Texas A&M University-Corpus Christi
CHEM4402 Biochemistry II Laboratory
Laboratory 6: Ligation & Bacterial Transformation

While PCR is a useful technique for producing nanogram to microgram levels of product, for most investigations it is useful to have a *cloned* copy of a particular sequence in some type of DNA vector. Cloning essentially means "to make a copy of". By placing the PCR-amplified DNA into a cloning vector, and then transferring that vector to a bacterial host cell, we have a means of "storing" our PCR amplified target sequence. Storage of the fragment is convenient for when you need to use the fragment at a later date for procedures such as restriction digest analysis, DNA sequencing, mRNA or protein expression.

Most cloning vectors are small, circular DNA molecules ("plasmids") originally isolated from bacteria (figure 1). Plasmids are essentially small (2000-10,000 bp), circular chromosomes. They contain all the necessary signals for binding the proteins involved in DNA replication and RNA transcription, which are found in the host cell along with the required nucleotides and coenzymes.

We will begin the cloning process today by joining our PCR-amplified target sequence to a plasmid cloning vector (ligation). Ligation reactions utilize a specific enzyme (DNA ligase) to covalently join DNA fragments (figure 2). We will next use the ligated PCR product/vector DNA to *transform* a bacterial host (E.coli) for the production of both DNA and protein.

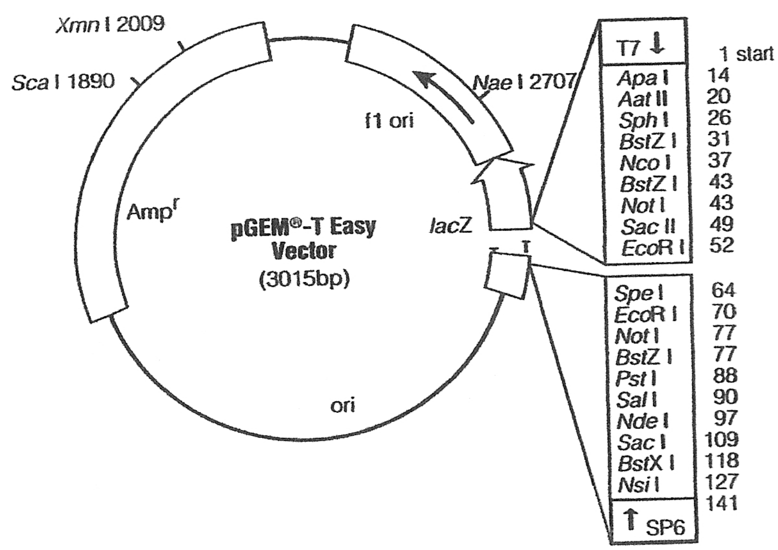

Figure 1. Map of Cloning vector pGEM T-Easy (bacterial plasmid DNA)

