

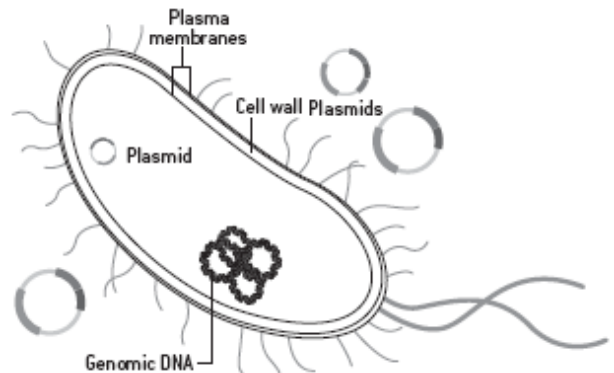
# Lab 5: Transforming *Escherichia coli* with a Recombinant Plasmid

**Thus far, you've produced ligated,** recombinant plasmids. Hopefully, some of these DNA recombinants will have the 702bp pKAN-R fragment (the *rfp* gene) ligated into the large pARA restriction fragment. This plasmid is referred to as pARA-R. Now, we want to get these recombinant plasmids into bacterial cells so that we can get the cells to express *rfp* gene and make the mutant fluorescent protein.

The process of taking up foreign pieces of DNA, like a plasmid, into a bacterial cell is called transformation. Transformation is a process that occurs in nature, although it is probably somewhat rare. A British medical officer, Frederick Griffith first studied the process, in 1928. Bacteria usually pass on extra chromosomal genetic material, like plasmids, during conjugation (bacterial sex) rather than relying on luck. But taking up plasmids can provide bacteria with certain genes that confer selective advantage, for example, antibiotic resistance. Under experimental conditions, however, it is possible to prepare cells so that about one cell in a thousand will take in a plasmid from the surrounding environment.

There are several factors that determine transformation efficiency. Two of these are related directly to the plasmid used for transformation. The larger the plasmid, the less likely it will be taken up by the bacterium. Remember, in order for the bacterium to take in foreign DNA, the plasmid must pass through bacteria's plasma membrane and cell wall.

Therefore, small plasmids are more likely to pass through the bacterium's plasma membranes (*E. coli* has two) and its cell wall than large plasmids.



Plasmids can assume different shapes. The supercoiled form is the easiest to get into the cell while the nicked-circle or the multimer, two or more plasmids linked together, are more difficult. The ligation tube, containing the recombinant plasmids you prepared, does not contain any supercoiled plasmids. Supercoiling of a plasmid requires an enzyme that is found in the bacterial cell; it was not included in your ligation tube. The recombinant plasmids you prepared are primarily nicked-circled, but there is a wide variation in sizes.

In nature, transformation is a relatively rare event. To increase our chances of getting our recombinant plasmids into bacterial cells, we will use “competent” cells. When cells are “competent,” it means that they are ready to receive plasmids. For the most part, you don't find competent cells in nature; instead, cells have to be made competent in the laboratory. One common way this is done is by soaking the cells in calcium chloride.

Remember that DNA is negatively charged. Do you remember why? The plasma membranes surrounding the bacterial cell also contain phosphate groups and are negatively charged. The problem of trying to get negatively charged DNA past a negatively charged membrane is that like electrical charges tend to repel each other. When cells are made competent, they are suspended in a solution of calcium chloride because calcium ions (positively charged atoms

of calcium, Ca<sup>++</sup>) help to neutralize the negative electrical charges of the plasma membrane and the plasmid. With these repulsive charges neutralized by the calcium ions, the plasmid DNA has an easier time passing by the plasma membrane of the bacterial cell. The cells have already been made competent for you and your teacher will give you an aliquot. You will, however, need to do the next step.

Now that we have the negative charges on the DNA and the plasma membranes neutralized, we need to create a bit of a pressure difference between the inside and the outside of the bacterial cell. This is done by first getting the bacteria really cold and then quickly putting them into warm water. This is called “heat shock,” and it creates a situation in which the pressure outside the cell is a tiny bit higher than inside the cell. This pressure gradient will help to move the plasmid DNA from the outside to the inside of the bacterial cell. Following this brutal treatment, we’ll need to feed our bacteria and let them recover for a few minutes before we spread them onto agar plates.

Once the cells have recovered, you’ll take samples of these cells and spread them on a series of sterile agar plates. One of these plates will contain only bacterial food; the plate contains no antibiotic. This plate is marked “**LB**.” A second plate contains LB and ampicillin; this plate is marked “**LB/amp**.” The third plate contains LB, ampicillin and a simple sugar called arabinose; this plate is marked “**LB/amp/ara**.”

