maximum amount of complete ligation product that could be produced?

 - c. Which is part is limiting?

 - d. If you transform all of the above ligation into 50 μL of competent cells with a density of 1×10^{12} cell per mL, what is the maximum fraction of cells that can get a complete plasmid

 - e. If you then move the entire transformation mix into 430 μL S.O.C. (final volume of 500 μL), let it build antibiotic resistance (assume no cell division in this time) and then plate the 100 μL of the mix.

What is the transformation efficiency (i.e. the percentage of ligation products recovered in cells)... (Hint: Think about how many ligation products would you expect a single colony to represent)

i. ...If you obtained 100 colonies on the plate

ii. ...If you obtained 10,000 colonies on the plate

References & Additional Reading