

Bio 6 – DNA Cloning Lab

Objectives

In the next four laboratory sessions you will attempt to “**clone**” a fragment of DNA by **ligating** the DNA fragment with a plasmid vector, **transforming** bacterial cells with the recombinant plasmid, **isolating** plasmid DNA from potential bacterial clones, and **analyzing** each plasmid clone for the DNA fragment by restriction enzyme digestion and agarose gel electrophoresis.

Introduction

The analysis of a gene, or any DNA fragment, requires that one **clone** the DNA sample. The term **DNA cloning** refers to the process of recombining a DNA fragment of interest with a DNA **vector** carried in a host microbe such as a bacterium or virus. In bacteria, the DNA cloning vector is typically a **plasmid**, a nonessential piece of DNA that is extrachromosomal and circular. In viruses the vector is the viral DNA itself. The cloning of relatively small DNA fragments (less than several thousand base pairs) is typically accomplished using plasmid vectors in bacteria. Larger DNA fragments require viral vectors or vectors called artificial chromosomes that are used in yeast or bacterial hosts. Regardless of the vector, once the process is complete a **clone** of the host containing the recombinant vector can be cultured to produce essentially unlimited amounts of DNA containing the fragment of interest.

You will be cloning a relatively small DNA fragment in a plasmid vector using a bacterial host, so we will focus on this particular method which involves three general steps:

- 1) **LIGATION** – combining the DNA fragment of interest with the plasmid vector
- 2) **TRANSFORMATION** – introduction of the recombinant vector into a bacterial host
- 3) **SCREENING** – identifying bacterial clones carrying the DNA of interest

The first two steps will be accomplished in a single lab session. The process of screening for desired bacterial clones will involve three separate lab sessions.

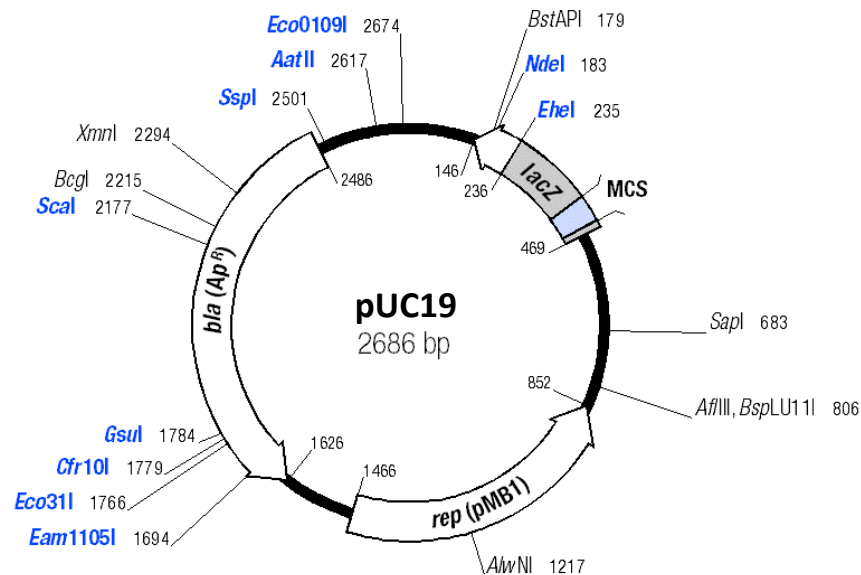
Part 1: LIGATION

Before you carry out a ligation of the plasmid vector with the DNA fragment to be inserted, it is important to review the properties of a plasmid vector, and how both the plasmid and DNA to be inserted must be treated before ligation can occur. Let’s begin with the plasmid vector...

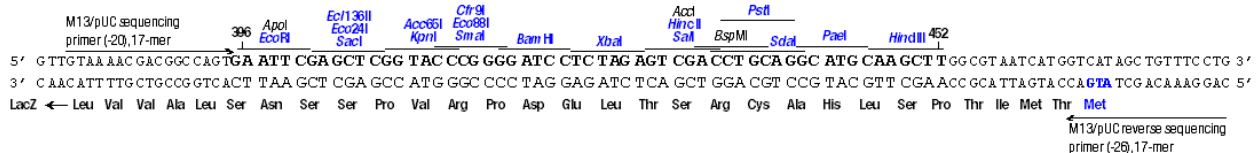
The Plasmid Vector

The plasmid vector you will use is called pUC19, a very common cloning vector used in labs throughout the world. This plasmid has four important characteristics that make it useful as a cloning vector:

- 1) origin of replication
- 2) ampicillin resistance gene
- 3) *LacZ* gene encoding the enzyme β -galactosidase
- 4) multiple cloning site (MCS) within the β -galactosidase the gene



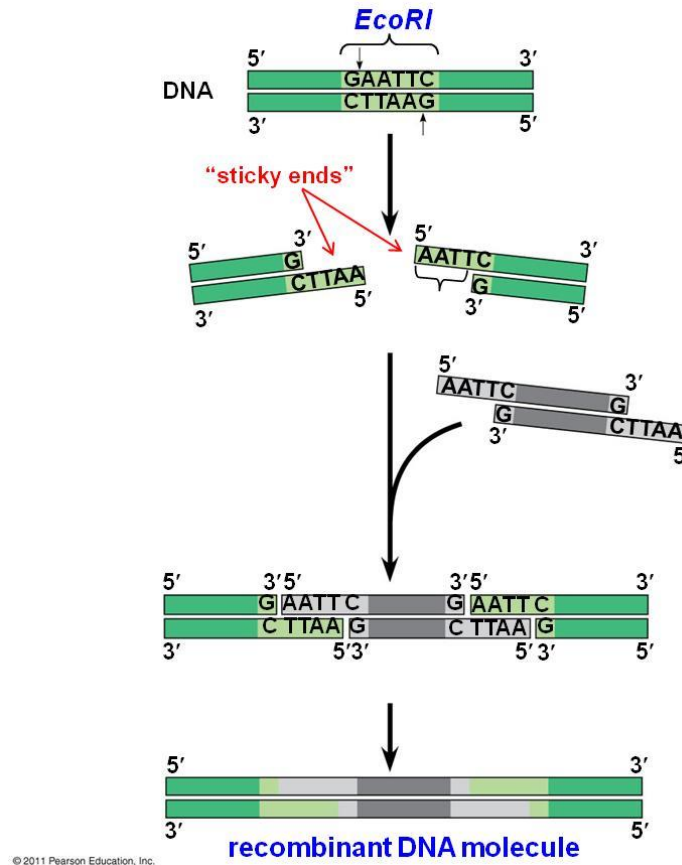
pUC19 MCS



An **origin of replication** (“rep” in the map above) is a DNA sequence that is essential for any plasmid. Without it the plasmid would never be copied by DNA replication in the bacterial host and thus never passed on to daughter cells. The **ampicillin resistance gene** (*Ap^R*) encodes an enzyme that degrades the antibiotic **ampicillin**. This ensures that only bacteria containing the plasmid will survive exposure to ampicillin which is extremely useful in the screening process. The **multiple cloning site** (MCS) consists of a short stretch of DNA containing a variety of unique restriction sites. This is where a DNA fragment of interest will be inserted into the plasmid. The *LacZ* gene produces the enzyme β -galactosidase which is involved in the catabolism of the sugar lactose. β -galactosidase can also catalyze the hydrolysis of an artificial substrate called **X-gal**, a molecule that resembles lactose and yields a blue product when hydrolyzed. Colonies of bacteria containing the plasmid will therefore be blue if X-gal is in the medium. The location of the MCS within the *LacZ* gene is extremely useful for the screening process since a DNA fragment inserted into the MCS will disrupt the *LacZ* coding region and thus no functional enzyme is produced. As a result, bacteria containing the plasmid with DNA inserted into the MCS should have a white color. This is the basis of what is commonly called “blue/white selection” in the cloning process.

Preparing the insert DNA and plasmid vector

Ligation of a plasmid vector with a DNA fragment is accomplished using the enzyme **DNA ligase**. Simply mixing the DNA molecules to be joined with DNA ligase, however, is not sufficient to get the job done. The two DNA molecules to be joined must be cut with restriction enzymes to create **compatible** DNA ends. This will also direct ligation to the desired location in the vector. For most ligations this means cutting the plasmid vector and the DNA insert with the same restriction enzyme as illustrated below:



If the plasmid and insert DNA are cut with the same restriction enzyme, the ends of each fragment will be the same and thus compatible. This is true whether the enzyme produces 5' overhangs, 3' overhangs or blunt ends. Compatible 5' or 3' overhangs, though, provide an extra advantage in that the overhangs are complementary creating "sticky ends" which greatly enhance the efficiency of ligation.