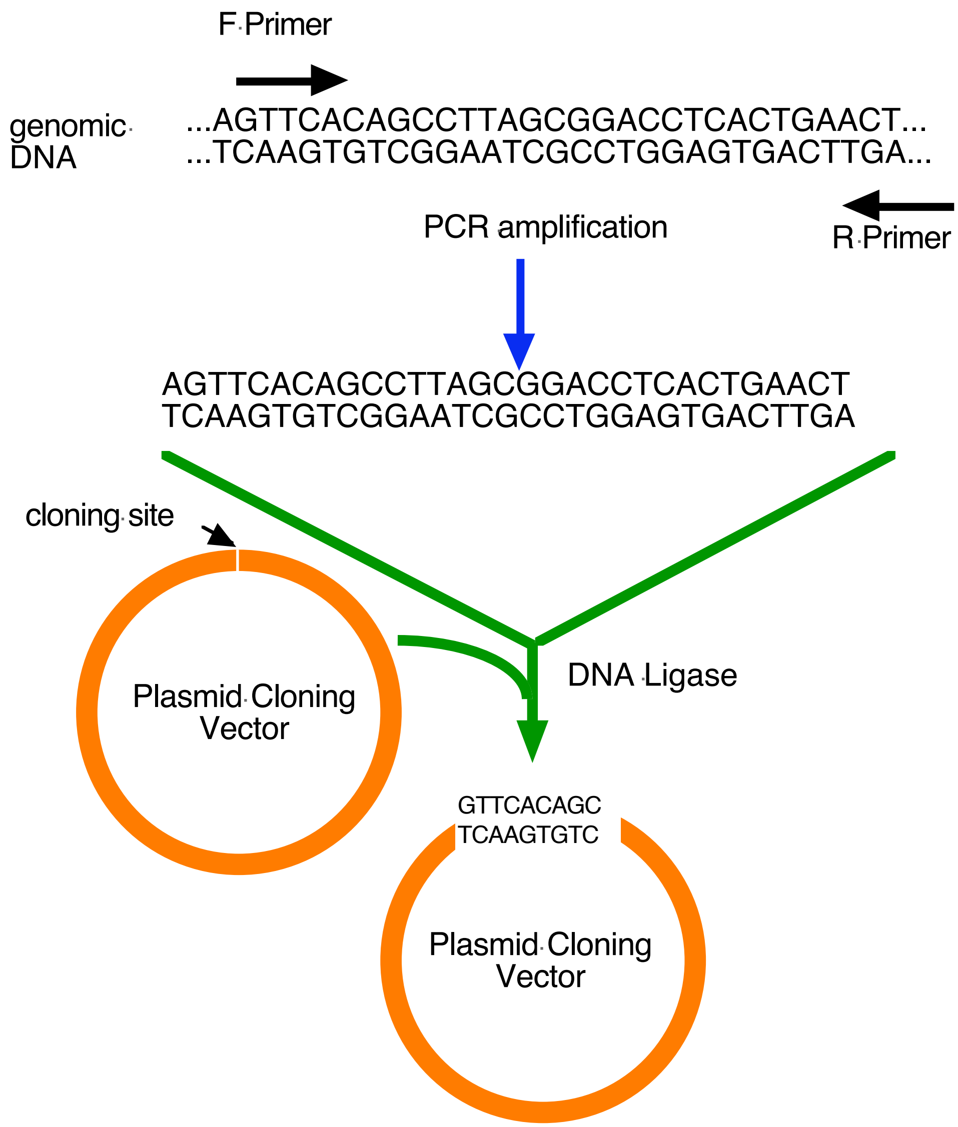

Figure 2. Ligation of PCR product into bacterial cloning vector (plasmid)

Materials

0.65 ml tube
2X rapid ligation buffer
Clean PCR product
T4 DNA ligase
Plasmid DNA cloning vector

Procedure

1. Label a 0.65 ml microcentrifuge tube with one lab partner's initials, and add the following components

2X rapid ligation buffer	5 ul
Plasmid DNA cloning vector (**instructor**)	1 ul
Cleaned PCR product	3 ul
DNA ligase (**instructor**)	1 ul
Total 10 ul	

2. Mix by gently stirring with pipet tip

3. Incubate on bench top for 60 minutes.

4. Proceed to "Bacterial DNA Transformation"

Bacterial DNA Transformation

If the ligation reaction was successful, the T4 DNA ligase will have joined your PCR product to the plasmid cloning vector to make one, continuous "recombinant" DNA molecule. In order to make multiple copies of this recombinant DNA molecule, we will insert it into bacterial *Escherichia coli* cells and allow them to grow under optimal nutrient, oxygen and temperature conditions. *Transformation* is the name given to this insertion process (figure 3). It involves the transient creation of small pores in the *E. coli* cell membrane, which allows recombinant DNA molecules to move inside. The creation of pores ("shocking) is accomplished by moving a solution of cells and recombinant plasmids from ice (4^{o} C) to a water bath at 42^{o}C. Once in, the plasmids are replicated along with the bacterial cell's genomic DNA. After 24 hours or so, cellular division results in the production of over a billion cells. Isolation of the plasmid DNA from these cells, which we will perform next week, will provide us with the necessary quantity of DNA for biochemical techniques such as restriction enzyme mapping and DNA sequencing.

