

### Cloning a DNA fragment in *E. coli*

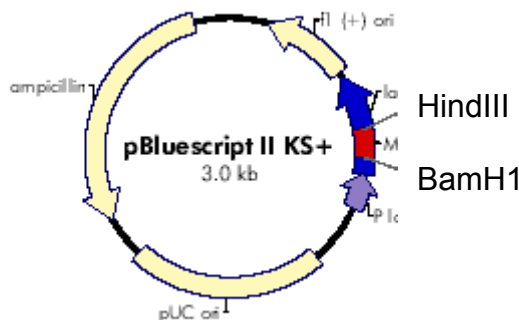
Report due Nov. 10/11.

**Reading:** Hartwell Chapter 9 pp. 303-326.

**Objectives:** In this lab we will clone a fragment of DNA that carries the information to encode an antibiotic resistance gene into a plasmid. As a result we will have a DNA circle that can replicate in *E. coli* and express the protein for antibiotic resistance. The objective is to familiarize you with the process involved in this technique and to expose you to the information components needed to express a foreign gene in an organism.

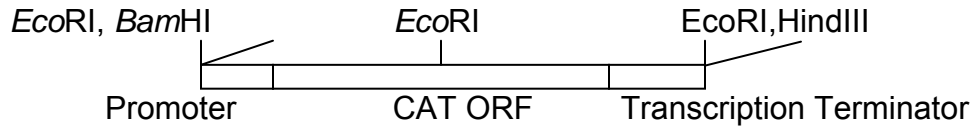
**Introduction:** We are cloning an antibiotic resistance gene (CAT) with plant-specific expression sequences into a bacterial plasmid. This would be one step in building a plant transformation vector that would allow us to select transformed plants. I have chosen this cassette because it is flanked by restriction sites, making it easy to cut out of the plasmid that is used to propagate the DNA. We will isolate this *Bam*HI-*Hind*III fragment from its current vector and ligate it to another plasmid that will replicate in *E. coli*. The plasmid must carry an *E. coli* compatible replication origin in order to replicate. The recipient plasmid will be pBluescript. This was designed by the company, Stratagene. It has an ampicillin resistance gene so that you can identify bacteria that carry it. It also has a multiple cloning site with many restriction enzyme target sites following one another closely. The multiple cloning site is in the middle of the *lacZ* gene, between the promoter and most of the protein coding sequence. It is inserted so that the *lacZ* protein is still properly expressed. However, insertion of foreign DNA into the multiple cloning site disrupts expression of the *lacZ* protein. Since colonies expressing LacZ protein can metabolize a white substrate (X-gal) to blue indigo, the Bluescript vector allows "Blue-White" selection of plasmids with inserts. Ampicillin resistant bacteria that grow as white colonies on media containing X-gal have an insert and blue colonies do not.

Map of pBluescript



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## Map of CAT cassette

**Materials:**

Gloves

Sterile eppendorf tubes

Pipetmen and sterile tips

Sterile distilled water

Restriction salts NEB2      10  $\mu$ l per groupLigation salts                      10  $\mu$ l per group

Ligase

*Bam*H1 enzyme (TAs should have extra enzyme and buffers available in case*Hind*III enzyme                      students have trouble pipetting small volumes)Purified cut vector at 20 ng/ $\mu$ l      5  $\mu$ l per groupPurified cut insert (CAT cassette) at 20 ng/ $\mu$ l      5  $\mu$ l per groupUncut vector 10 ng/ $\mu$ l in 10 mM Tris buffer      25  $\mu$ l per groupUncut insert plasmid 10 ng/ $\mu$ l in 10 mM Tris buffer      20  $\mu$ l per group1 kb ladder      10  $\mu$ l

Cut vector and cut insert plasmid (not purified) 100 ng each

Competent cells (3 tubes per group)

LB 100  $\mu$ g/ml ampicillin X-gal plates (4 per group)LB 30 $\mu$ g/ml chloramphenicol plates (1 per group)LB 100  $\mu$ g/ml ampicillin (1 per group)

LB plates (1 per group)

Alcohol lamps

Spreaders

Sterile toothpicks

Sterile 1.5 ml eppendorf tubes

Floats for water baths

Water baths at 37 degrees

10X gel loading dye

10 X TAE buffer

agarose

10 mg/ml Ethidium Bromide solution in water (EtBr)

gel tanks

gel molds

Power supply

Transilluminator

**Recipes:** TA prepares LB amp X-gal plates before class

LB amp X-gal plates

Dissolve X-gal in DMF (dimethylformamide) to 4% weight per volume

Add 800  $\mu$ l to 500 ml of melted LB agar (cooled to 60 degrees), add ampicillin to 100  $\mu$ g/ml (500  $\mu$ l of 100mg/ml stock).