When the plasmid vector is cut or **linearized** with a single restriction enzyme, it can easily be ligated with itself to restore the original plasmid without any inserted DNA. This is a ligation product you clearly want to avoid and can do so by treating the cut plasmid DNA with a type of enzyme called a **phosphatase**. Phosphatases catalyze the removal of phosphate groups from a substrate, and treating the cut plasmid vector with a phosphatase will remove the 5' phosphates from each end of the DNA molecule. Without the 5' phosphates, the linearized plasmid cannot be ligated to itself. The DNA insert, however, is not treated with phosphatase and can still be ligated with dephosphorylated plasmid vector.

This issue can be avoided by cutting the plasmid with 2 different restriction enzymes and cutting the ends of the insert with the same 2 enzymes (this is the case for the vector and insert you will be ligating). With 2 different ends the plasmid can't ligate with itself so dephosphorylation is not necessary.

Planning a ligation reaction

As with any other enzymatic reaction, it is essential that you plan a ligation reaction first to ensure that you assemble the correct amounts of each component. First of all, it is important to identify the components that go into a ligation reaction:

prepared plasmid DNA
prepared insert DNA
10X DNA ligase buffer
H₂O (ultrapure)
DNA ligase

A typical ligation reaction will be carried out in a total of 20 μ l with \sim 2 units of DNA ligase and 50 ng of prepared plasmid DNA. The molar ratio of insert to plasmid DNA is typically 3:1. This ratio maximizes the yield of ligation products with only one copy of the insert DNA. Assuming a reaction will have 50 ng of plasmid DNA, a 3-fold molar excess of insert DNA can be calculated as follows:

$$50 \text{ ng of plasmid} \times \text{length of insert (bp)} / \text{length of plasmid} \times 3$$

For example, if the plasmid is 3000 bp and the insert is 600 bp, the calculation would be:

$$50 \text{ ng} \times 600 / 3000 \times 3 = 30 \text{ ng of insert DNA}$$

Once assembled, the reaction is incubated at room temperature for 1 $\frac{1}{2}$ hours. Although the ligase works best at 37 $^{\circ}$ C, this is too warm for the sticky ends to effectively base pair. Room temperature is cool enough for the base pairing of complementary sticky ends and results in a more efficient ligation.

Exercise 1 – Ligation of plasmid and insert DNA

You will ligate 50 ng of pUC19 (2686 bp) with a 3-fold molar excess of a 650 bp DNA insert, both of which have been cut with the restriction enzyme EcoRI. Working with the other students at your table, plan and carry out the ligation of the two fragments as described below:

1. Refer to the guidelines above and plan a ligation using 2 units of DNA ligase and a total reaction volume of 20 μ l. The plasmid and insert DNA are both at a stock concentration of 10 ng/ μ l, and the DNA ligase stock is 5 units/ μ l.
2. Assemble your reaction, being sure to add the enzyme last, mix gently by tapping and quick spin.
3. Incubate the reaction at room temperature for 1 $\frac{1}{2}$ hours.

Part 2: TRANSFORMATION

Once your ligation reaction is complete, you are ready to induce bacterial host cells to internalize the ligation products, a process called **transformation**. This is not a trivial process since the bacteria you will be transforming have to transfer the DNA across a rigid cell wall sandwiched between two membranes. However when chemically pretreated to make them **competent** and exposed to a brief heat shock, the bacteria readily internalize DNA from their immediate surroundings. If the internalized DNA is a circularized plasmid, it will be copied and transmitted to all daughter cells (bacteria can complete DNA replication only if the molecule is circular).

Your ligation reaction will produce a very heterogeneous collection of DNA molecules. Most molecules will be linear plasmid and insert DNA along with various ligation products that did not circularize. If internalized by bacteria, none of these DNA fragments will be copied or passed on to daughter cells. The only ligation products that can successfully transform bacteria are recircularized plasmids, regardless of the presence of the DNA insert.

Exercise 2 – Transformation of bacteria with ligation products

1. Completely thaw a tube of competent bacterial cells on ice.
2. Add 1 μ l of your ligation reaction to the thawed competent cells, tap *gently* to mix, and place on ice for 30 minutes.
3. Place the tube in a 42° C water bath for 30 seconds, then immediately place on ice *without mixing* and leave for 5 minutes.
4. Transfer all 50 μ l of the heat-shocked bacteria to tube containing 1 ml of sterile LB medium and place the tube in a 37° C shaker for 1 hour.
5. Use a P200 with a sterile tip to drip 100 μ l of the transformed bacteria on the surface of an LB agar plate containing ampicillin and X-gal. Sterilize a plate spreader with alcohol and a brief flaming and spread the transformed bacteria on the plate as demonstrated by your instructor.
6. Label the plate and place *lid down* in a 37° C incubator overnight.

