

Experiment 4

Transformation of *Escherichia coli* With Chimeric Plasmid

Now that we have generated recombinant molecules, we must next amplify them by inserting them into an acceptable host so that they may be analyzed or manipulated in some way. Insertion of exogenous naked DNA into cells is called *transformation*. Some bacteria, like *Bacillus*, *Neisseria*, and the pneumococcus possess genetic and enzymatic systems that permit them to transport DNA across the cell wall and to incorporate it into their genomes. Indeed, transformation is the normal means of genetic exchange employed by these organisms. Such organisms are said to be *competent*. *E. coli*, however, employs sexual interaction to exchange DNA and does not normally take up exogenous DNA. However, *E. coli* can be forced to become competent by treating it with CaCl₂. This technique is the first such technique worked out for *E. coli* but it is not the only transformation regimen available. Others provide higher levels of DNA uptake, but are more complex to use and, in our case, unnecessary.

The transformation process is extremely inefficient and normally, only a fraction of a percent of cells actually becomes transformed. Thus, picking out transformed cells in a nearly infinite population becomes a major problem. This can be circumvented by designing a selection technique whereby the cells in which we are interested will grow at the expense of those in which we are not interested. We have already seen in experiment 1 that our vector, pUC9 carries a gene for ampicillin resistance. Thus, if we put ampicillin into the plating medium, only those cells that have picked up exogenous DNA can grow.

Once we have selected for transformed cells, we must then determine which of the transformed cells carries an insert into the vector. This can be done by employing a *screen*. The type of screen employed varies depending on the vector used and the nature of the intended target. In this case, we have seen that pUC9 carries a fragment of the *lacZ* gene in addition to the *bla* gene. Insertions into the multiple cloning site within *lacZ* leads to inactivation of β -galactosidase, and a Lac⁻ phenotype. On MacConkey agar, cells that are Lac⁺ secrete lactic acid as a waste product of lactose fermentation. This causes a localized drop in the pH around the colony, which, in turn, causes a pH indicator dye to turn bright pink. Lac⁻ cells cannot ferment lactose and therefore do not cause a change in the pH. These

colonies appear white on MacConkey agar, and the plate background turns yellow from the basic waste products normally produced by cells.

In this experiment you will transform your ligation mixes into *E. coli* and recover transformed, AMP^r colonies that will be either red (-insert) or white (+insert). The plasmids carried by these cultures will be characterized in Experiment 5.

Procedure

1. Inoculate 20 ml of fresh LB broth in a sterile side-arm flask with 1 ml of an over-night culture of *E. coli* strain JM83. Incubate shaking at 37^o until the cell density reaches about 5×10^7 cells/ml (OD₆₀₀ = 0.2. You will eventually be able to judge this by eye). Incubation will take approximately 2 hours.
2. Transfer 10 ml of cell culture to a sterile 25 ml glass screw-cap centrifuge tube and centrifuge for 10 minutes at 7,000 rpm. Be sure to place the tube in a rubber adapter. If you don't, the tube will shatter (at \$17.00 per tube!). Note the shape and consistency of the cell pellet. Carefully pour off the supernatant.
3. Resuspend the cells in an equal volume of *ice cold* 50 mM CaCl₂ and allow to stand on ice for 20 minutes. The CaCl₂ is hypotonic and causes the cells to swell and become spheroplasts.
4. Centrifuge the cells again and note the pellet. Since the cells are spheroplasts, the pellet will look quite different than in step 2. Typically, the cells will appear as a diffuse ring rather than as a solid pellet. If the pellet appears as described, then the cells have successfully been rendered competent. If they do not appear as a diffuse ring, the transformation will not work and should be aborted.
5. Resuspend the cells in 1.5 ml of ice cold 50 mM CaCl₂.
6. Transfer 200 μ l of competent cells to each of four sterile tubes and set up the following transformations:
 - 1 10 μ l ligation mix #1
 - 2 10 μ l ligation mix #2
 - 3 5 μ l uncut pUC9
 - 4 0 DNA control

