

## Session 6

### Ligation and Transformation

#### ***Learning Objective:***

In this lab you will become familiar with the procedures for ligating DNA pieces together and for transforming an assembled plasmid into bacteria. You will see and understand the importance of antibiotic resistance and selection in the lab.

#### ***Introduction***

In previous sessions we have seen how we can manipulate pieces of DNA by isolating them from cells, cutting them with restriction enzymes, and amplifying them with PCR. Today we will put the DNA pieces back together with a process called ligation. Ligation permanently attaches strands of DNA, so that they can be put into a cell via transformation, and used in further experiments. After transformation, the cells containing the plasmid are selected with an antibiotic, completing the cloning process.

#### ***Background:***

##### **Ligation: Joining DNA Fragments**

In earlier weeks you learned how DNA can be cut in specific locations by restriction enzymes. The opposite process, ***ligation***, joins DNA strands together. Enzymes called ligases can join the ends of two different DNA fragments, or join the two ends of a single fragment. There are two types of ligation, called ***sticky-end*** ligation and ***blunt-end*** ligation, whose names refer to the presence or absence of short 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.

Recall that compatible sticky ends can stick to each other temporarily by base-pairing. This base-pairing is responsible for the specificity of sticky-end ligation; it ensures that only the “correct” ends will come together, if we have chosen our restriction enzymes appropriately. However, after the temporary base-pairs form, the sugar-phosphate backbones of the two pieces of DNA are still separate, and they may be pushed apart by other molecules moving around. The ligase enzyme's job is to repair these gaps, linking up the two fragments into a single piece of double-stranded DNA that will not fall apart.

In this lab session you will be ligating a vector (V-A or V-B) with an insert containing pBad and a gene for red fluorescent protein, as well as a constitutive promoter and the gene for the *araC* transcription factor, which is required for pBad to function. Both parts have been cut with EcoRI and PstI, which form incompatible sticky ends. Thus, in theory, the insert cannot go into the vector “backwards”, and neither the vector nor the insert can ligate with itself. In practice, self-ligation of vector DNA is always a problem. To fight this, we treated the vector DNA with a phosphatase enzyme, which cuts off phosphate groups from the very ends of the sugar-phosphate backbone of the DNA molecule. The

ligase enzyme requires these phosphate groups on at least one of any pair of sticky ends before it can ligate them together; if neither sticky end has a phosphate, those two ends cannot be ligated. Thus, because both ends of the vector have had their phosphates removed, the vector cannot ligate with itself.

## **Transformation: Putting Foreign DNA into a Cell**

In nature, *transformation* is a process by which cells pick up foreign DNA from their surroundings and move it into their cytoplasm. Scientists and engineers can also transform cells with any DNA they wish to use in an experiment or a biological device. For a transformation to be successful, the cells must be *competent*, meaning that there must be some pore or gap in the cell wall and cell membrane, through which DNA can enter. (Recall that the interior of the lipid bilayer membrane is made of non-polar molecules. A polar or charged molecule such as DNA cannot pass through unless there is a hole in the membrane, or a specific pore protein embedded in the membrane.) Some species of bacteria are naturally competent, but the strain of *E. coli* we use in the lab must be artificially made competent before we can transform it.

There are two types of competent cells, *electrocompetent* and *chemically competent*. To make the cells competent, they are treated using cold salt solutions and glycerol. The cells 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. Chemically competent cells are rapidly heated for a short time (“heat-shocked”), and then quickly cooled. Both of these processes disrupt the cell membrane, forming holes for DNA to enter the cell.

Immediately after transformation, the cells are very fragile. They are grown in rich media for a few hours to allow them to recover from their damaged state. If they contain the proper plasmid DNA, they begin to build up selective resistance against an antibiotic. After this recovery period, the cells are plated on selective media containing that antibiotic, so that only cells which took up the DNA can survive. After incubation, single monoclonal colonies can be selected which contain the DNA, and these cells can be grown up for further experiments. Frequently, some DNA is extracted and sequenced (or run through a “diagnostic” restriction digest and gel) to verify that the cloning process produced the plasmid exactly as desired.

The two vectors we have given you, V-A and V-B, both include a gene for *ampicillin* resistance. In addition, one has *chloramphenicol* resistance and one has *kanamycin* resistance. After transformation, you will plate your cells on four sets of plates: no antibiotic, ampicillin, chloramphenicol, and kanamycin. On the “no antibiotic” plate, all the bacteria should survive, and they should grow into a *lawn* that covers the whole plate uniformly. On the antibiotic plates, only the few cells that took up the DNA will survive, and each of these will form a *colony* descended from a single cell. The number of colonies will tell you about the *efficiency* of the ligation-and-transformation procedure. The cells on the wrong-antibiotic plate should all die, and you will be able to figure out which vector you were given.