Besides the molecular signals necessary for DNA replication, RNA transcription and protein translation, our plasmid DNA vector contains two unique genes, Amp^{r} and *lacZ* (figure 1). Both produce proteins that will aid the identification of bacterial colonies that contain our PCR product. The Amp^{r} gene encodes a protein that confers resistance to the antibiotic ampicillin. Including ampicillin in the LB-agar plate media will prevent cells that did not take up a copy of the plasmid from surviving. Antibiotic resistance thus provides one means of *selecting* for colonies that have taken up the plasmid.

For a variety of reasons, however, every cell that takes up a plasmid DNA does not necessarily mean that it contains our PCR product. Some cells may have been transformed with plasmid vectors from failed ligation reactions that either contained no PCR product or small DNA fragments (e.g. oligonucleotide primers). For this reason, we require an additional selectable marker to choose only those cells that possess a plasmid that also contains a rather large (>100 base pairs) fragment. This second, selectable "marker" is produced by the activity of the *lacZ* gene (actually, lack thereof). The *lacZ* gene encodes the enzyme *β-galactosidase* that catalyzes the hydrolysis (cleavage) of sugars containing a galactose residue. Scientists have exploited this property by attaching a dye molecule to a galactose monosaccharide. When this synthetic sugar is cleaved, the freed dye produces a blue color. Colonies of cells with a functional *lacZ* gene, producing active β-galactosidase will show up as blue spots on an LB-agar ampicillin plate. So how is this activity of this enzyme used as an indicator? The site for plasmid insertion of DNA fragments occurs right in the middle of this gene. Insertion of large pieces of DNA disrupts the β-galactosidase coding sequence, inactivating the gene. With no functional β-galactosidase, the transformed *E.coli* cells are unable to cleave the dye-containing sugar. Colonies with disrupted *lacZ* genes will not turn blue but show up as white spots on a LB-agar-ampicillin plate.

We therefore have a twofold selection procedure: (1) selection for cells which contain the plasmid by testing for resistance to an antibiotic, and (2) selection for plasmid-containing cells (antibiotic resistant), which contain a relatively large DNA fragment, by examining their ability to hydrolyze a dye-containing sugar. We will select colonies that are unable to hydrolyze the dye (white = disrupted *lacZ*) for culture and plasmid DNA isolation.

Materials

ligation reaction
LB-agar-Ampicillin plate
E.coli competent cells
SOC media
styrofoam cup w/ice
galactose dye screening solution (20 ul)
42ºC water bath
glass plate spreader
37ºC incubator oven (for plates)
15 ml snap-cap tube

Procedure

1. After your ligation has finished incubating, add 2 ul of the reaction directly to the bottom of a 15 ml "snap-cap" tube. Stick the tube in an ice-containing styrofoam cup.

2. ***Carefully*** transfer 50 ul of *competent* (able to be transformed) E.coli cells **directly to the 2 ul of ligation in your "snap-cap" tube**. **Gently flick 1-2x to mix.** Mark tube with your initials and return to ice.

3. Incubate on ice for 20 minutes

4. Heat ("shock") your transformation for 45 seconds in a 42ºC water bath. Do not shake.

5. Return to ice for 2 minutes.

6. Add 950 ul of room temperature SOC (growth media) to your tube.

7. Incubate for 50 minutes at 37ºC with shaking (~250 rpm - see instructor).

8. About 20 minutes before the 37ºC incubation is finished, make a plate spreader, from a disposable glass pipet, as demonstrated by your instructor. Add 20 ul of galactose-dye screening solution to a LB-agar-ampicillin plate. Spread gently around the **entire** plate with your spreader. Mark plate with your initials, section no. and date. Allow plate to absorb screening solution on your bench top until you are ready to plate your transformation.