7. Allow all four tubes to stand on ice for 15 minutes. During this time the DNA forms a complex of hydroxyl-calcium phosphate, which adheres to the cell surface. This complex is DNase resistant.
8. Heat-shock the cell + DNA mixes by placing in a 37° water bath for 2 minutes. This will induce the cells to take up the DNA complex. It is easy (and fatal) to forget this step if you are in a hurry.
9. Add 2 ml of LB broth to the cell suspensions and incubate shaking at 37° for 90 minutes. This incubation will allow the spheroplasts to regenerate and permit time for the expression of the newly incorporated genes.
10. During the incubation, prepare four sets of six sterile dilution tubes, each containing 0.9 ml of LB broth. Use glass tubes. Do not use microfuge tubes.
11. Serially dilute the transformed cells and plate on LB and on the MacConkey + amp plates according to the table below. The table shows the final dilution factor *ON THE PLATE*. All dilutions indicated by a “+” should be plated. Refer to the diagram on serial dilution on page 54 if you need to brush up.

| <u>Media</u> | <u>Dilution</u> | <u>0 DNA</u> | <u>pUC9</u> | <u>Lign #1</u> | <u>Lign #2</u> |
|--------------|------------------|--------------|-------------|----------------|----------------|
| Mac | 10 ⁻¹ | + | | + | + |
| “ | 10 ⁻² | | + | + | + |
| “ | 10 ⁻³ | | + | + | + |
| “ | 10 ⁻⁴ | | + | | |
| XXX* | 10 ⁻⁵ | | | | |
| LB | 10 ⁻⁶ | + | + | + | + |
| “ | 10 ⁻⁷ | + | + | + | + |

*XXX means that the dilution is not plated on any medium

12. Invert your plates and place them in the 37° incubator until the following day.

13. On the following day, count your colonies and record numbers in your notebook according to the data sheet below. You should see red and white colonies. Red colonies indicate that no insertions into *lacZ* were made, and that the cells are fermenting lactose. White colonies indicate insertional inactivation of β -galactosidase.

| Dilution Factor | | 0 DNA | pUC9 | Lign #1 | Lign #2 |
|----------------------------------|-------|-------|------|---------|---------|
| 10 ⁻¹ | red | | | | |
| | white | | | | |
| 10 ⁻² | red | | | | |
| | white | | | | |
| 10 ⁻³ | red | | | | |
| | white | | | | |
| 10 ⁻⁴ | red | | | | |
| | white | | | | |
| 10 ⁻⁵ | red | | | | |
| | white | | | | |
| 10 ⁻⁶ | red | | | | |
| | white | | | | |
| 10 ⁻⁷ | red | | | | |
| | white | | | | |
| Total Viable Count (LB) | | | | | |
| Total Transformants | | | | | |
| Transf Frequency (r+w)/viable | | | | | |
| insert frequency w/(r+w) | | | | | |
| transformants per μ g DNA | | | | | |

14. Recover colonies for Experiment . Prepare 14 tubes of LB + amp, 2 ml per tube. Use sterile swab sticks to pick 10 white colonies and 4 red colonies and inoculate one in each tube. After inoculating the tube, use the same swab to make a plate culture as described in step 15. Incubate the tubes standing over night at 37° for use the following day. Make sure that each tube is numbered.

15. At the same time that you make your broth cultures, make plate cultures as well. Mark several pie-shaped sectors on the back of a few MacConkey plates and number them. Pick a transformant from one of your plates, dip it into one of the tubes, and then streak it across one of the numbered pie-shaped sectors. These will become your permanent stock cultures. You must be able to correlate a particular broth culture with a particular solid culture. Eventually, you will also need to be able to correlate the cultures with lanes on a gel. *Record keeping is very important!!* Incubate the plates inverted at 37° over night and then seal with parafilm.