Ampicillin is an antibiotic that prevents bacteria from fully forming its cell wall. Cells that are not ampicillin resistant cannot grow in

its presence; the new cells simply rupture or lyse. If a cell receives an ampicillin-resistant gene, amp<sup>r</sup>, it will produce a protein that will chemically destroy ampicillin and, therefore, will be able to grow with ampicillin in its environment.

Arabinose, a simple sugar, is needed by the bacterium to express the rfp gene. If a bacterium takes up pARA-R, arabinose helps the enzyme RNA polymerase, needed to transcribe the rfp gene, to align itself correctly on the plasmid.

Although the E. coli strain that you are using in these labs is relatively benign, it is important that you use proper techniques when handling them.

#### Materials

#### **REAGENTS AND CULTURES**

LIG tube (recombinant plasmids)

100 µL of competent cells (LMG)

300 µL of LB broth (sterile)

Crushed ice

Agar plates 1 LB, 1 LB/amp, 1 LB/amp/ara

#### **EQUIPMENT & SUPPLIES**

P-20 and P-200 micropipette and tips

42° C water bath

1 pack cell spreaders (shared)

Plastic microfuge tube rack

Two 1.5 mL microfuge tubes

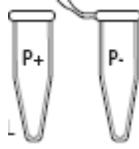
Marking pen

Timer

# Methods -Transformation

In order to make this lab run smoothly, it's important that you and your partner share the work load and communicate clearly with each other.

1. Locate two clean microfuge tubes. Label one "P+" and the other "P-." Place your labeled P+ and P- tubes into your blue ice cup.



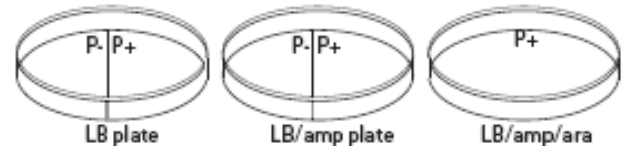
2. The bacteria cells that we are using today have been made competent (able to take up plasmid). It is important that the competent bacteria cells remain in the ice at 0°C.

3. Pick up your ligated plasmids (LIG tube) from the microfuge tube rack at the front of the room.
4. Set the P-200 micropipette to 50  $\mu\text{L}$  (set to "0-5-0") and place a clean tip onto its barrel. Very carefully resuspend (mix) the competent cells by **gently** pumping the cells in and out (going only to the first stop) two times. Hold the tube by the upper rim to avoid warming the cells with your fingers.
5. Aliquot 50  $\mu\text{L}$  of the resuspended cells into the prechilled P+ and P- microfuge tubes. Immediately return the aliquoted cells to the ice.
6. Using the smaller P-20 pipette, add 10  $\mu\text{L}$  (set micropipette to "1-0-0") of your ligated plasmid (LIG tube) to the tube labeled "P+." Gently mix the plasmid with the cell suspension by pumping the cell suspension two times. Immediately return the P+ tube into the ice. **Do not add plasmid to the P- tube.** The cells in the P- tube will serve as the "plasmid control."

7. Keep your bacteria cells in ice for 15 minutes (timer).
8. While the cells are incubating in ice, obtain the following: One **each** of these agar plates: **LB**, **LB/amp** (LB + ampicillin) and **LB/amp/ara** (LB + amp + arabinose)
9. Writing small and toward the edges of the plates, label the **bottom** (smaller plate containing the agar) of all three plates with your

group number and class period. Then divide the LB and amp plates down the middle using two lines. Label one half of each plate "P+" and the other half with a "P-." See the diagram below.

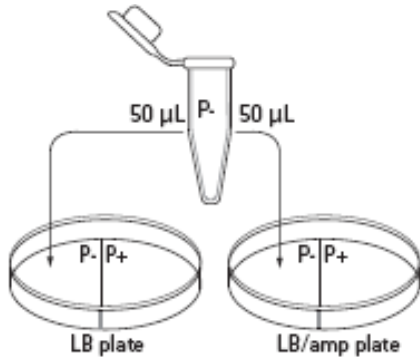
**Do not divide the ara plate.**



10. Following the 15-minute incubation in ice, **carry the ice cup** containing the P+ and P- tubes **to the 42°C water bath**. Take both tubes out of the ice and immediately hold them in the water bath for **45 seconds**. After the 45-second heat shock, place them **back into the ice immediately** for at least one minute.
11. After one minute, use the P-200 pipette to add 150  $\mu\text{L}$  (set to "1-5-0") of LB broth to the P- tube. Cap the tube and gently flick the lower portion of the tube two or three times to mix.
12. Use a new tip and transfer 150  $\mu\text{L}$  of LB broth to the P+ tube. Close the cap and gently flick the tube to mix.
13. You are now ready to spread your bacterial cells onto the sterile agar plates.
  - a. Using the P-200 pipette (set to "0-5-0"), gently pump the pipette two or three times to resuspend the cells then aspirate (take up) 50  $\mu\text{L}$  of cells from the **P- tube**. Open the lid from the LB plate like a "clamshell." Dispense these cells **on the half of the plate marked "P-."** Close the lid.
  - b. Resuspend the cells by gently pumping the pipette then aspirate a second 50  $\mu\text{L}$  aliquot of P- for the LB/amp plate. Remember, you