9. When your transformation has finished incubating, transfer 1ml of the contents to a 1.5ml microcentrifuge tube. Set the centrifuge to **1.5 (1500 rpm)** and Centrifuge for 2 minutes. Pour off supernatant into a waster beaker containing a 10% bleach solution.

10. Add 100 ul of SOC to the cell pellet. Resuspend as demonstrated by your instructor.

11. Plate 100 ul of your resuspended transformation mixture onto the LB-agar-ampicillin plate. Spread gently around entire plate with a **COOL,** sterilized (ethanol and flame) spreader, but **DO NOT continue to spread transformation mixture until it is completely absorbed - this is over-spreading and will kill the cells**.

12. Let plates sit on the bench for 5-10 minutes to allow cells to be absorbed. Invert and place in the 37ºC oven to incubate overnight.

13. The next morning, one of the lab partners will need to return to the lab to take their plate out of the incubator. GFP PCR product-containing cells will show up as white spots. Count both the number of white colonies and the number of blue colonies on your plate. Wrap your plate with a strip of parafilm (instructor demonstration) and place in the refrigerator until next week (be sure to answer question 1 first). If distinguishing between light blue and white colonies is difficult, wrap your plate and place it in the refrigerator. After a few hours, return to make your count. The cold will aid color development.

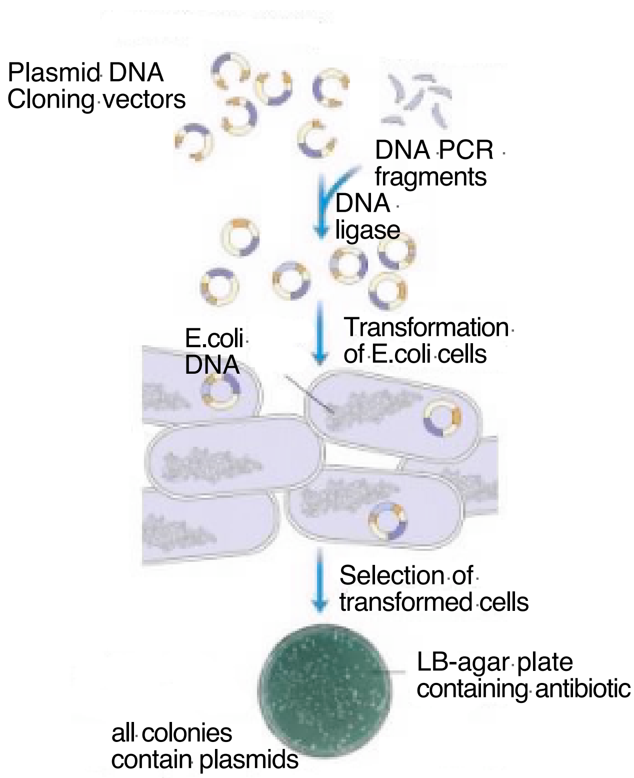

Figure 3. Transformation of E.coli cells with recombinant DNA plasmids

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READING ASSIGNMENT: Lehninger 9.1-9.2 (DNA cloning: The Basics, From Genes to Genomes)

1. How many white colonies did your plate contain (estimate if there are more than a few dozen)? How many blue colonies? What is the efficiency of your ligation reaction (No. white colonies/ total no. of colonies). (2 pt)

2. Refer to your text. List some of the features of useful cloning vectors here. (2 pt)

3. What is the purpose of the antibiotic? What is the purpose of the dye-containing galactose sugar? Be specific (2 pt).

4. Refer to figure 1. Use your textbook or other sources (library resources, internet, etc.) to define the features found on our cloning vector (4 pt).

    a. T7 promoter

    b. *Ori* (origin)

    c. f1 *ori* (origin)

    d. EcoRI site

5. Lab performance (2 pt)