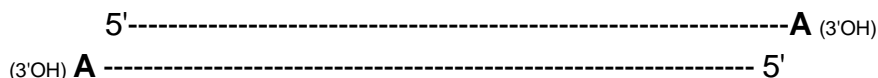


CLONING IN *ESCHERICHIA COLI*

Introduction:

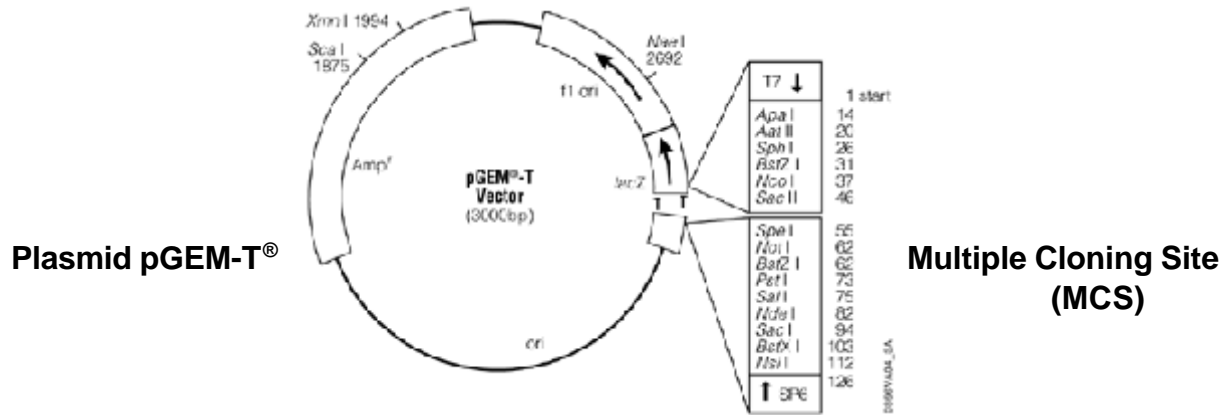
In this laboratory, you will carry out a simple cloning experiment in *E. coli*. Specifically, you will first create a recombinant DNA molecule by carrying out a **ligation reaction**, with the goal of connecting a linear DNA molecule generated by the polymerase chain reaction with a bacterial plasmid called pGEM-T[®]. Next, you will introduce the products of this ligation reaction into *E. coli* cells in a standard technique called a **transformation** experiment, and **select** for cells that have taken up plasmid DNA, based on an ampicillin resistant phenotype conferred to the cells by a plasmid-encoded gene (Amp^r – see plasmid image on next page). Finally, you will **screen** putative transformants to identify ones that have the recombinant plasmid, isolate plasmid DNA from these cells, and characterize the DNA by restriction endonuclease digestion and agarose gel electrophoresis.

The **polymerase chain reaction (PCR)** is one of the most useful techniques in molecular biology. With just a small amount of sequence information about a target DNA molecule, a pair of short single-stranded primers complementary to the ends of this DNA molecule, and a special DNA polymerase enzyme, the PCR technique can amplify the sequence to produce enough DNA for most standard cloning and gene expression experiments (see Hartl and Jones, pages 237 - 239). PCR depends on the use of a special class of DNA polymerase, isolated from thermophilic bacteria such as *Thermus aquaticus*. *Taq* polymerase is heat-stable, which is essential for its use in the PCR amplification reaction. It is also error prone because it lacks proof-reading activity. Another frequent mistake this enzyme makes is to add an additional adenine nucleotide to the 3' ends of the DNA molecule it is producing, *i.e.*,



These single nucleotide overhangs can be exploited for the creation of recombinant DNA molecules, for example, the addition of a linear PCR product into a plasmid sequence. Plasmids such as pGEM-T[®] (shown below) have been created by biotech companies (*e.g.*, Promega) to carry single thymine nucleotides at their 3' ends – these single nucleotide overhangs are complementary to the single A's at the ends of the PCR product. Base pair complementarity can orient the PCR product with respect to the plasmid sequence. The enzyme DNA ligase can then be used to generate the phosphodiester bonds between 3' hydroxyls and 5' phosphates to connect the DNA molecules and create a recombinant plasmid. You will carry out the ligation reaction “*in vitro*” (literally “in glass”; in our context, in a tube). Some of the ligation products will be the desired recombinant plasmid (the pGEM-T[®] plasmid + PCR product). There are other possibilities, including a non-recombinant form of the pGEM-T[®] plasmid whose ends have

joined together. All cells that successfully take up the pGEM-T[®] plasmid (recombinant or non-recombinant) will be ampicillin resistant. Indeed, you will **select** for cells that have taken up plasmid DNA by plating them after transformation onto agar plates containing this antibiotic. Subsequent steps of the experiment described below will allow you to distinguish between those cells that carry the desired recombinant DNA molecule and those that only carry the non-recombinant pGEM-T[®] plasmid.