Part 3: SCREENING FOR POSITIVE BACTERIAL CLONES

Inoculating bacterial clones

At this point in the DNA cloning process you should have an agar plate containing both blue and white colonies. It is important to realize that each colony is derived from a single bacterial cell and thus all the bacteria in a given colony are clones of the original cell. In addition, the only bacterial cells capable of growing into a colony are those that acquired a plasmid with ampicillin resistance during the

transformation process. Recall that blue colonies should consist of bacterial clones transformed with a plasmid that does not contain a DNA insert in the MCS. Conversely, each white colony should consist of bacterial clones that do have a DNA fragment inserted in the MCS.

The first step in the screening process is the inoculation of randomly selected colonies from which you will purify plasmid DNA to test by restriction enzyme digestion...

Exercise 3A – Inoculation of selected bacterial clones

For this exercise you will randomly choose 3 white colonies and 1 blue colony for inoculation. The white colonies are clones that potentially contain your 650 bp DNA insert. The blue colony is a negative control.

1. Obtain 4 sterile plastic culture tubes and label them 1 to 4.
2. Use a sterile pipette to add 3 ml of sterile LB medium with ampicillin to each tube.
3. Inoculate 3 random white colonies into tubes 1 to 3 as follows:
 - sterilize a pair of metal forceps by dipping in alcohol and briefly flaming (*be careful not to drip flaming alcohol!*)
 - grab a sterile toothpick with the forceps and touch a white colony with one end of the toothpick
 - place the entire toothpick, inoculated end down, into the corresponding labeled tube
4. Inoculate a single blue colony into tube 4 in the same manner.
5. Make sure the cap of each tube is loose, and incubate the tubes in 37° C shaker overnight.

Purifying plasmid DNA

You are now ready to purify the plasmid DNA from each of the six bacterial cultures you inoculated in the previous laboratory session. In most laboratories this is referred to as doing “plasmid mini-preps” since you are isolating plasmid DNA on a small scale. There are many different approaches to doing plasmid mini-preps, many of which involve commercially produced kits for this purpose. Commercial kits are very useful and reliable, though they can be expensive. Luckily there are several very effective plasmid mini-prep protocols that require relatively inexpensive materials common to most laboratories. You will be carrying out one such protocol which is referred to as the “boiling method”.

In principle, the process is rather straightforward. The bacterial cells are lysed in a solution containing a detergent plus **lysozyme**, an enzyme that degrades the bacterial cell walls. The lysate is then briefly boiled to coagulate and remove the proteins and chromosomal DNA from solution. The much smaller plasmid DNA remains in solution and can be easily purified by **alcohol precipitation**, a process by which DNA is made insoluble by adding the right amount of alcohol.

Exercise 3B – Plasmid purification (boiling method)

1. Label four microcentrifuge tubes 1 to 4 and set a beaker of water to boil on your hot plate.
2. Pour 1.5 ml of the corresponding bacterial culture into each tube, being careful not to spill or over fill.
3. Spin the tubes at full speed in a microcentrifuge for 30 seconds. This will pull the bacterial cells to the bottom of the tube.
4. Use a small fine-tipped transfer pipet to remove and dispose of the supernatant into a small beaker (this is biohazard waste which will be handled by the lab technician).
5. Repeat steps 3 and 4 to get rid of any remaining liquid.
6. Use a P1000 to *completely* resuspend each bacterial pellet in 300 μ l of STET/lysozyme solution, and leave at room temperature for *at least* 2 minutes. This will disrupt the bacterial membranes and cell wall releasing the cellular contents. STET/lysozyme solution contains the following:
 - 8% sucrose (S)
 - 5% Triton X-100 detergent (T)
 - 50 mM EDTA (E)
 - 50 mM Tris-HCl pH 8.0 (T)
 - 2 mg/ml lysozyme
7. Place all six tubes in a boiling rack and boil for **exactly 50 seconds**, then spin the tubes at maximum speed in your microcentrifuge for 10 minutes.
8. Use a sterile toothpick to remove the “goop” from the bottom of each tube and dispose of the goop in the biohazard waste.
9. Estimate the approximate volume of liquid remaining in each tube (refer to the markings on the tube) and add an equal volume of isopropanol to the remaining liquid in each tube, vortex to mix, and place on ice for 10 minutes to precipitate the plasmid DNA.
10. Spin the tubes at maximum speed in your microcentrifuge for 10 minutes to pellet the precipitated plasmid DNA.
11. Remove the liquid supernatant in each tube using a small fine-tipped transfer pipet and discard into the small beaker of biohazard waste. Quick spin and remove any remaining liquid using a P200. Air dry the DNA pellet (leave lid open) for ~5 minutes.
12. Resuspend each DNA pellet in 50 μ l of TE with 10 μ g/ml RNase (to degrade any RNA in the samples).