## **Laboratory Protocol**

The exercise: You will perform a ligation to put together your vector and insert DNA in a bio-bricks style assembly. Then, you will transform the ligated plasmid into chemically competent cells. You will then plate these transformed bacteria on antibiotic-containing plates to obtain colonies. A negative control will help to determine if the ligation and transformation were successful.

Materials: Vector, V-A or V-B (cut with EcoRI & PstI and treated with phosphatase)  
Insert, pBad-RFP (cut with EcoRI & PstI)  
T4 DNA Ligase  
T4 DNA Ligase Buffer (10X)  
1.5 mL Microcentrifuge Tubes  
Deionized water  
Chemically Competent Cells  
Ice Bucket  
Rich Media  
Petri Dishes with Solid Media (+/- antibiotics)

Equipment: Heat Block  
Microcentrifuge

Protocol: Part I – Ligation of Vector and Insert

1. Label two 1.5mL tubes with your team color and a plus or minus. The Plus tube will be your ligation and the Minus tube will be a negative control containing only vector DNA.
2. Add the appropriate volume of your gel purified DNA as told by the instructors. Remember to add vector DNA to both tubes, but add insert DNA to only the Plus tube. (If you need to dispense less than 2 $\mu$ L of any DNA, wait for the instructors; do not use your p20.)
3. Using an instructor's pipet (we will come to your bench), add 1 $\mu$ L of the 10X T4 DNA ligase buffer to each tube.
4. To each tube add 1 $\mu$ L of T4 DNA Ligase. Pipet slowly!
5. Mix by pipetting up and down. Collect liquid in the bottom of the tube by tapping against the bench or briefly centrifuging.
6. Bring ligation tubes to instructors to incubate at 16°C for two hours.

## Part II – Transformation of Ligation Product

1. Keep cells on ice at ALL times. NEVER rack the cells or pipette up and down to mix them. Competent cells are very delicate!
2. You will be given three 1.5mL tubes containing competent cells, labeled Ligation, Vector Only, and Water. Mark them with your group color.
3. Add 5 $\mu$ L of your ligation mixture (“+”) to the Ligation tube, add 5 $\mu$ L of unligated vector DNA (“-“) to the Vector Only tube, and add 5 $\mu$ L of sterile water to the Water tube.
4. GENTLY flick the tubes with your finger to mix.
5. Place transformation mixtures on ice for 30 minutes. During this time, come to the center of the room for another activity.
6. Prepare a timer or watch. Place tubes in 42°C heat block for exactly 30 seconds. When time is up, immediately place tubes on ice.
7. Add 250 $\mu$ L of SOC media to each tube. Be very careful to use proper sterile technique as there is no antibiotic selection at this point! (Make sure your pipet tip is clean, etc.)
8. Bring your tubes to the instructors to incubate at 37°C for 30 minutes. During this time, come to the center of the room for another activity.
9. Take three LB Agar + Ampicillin plates. Writing on the bottom of the plate (not the lid) and around the edge (not in the middle), label each plate with your group color, the date, and which transformation mixture it will contain (Ligation, Vector only or Water)
10. Pipet the entire volume of each transformation mixture onto the appropriate plate.
11. Use a plastic “hockey stick” spreader to spread the cells around the whole surface of the plate. Use a fresh hockey stick for each plate!
12. Bring plates to instructors to grow overnight at 37°C.

## Session 6: Post-Laboratory Exercises

Name: \_\_\_\_\_

Date: \_\_\_\_\_

- 1) Explain why it is critical to plate newly transformed bacteria on a plate containing antibiotics instead of a plate without antibiotics.
  
- 2) Back in Session 5, when digesting vector and insert DNA with restriction enzymes, we also treated the vector DNA with a phosphatase enzyme. Explain what the phosphatase does and why this helps our ligation/transformation work correctly.
  
- 3) Recall that your vector (V-A or V-B) is a mystery vector, with either chloramphenicol resistance or kanamycin resistance (and both vectors have ampicillin resistance). Next week, when you re-plate your transformed bacteria on Kan and Chlor plates, you will find out which vector is which. Predict whether you would expect to see a bacterial lawn, individual bacterial colonies, or no growth, for each of the following situations:

	<i>Vector:</i>	Chlor+Amp Resistant	Kan+Amp Resistant
<i>Plated on...</i>			
No antibiotic			
Ampicillin			
Kanamycin			
Chloramphenicol			

## ***References & Additional Reading***