It would be wise to save your transformation plates, wrapped in parafilm, until you know that you have recovered the necessary plasmids from this experiment

Luria Broth (LB) and Agar

| | |
|--------------------|------|
| Tryptone..... | 10 g |
| Yeast Extract..... | 5 g |
| NaCl..... | 10 g |
| Agar..... | 20 g |

per liter of water

MacConkey Agar

| | |
|-----------------------|---------|
| Peptone..... | 17 g |
| Proteose Peptone..... | 3 g |
| Lactose..... | 10 g |
| Bile Salts #3..... | 1.5 g |
| NaCl..... | 5 g |
| Agar..... | 13.5 g |
| Neutral Red..... | 0.03 g |
| Crystal Violet..... | 0.001 g |

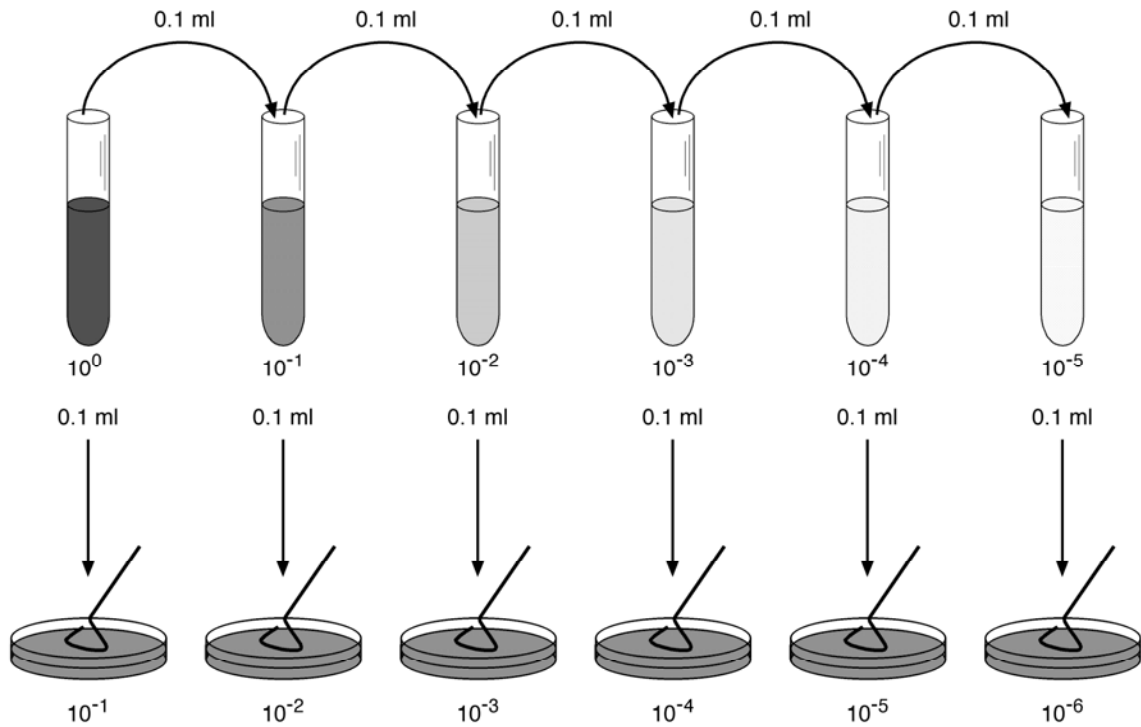
per liter of water

MacConkey agar comes as a pre-mixed powder. Therefore, it is necessary only to add 50 g to one liter of water.

Ampicillin

Stock ampicillin (= 25 mg/ml) is prepared by dissolving it in water and filter-sterilizing it. Do not add before autoclaving. After the medium has been autoclaved and allowed to cool, add enough ampicillin to make the final concentration 50 µg/ml.

Serial Dilution:



$$a \times b = c \quad a = c/b$$

Cell Concentration x dilution factor = # colonies

$$\text{cell concentration} \times 10^{-6} = 200 \text{ colonies}$$

$$\text{cell concentration} = 200/10^{-6}$$

$$\text{cell concentration} = 200 \times 10^6 = 2 \times 10^8$$