10X gel loading dye:

1 kb ladder for 100  $\mu$ l:

10  $\mu$ l commercial ladder

10  $\mu$ l 10X gel loading dye

80  $\mu$ l TE (10 mM Tris pH 8.0; 0.1 mM EDTA pH 8.0)

use 5  $\mu$ l per sample

NEB2:

10 mM Tris-HCl

10 mM MgCl<sub>2</sub>

50 mM NaCl

1mM Dithiothreitol

pH 7.9

Ligation salts:

50 mM Tris-HCl

10 mM MgCl<sub>2</sub>

10 mM Dithiothreitol

1 mM rATP

25  $\mu$ g/ml Bovine Serum Albumin

pH 7.5

1X TAE buffer:

40 mM Tris-acetate pH 8.0

1 mM EDTA pH 8.0

Agarose gel: need 200 ml per gel

For 200 ml:

20 ml 10X TAE

1800 ml H<sub>2</sub>O

1.4 g Agarose

Heat in a microwave to dissolve the agarose

Cool to 60 degrees

Add 10  $\mu$ l Ethidium bromide

Keep at 60 degrees for pouring in class

### **Methods:**

Wear gloves. Your hands are a source of degrading enzymes as well as contaminating DNA.

#### 1. Prepare LB ampicillin X-gal plates and warm them at 37 degrees:

Each group should label 4 LB ampicillin X-gal plates as follows

1. negative control, date and initials
2. positive control, date and initials
- 3, 4. test ligation, date and initials

Also take an LB plate and label it 5 growth control, with date and initials.

Warm this up as well

Put the 4 LB amp X gal plates and one LB plate at 37 degrees to warm up.

## 2. Ligation:

You will be given a tube of cut vector plasmid and a tube of purified CAT cassette.

Mix 1  $\mu$ l of vector with 7  $\mu$ l of CAT cassette. Add 1  $\mu$ l of 10X ligase buffer. Add 1  $\mu$ l ligase enzyme. Check your sterile tip to be sure you have 1  $\mu$ l, not more or less. Mix and let stand on your bench for 1 hour. Keep the enzyme cold in an ice bucket at all times. Take the tube out briefly to remove an aliquot and return the tube to the ice.

Fragments should be at 100 ng/ $\mu$ l for vector and 20 ng/ $\mu$ l for insert.

Negative control: Set up a ligation mix but replace the CAT cassette insert fragment and ligase with water. Do not add ligase.

Positive control: Add 9  $\mu$ l mixture of uncut vector (at 1 ng/10  $\mu$ l) plasmid to  $\mu$ l of 10X ligase buffer. Do not add ligase.

Let stand at room temperature for 1 hour.

## 3. Prepare a sample of restriction enzyme cut vector plasmid and cut the CAT cassette out of its plasmid.

Label the tubes for each restriction digest. One will be the vector plasmid cut with *Bam*HI and *Hind*III and the second will be the CAT cassette plasmid cut with the same.

To each tube add: 6  $\mu$ l sterile distilled water, 2  $\mu$ l 10X restriction enzyme salt, 10  $\mu$ l appropriate plasmid (at 10 ng/ $\mu$ l) and 1  $\mu$ l of each restriction enzyme. Add the enzyme to the mix last (always after the salt solution has been correctly adjusted). Keep the enzymes on ice when not in use.

Set up negative controls of each plasmid replacing the enzymes with water. We will add positive controls of precut plasmid to the gels.

(Note to TA: Have 50 ng of positive controls for each gel. Run 5  $\mu$ l of 1 kb ladder, a 10  $\mu$ l sample of uncut and a 10  $\mu$ l sample of cut for each plasmid at the left of each row of digests, Have each group's digests and controls (4 per group) following)

Incubate the digestion tubes at 37 degrees in an incubator for 1 hour.

After digestion is over, add 2  $\mu$ l of 10X dye.

Pipette sample into the well of a 0.7% agarose gel.

Apply power. The negative lead goes to the end of the gel that was loaded. The DNA has a negative charge and will run to the positive lead.

We will make a set of gels for the class during the class period. The gels will be made with 1X TAE buffer, agarose and water, heated in a microwave to dissolve all agarose and then cooled to 60 degrees. We will add ethidium bromide at 5 $\mu$ l per 100 ml. In the lab period, we will demonstrate how to assemble the gel form and pour the gel. Let it stand for 30 min. We will put the gels into gel tanks and cover them with TAE buffer. Each group will load samples their samples as instructed by the TA and the TA will apply electric current. The gel will need to run at 90 volts for approximately one hour.

4. Transform *E. coli* with ligation mix. Meanwhile, we will transform competent *E. coli* with the ligation mixture and controls. Each group will do three transformations.

The competent cells are stored on dry ice. When your ligations are finished incubating, get three tubes of competent cells and put them in your ice bucket. Label the tubes: 1 is for the negative control ligation. 2 is for the positive control ligation and 3 is for the test ligation mix. Allow the cells to thaw on the ice. Add 5  $\mu$ l of DNA mix from the appropriate ligation tube. Mix gently by stirring with the pipette tip, keeping the end of each tube in the ice. This allows the DNA to mix with calcium salt in the competent cell buffer to form a fine calcium precipitate that can enter the bacteria. Let the tubes stand on ice for 5 minutes and then plate.