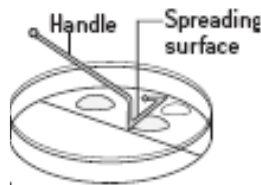


want to deposit the P- cells on the half of the plate you labeled "P-." Cover the plate.



- c. Open the package of sterile inoculating loops **at the end closest to the handles**. You will share this package with another group. Remove only one inoculating loop, keeping the others sterile. Hold the inoculating loop by the handle and do not allow the loop end to touch any surface, as this will contaminate the spreader. Close the package to avoid contaminating any of the other loops.

- d. Open the lid to the **LB plate**, like a clamshell, and gently using a light, gliding motion spread the cells across the surface of the agar, keeping the cells on the P- side of the plate. Try to spread the cells evenly over the surface of the plate.

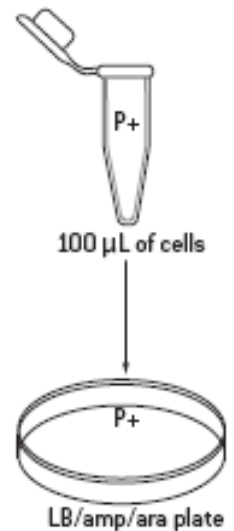


- e. Carefully spread the P- cells on the LB/amp plate using the same loop and technique.
- f. Place the used inoculating loop into the bleach solution.

- g. Repeat steps 13 a-e to inoculate the LB and LB/amp plates with the **P+ culture**. Be certain to use the "+" tube and a new inoculating loop to avoid contamination.

14. Now you're ready to inoculate the LB/amp/ara plate.

- a. Using the P-200 pipette (set to "1-0-0"), transfer **100 μL** of the P+ culture onto the surface of the LB/amp/ara plate. Deposit the 100μL of cells on several areas across the agar surface rather than a single spot.



- b. Lift the lid, clamshell style, and using the loop spread the cells evenly over the surface of the plate.
- c. Cover the plate when finished.

15. Allow all three plates to sit right-side up for five minutes.

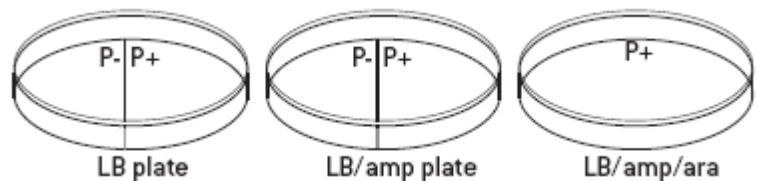
16. Move all three plates into one stack. Bind the stack together using one piece of tape that loops from the top dish to bottom dish then back up to meet the start of the tape. Place your stack of petri dishes in the incubator, **gel-side up**. Be certain that you have clearly labeled your plates with your group number and class period. You can mark the tape to help you find them for the next lab.

17. Discard cell-contaminated waste: loops, cell tubes (open), and pipette tips, by placing them into the bleach solution.

## Lab 5 Pre-Lab Questions

1. The plasmid we are trying to make is pARA-R. What does the p in pARA-R mean? What does ARA refer to? R?
2. Use Ch 16.1 to describe Fredrick Griffith's experiment with transformation and pneumonia causing bacteria.
3. Under experimental conditions, how likely is it that a cell will take up a plasmid?
4. T/F Most of our plasmids in the LIG tube will be supercoiled. Explain
5. What three factors can lead to more successful transformation? explain how each help the process.
6. Why are we attempting to grow bacteria on ampicillin plates? (Two of the three plates contain ampicillin, why?)

7. Predict the growth, if any, on the following plates. Remember the cells from the P+ culture were given recombinant plasmids while those from the P- were not. Use a "+" if you expect growth and a "-" if you expect no growth. Of the plates that do show growth, will the amount of bacterial growth differ? Explain



- a. What do all of the cells growing on the LB/amp and LB/amp/ara plates have in common?
  - b. What single *restriction fragment* must they all contain to grow on plates with ampicillin?
- 8a. Would you expect that all of the cells growing on the LB/amp/ara plate were transformed with the same plasmid? Explain.
  - 8b. How might you determine which of the cells on the LB/amp/ara plate contain pARA-R, the recombinant plasmid that you've made by ligating the *rfp* gene with the large pARA restriction fragment?