Analyzing plasmids by restriction enzyme digestion

To see if any of your bacterial clones contain a plasmid with a DNA insert of the desired length, all you need to do is cut a sample of the plasmid DNA with an appropriate restriction enzyme and subject the resulting DNA fragments to agarose gel electrophoresis. If the restriction site used to ligate the insert DNA with the plasmid vector was recreated when the fragments were

joined together, then you can simply cut the DNA with the same restriction enzyme. On occasion the cloning restriction site is not recreated and therefore you would need to examine the MCS and cut with restriction enzymes that are on either side of the original cloning site.

Exercise 3C – Restriction enzyme digestion and agarose gel electrophoresis

Any plasmid clones containing the desired DNA insert should have recreated EcoRI restriction sites on each side of the DNA insert. Digestion of a positive plasmid with EcoRI should therefore release the 650 bp DNA insert which will be detected by agarose gel electrophoresis:

1. Plan *EcoRI* digests of your four plasmid samples such that each reaction contains 5 μ l of plasmid DNA and 5 units of each enzyme in a total volume of 30 μ l (refer to Lab 11 if necessary).
2. Pool all common components (everything but the plasmid DNA) such that you have enough for one extra reaction and assemble your digests (refer to Lab 9 if necessary).
3. Incubate each reaction at 37^o C overnight.
4. Prepare and pour 60 ml of a 1% agarose gel with 1X TAE.
5. Add 3 μ l of gel loading dye to each reaction, mix and quick spin.
6. Load all 33 μ l of each sample on the agarose gel along with 10 μ l of 1 kb DNA ladder and 10 μ l of uncut plasmid DNA and run at 100 volts for \sim 1 ½ hours.
7. Stain the gel with an InstaStain ethidium bromide card as described in Lab 11.
8. Produce a photographic record of your gel and place in your notebook.
9. Examine the length of each DNA fragment in each lane. A positive clone should produce a 2686 bp band corresponding to the linearized plasmid, and a 650 bp band corresponding to the DNA insert you attempted to clone.

NOTE: Positive clones on your gel should look just like the digests you did in the Restriction Enzyme Digestion lab.

Once a positive clone has been identified, the next step would normally be to sequence the DNA insert in the plasmid to confirm its identity. Once confirmed, a culture sample of the positive clone is preserved and can be used to grow as much of the plasmid as needed.

NOTE: For this laboratory you will turn in a formal lab report.

DNA Cloning Lab– Study Questions

Name:

1. What is a plasmid?
2. What is a vector? What vector was used in this experiment?
3. Explain how having a MCS (Multiple Cloning Site) in the middle of the Lac Z gene of pUC19 allows for the selection of bacteria containing plasmids with or without an insert.
4. What are the roles of X-Gal and ampicillin in the cloning process?
5. Define the terms **ligation** and **transformation**.
6. What enzyme is required to covalently bond a linear DNA fragment into a plasmid?

7. Explain what is meant by the term “competent cell” as it relates to transformation.
8. Diagram what you would expect to see on your agarose gel for *EcoRI* digests of plasmid DNA from white colonies vs blue colonies in your cloning project.
9. Pretend you cloned a DNA insert cut with *SpeI* into the *XbaI* site of pUC19 resulting in the destruction of the *XbaI* site (*SpeI* and *XbaI* have slightly different restriction sites that produce the same sticky ends which when joined produce a sequence that can't be cut by either enzyme). What restriction enzymes would you cut the plasmid samples with to release the insert?
- HINT: Refer to the pUC19 MCS sequence on the second page of this lab (page 158) and the New England Biolabs catalog to find compatible enzymes for a double digest.*
10. What are the roles of **lysozyme** and **phosphatase** in the cloning process?