www.promega.com/catalog/CatalogProducts.asp?catalog%5Fname=Promega%5FProducts&category%5Fname=&description%5Ftext=pGEM%3Csup%3E%26%23x00AE%3B%3C%2Fsup%3E%2DT+Vector+System+II&product%5Fid=A361

pGEM-T[®] has many of the basic features of bacterial plasmids. It is a relatively small circular DNA molecule that has its own origin of replication (“f1 ori”) so that it can be replicated in cells to high copy number, a selectable marker (“Amp^r”) that confers antibiotic resistance, and a “multiple cloning site” – a sequence that carries cleavage sites for many different restriction enzymes (shown vertically on plasmid map above).

pGEM-T[®] has some unique properties that make it useful for this experiment. Its single T overhangs and the multiple cloning site are positioned within the lacZ gene. As you may recall, lacZ encodes the enzyme β -galactosidase. This enzyme cleaves β -galactoside bonds, such as those between the glucose and galactose subunits of the disaccharide lactose. Ligation of a PCR product into pGEM-T[®] interrupts the lacZ sequence. Cells that take up this recombinant plasmid will not produce the β -galactosidase enzyme. Cells that take up the non-recombinant pGEM-T[®] plasmid will have an intact lacZ gene and a functional β -galactosidase enzyme. You will be able to distinguish between cells that have these two different forms of plasmid based on a colorimetric assay for β -galactosidase activity. A colorless substrate (abbreviated “X-gal”) will be added to the agar plates onto which the transformed *E. coli* cells will be plated. The X-gal molecule also has a β -galactoside bond within it. Cleavage of this bond by β -galactosidase converts the colorless molecule to one that is dark blue. When *E. coli* colonies are grown on agar plates containing X-gal, those with a functional lacZ gene (from the non-recombinant pGEM-T[®] plasmid) will be blue, whereas those that have an interrupted lacZ gene (from the recombinant plasmid) will be white. This color **screen** will allow you to identify *E. coli* cells that have the desired form of plasmid DNA within them.

(Compare this with the information on pages 417 - 418 in Hartl and Jones).

The addition of ampicillin and X-gal to the agar plates onto which you will plate the transformed *E. coli* cells provides a combined selection (for the ampicillin resistance phenotype) and screen (for the interrupted lacZ gene) for cells carrying the recombinant plasmid. The final steps of the experiment require the isolation of plasmid DNA from ampicillin resistant, white *E. coli* colonies and the characterization of this plasmid DNA by restriction digestion and agarose gel electrophoresis. For plasmid isolation, you will use a quick and easy “mini-prep kit” (QIAprep[®] kit) created by the biotech company Qiagen. In less than an hour, starting with less than 2 mls of cell culture, this protocol should yield sufficient plasmid DNA for restriction endonuclease analysis. The QIAprep[®] procedure involves concentrating a small volume of cell culture into a pellet, lysing these cells with detergent and an alkaline buffer, separating out chromosomal DNA and cell debris by centrifugation, and purifying plasmid DNA by column chromatography (on silica gel columns). After you have purified plasmid DNA from a number of putative transformant cell colonies, you will digest this DNA with a restriction enzyme to confirm the presence of the PCR product within the pGEM-T[®] plasmid. Note on the image of the pGEM-T[®] plasmid shown above that the multiple cloning sites flank the sequence into which the PCR product is ligated. Cleavage of a recombinant plasmid (pGEM-T[®] + PCR product) with EcoRI, for example, will yield at least two linear DNA molecules – one 3015 bp’s in length (the linear size of pGEM-T[®]) and others (whose size you will determine) that will be reflective of the size and sequence of the PCR product. Cleavage of non-recombinant plasmid with EcoRI will produce a single linear DNA molecule 3015 bp’s long.

Protocol:

WEEK ONE

I. Ligation Reaction

1. WEAR GLOVES AT ALL TIMES!!! USE GOOD STERILE TECHNIQUE WHEN HANDLING BACTERIAL CULTURES AND BACTERIAL GROWTH MEDIA (liquid and agar plates)!!! Recombinant DNA Guidelines requires all glass- and plastic-ware that come in contact with recombinant DNA and transformed cells be decontaminated before disposal. All used pipet tips, toothpicks, *etc.* should be placed in a beaker with bleach after you use them.

2. Set up the following ligation reactions in microcentrifuge tubes (volumes in microliters). Use the P20 pipetman. Make sure you measure volumes accurately!!! One microliter of liquid fills only the very end of the pipette tip.