Plate the cells on media: Plate  $\frac{1}{2}$  or 50  $\mu$ l of the negative control transformation on a prewarmed LB plate (plate 5), and the other half (50  $\mu$ l) on one of the prewarmed LB ampicillin X-gal plates (plate 1). Plate 50  $\mu$ l of the positive control transformation on another LB ampicillin X-gal plate (plate 2) and plate 50  $\mu$ l (or  $\frac{1}{2}$ ) of the transformation with your ligation onto each of 2 LB ampicillin X-gal plates (plates 3 and 4). Incubate the plates at 37 degrees. Transferring the cells from ice to warm plates gives them the heat shock necessary to take up DNA.

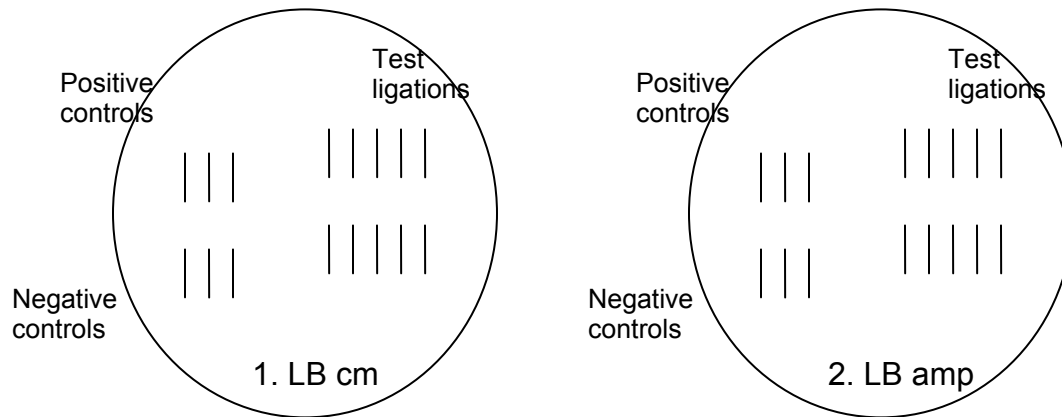
Day 2: Examine the transformation plates: Count the number of white and blue colonies on the LB amp 50 Xgal plates. Compare the number of white colonies on the negative control plate to the test ligation plates. Note if the LB plate has a confluent lawn of bacteria or not.

Check your transformants from the vector plus insert ligation reaction to see if they are now chloramphenicol resistant.

Set up an LB chloramphenicol plate and an LB ampicillin plate. You will make them duplicates. On one side you will streak controls and on the other side you

will test 10 individual colonies from the transformation plate for resistance to chloramphenicol. Set up your plates according to the diagram below.

### Duplicate Plates to test chloramphenicol resistance



Start with the positive control. You will be provided with a plate containing colonies that are resistant to both antibiotics. Touch the end of a sterile toothpick into an isolated colony. Make a small streak on the chloramphenicol plate. Then make a small streak at the same place on the ampicillin plate. Underneath the plate write + cont. under the streak. Use any colonies growing on your negative control transformation ampicillin plate to make a negative control for this test. (We will also provide a negative control plate in case you have no colonies on yours). Make a streak in the same place on both plates and mark – cont. underneath.

On the other side of each plate make a streak of 10 different individual colonies from the vector + insert ligation transformation plate. Always streak onto the chloramphenicol first. The ampicillin plate is a control to be sure you had bacteria on the toothpick.

Day 3 Examine your ampicillin and chloramphenicol plates from Day 2. The positive control should grow on both plates, the negative control should only grow on the ampicillin plate. How many of your 10 test transformants grew on both plates?

In real life the next step would be a simple procedure to purify the cloned plasmid DNA, cutting it with the appropriate restriction enzymes to be sure the insert and vector were the expected size.

**Report:** Restriction enzymes: We will post a photo of the restriction enzyme digestions on the lab web site. Please include a picture of your cut samples and

label it to explain what the bands represent (cut plasmid: uncut plasmid: open circle form, supercoiled form).

Transformation: Check to be sure you have a lawn of colonies on the LB plate. You do not need to count them.

Make a table of how many ampicillin resistant colonies came from each transformation. Take the average of the two test ligation plates. Report the percentage of ampicillin resistant colonies that were blue and white for each transformation.

Describe your test to determine if the test ligation colonies were resistant to both ampicillin and chloramphenicol. What proportion of the ampicillin resistant colonies you plated were also resistant to chloramphenicol? What were the results with your positive and negative controls on the chloramphenicol and LB amp plates? Did the test for chloramphenicol resistance work as expected?