	Ligation Reaction (PCR Product + pGEM-T®)	Control Ligation Reaction (pGEM-T® Alone)
2X Ligation Buffer	5 : 1	5 : 1
pGEM-T DNA	1 : 1	1 : 1
PCR Product	3 : 1	–
T4 DNA Ligase	1 : 1	1 : 1
deionized H ₂ O	–	3 : 1

- Mix the samples by spinning for 10 - 20 seconds in the microcentrifuge.
- Incubate the ligation reactions for 1 hour at room temperature.
- While the ligation reactions are incubating, prepare 4 plates onto which you will plate the cells transformed with the two different ligation reactions in the following manner: With the P200 pipetman, pipet 40 : l of X-Gal (20 mg/ml in dimethylformamide) onto the surface of an LB + ampicillin plate. With the P20 pipetman, pipet 4 : l of IPTG (200 mg/ml in H₂O) onto the surface of the plate. [IPTG serves as an inducer of the lacZ promoter, in a manner analogous to lactose, and will turn on transcription of the lacZ gene]. Immediately spread the two solutions across the surface of the agar plate using a spreader that has been surface sterilized by dipping it in ethanol and then passing it through a bunsen burner flame.
- Cover the plates and allow their surfaces to dry.

II. Transformation of *E. coli* Cells

- Briefly (10 - 20 seconds) spin down the ligation reaction tubes in the microcentrifuge.
- Transfer 5 : l of each ligation reaction to a new tube. Make sure to label the tubes so you know which sample is in which tube.
- Carefully pipet 50 : l of JM109 “Competent” Cells into each of the ligation reaction tubes. “Competent” cells have been treated such that they are permeabilized and can take up exogenous DNA. Because they are permeabilized, competent cells are quite fragile and must be handled carefully and gently throughout the rest of the experiment.
- Gently flick the tubes to mix and place them on ice for 20 minutes.

5. Heat-shock the cells for 45 - 50 seconds in a water bath at exactly 42°C. BE ACCURATE IN THIS TIMING! DO NOT SHAKE THE TUBES. In some unknown manner, this heat-shock step induces cells to take up exogenous DNA.

6. Immediately return the tubes to ice for 2 minutes.

7. Add 0.9 ml of LB medium to each tube. USE GOOD STERILE TECHNIQUE SO YOU DO NOT CONTAMINATE THE LB MEDIUM.

8. Incubate the tubes for 1 hour at 37°C with shaking.

9. Using good sterile technique, spread plate 100 : l of each of the transformations onto the surface of two LB + ampicillin + X-gal + IPTG plates that you have prepared. (2 plates for the Ligation Reaction - PCR Product + pGEM-T[®]; 2 plates for Control Ligation Reaction - pGEM-T[®] Alone). Make sure the bottoms of the plates are labeled.

10. Incubate the plates overnight at 37°C. The plates will be transferred to 4°C after this overnight incubation. You will need to score these plates before your next lab session. Count and record the total number of white and blue colonies on each plate. Transfer 5 ampicillin resistant, white colonies from the Ligation Reaction (pGEM-T[®] + PCR Product) transformation plates to 2 mls of LB + ampicillin liquid medium the evening before your next lab period. Incubate these cultures at 37°C overnight with shaking.

WEEK TWO

I. QIAPrep[®] Miniprep of Putative Transformants

1. **The evening before this lab session**, you will have counted and recorded the total number of white and blue colonies from each of your transformations. In addition, you will have inoculated 5 ampicillin resistant, white colonies from the Ligation Reaction transformations (pGEM-T[®] + PCR Product) into 2 mls of LB + ampicillin liquid medium and grown these cultures overnight at 37°C.

2. Take your 5 cultures out of the 37°C incubator and transfer 1.5 mls of each to a labeled microcentrifuge tube.

3. Spin down the cultures in a microcentrifuge, at top speed for 10 minutes. Use a balance tube filled with 1.5 mls of H₂O so you have an even number of tubes in the microcentrifuge.

4. Carefully remove and discard the supernatant (LB + ampicillin medium) into a beaker.

5. Add 0.25 ml of Buffer P1 to each tube and resuspend the cell pellets in this buffer. Make sure that no cell clumps remain.

6. Add 0.25 ml of Buffer P2 to each tube. Gently invert the tubes 4 - 6 times to mix.

NOTE: Buffers P1 and P2 lyse the cells. P1 (probably) contains an enzyme such as lysozyme (it's a company secret) that helps to digest away the bacterial cell wall. P2 is an alkaline buffer containing a detergent. Together, they are effective in lysing cells.

7. Add 0.35 ml of Buffer N3 to each tube. Gently invert the tubes 4 - 6 times to mix. The solution will become cloudy and viscous after the addition of Buffer N3. The bacterial cells should be lysed at this point of the procedure. Chromosomal DNA and RNA released from the cells makes the solution viscous. You must treat the samples gently at this point to avoid shearing chromosomal DNA which would contaminate your plasmid DNA preparation.

NOTE: Buffer N3 contains acetic acid, together with other components that are also company secrets. It is used as a neutralizing agent in this prep.

8. Spin tubes in microcentrifuge at top speed for 10 minutes. A tight white pellet should form.

9. Carefully transfer the supernatant of each tube to a QIAprep[®] Spin Column that is positioned in its associated plastic tube.

10. Spin the QIAprep[®] Spin Columns (in their tubes) in the microcentrifuge for 30 - 60 seconds. Discard the flow-through.

NOTE: Your plasmid DNA should adhere to the silica of the column at this point of the preparation.

11. Wash the QIAprep[®] Spin Columns by adding 0.75 ml of Buffer PE and spinning for 30 - 60 seconds. Discard the flow-through. Spin an additional 1 minute to remove residual Buffer PE.

NOTE: The PE Buffer serves to wash the column of materials that are non-specifically stuck. Plasmid DNA should remain attached.

12. Place the QIAprep[®] Spin Columns into clean, labeled microcentrifuge tubes. With the P200 pipetman, add 50 : l of Buffer EB to each column. Let stand for 1 minute, and then spin in the microcentrifuge for 1 minute.

NOTE: Buffer EB is a low salt buffer. Plasmid DNA should lose its affinity for the silica of the column in this buffer and will elute from the column. If you have done everything correctly, you should have a significant amount (micrograms) of plasmid DNA in the 50 : l of EB buffer.

II. Restriction Digestions

1. Set up restriction digestions of each of your plasmid DNA samples in the following manner:

	Volume
Plasmid DNA Samples	5 : l
EcoRI	1 : l
XhoI	1 : l
10X Buffer	2 : l
Deionized H ₂ O	11 : l

2. Incubate digestions at 37°C overnight. After this overnight incubation, your samples will be transferred to the freezer until your next lab period. Save the remainder of your DNA samples – you will be using some later in this experiment.

WEEK THREE

Agarose Gel Electrophoresis of Restriction Digestions

1. Prepare an agarose gel and set up the electrophoresis chamber as you did in the Restriction Analysis of Plasmid pBR322 lab. Use the comb with 12 wells for this gel.
2. Obtain your digests from your TA and allow them to thaw.
3. Add 4 : l of 6X loading dye to each of your samples. Spin briefly in the microcentrifuge to mix.
4. From each of your five original DNA samples, pipet out 10 : l and transfer each to a new, labeled microcentrifuge tube. Add 2 : l of 6X loading dye to each of these samples. Spin briefly in the microcentrifuge to mix. These will be your “uncut” DNA samples
5. Before loading your samples into the wells of the agarose gel, make sure that the gel is completely solidified, that the wells are intact and cleared of excess agarose, and that there is sufficient electrophoresis buffer in the gel apparatus to cover the gel.
6. Carefully pipet 20 : l of each digestion sample and 10 : l of each uncut DNA sample into the wells of the agarose gel. Record the order of your samples. As controls, pipet 10 : l of pGEM DNA markers, and 10 : l of pGEM-T[®] DNA (linear).
7. Attach electrodes to the gel apparatus, making sure that the negatively charged DNA

will electrophorese toward the anode (+ electrode).

8. Turn on the power supply and adjust the voltage to 100 volts. Allow the electrophoresis to proceed for approximately 1 hour or until the leading dye marker is about 1 cm from the bottom of the gel.

9. At the end of the run, turn the voltage to zero, turn the power supply off, and detach the leads from the power supply. Use a razor blade to cut the upper left corner of the gel to indicate the position of your first sample, and use a pipette tip to punch a hole in the edge to identify the gel of your group. Remove the gel from the electrophoresis chamber.

10. Your TA will place the gel into the EtBr solution (1: g/ml EtBr) for staining. After 10 minutes in the EtBr, the gel should be transferred to a water rinse for 10 minutes to remove excess EtBr from the gel.

11. View the destained gel under UV light for visualization of the DNA, and photograph the gel using the Photodyne apparatus. Be sure to follow the TA's instruction regarding contact with the EtBR stained gel. Use gloves, goggles, and long sleeves to prevent UV burns to skin and eyes.

12. Examine the photograph for the presence of bands. Observe their positions and the differences between the cut (digested) and uncut DNA samples. Determine the size (in base pairs) of the DNA in each band. Of the five DNA samples you chose, how many are recombinant? non-recombinant? What is the approximate size of the PCR product? If you see more than one band in an uncut sample, what do you think these represent? Review the number of ampicillin resistant white and blue colonies that you got from the two transformations. How do the numbers compare? How do you explain this result?