

Experiment 5

Screening of Transformants for Chimeric Plasmids

In Experiment 4 you transformed *E. coli* with the recombinant plasmids produced in Experiment 3, and have now recovered transformants with and without inserts. When a circular plasmid is cut once, it becomes linearized. When two linear fragments fuse to form a new circle, they can join in one another, as we have seen in Experiment 1, in two possible orientations, each of which produces a unique pattern of restriction fragments. In order to characterize the chimeric plasmids, which you have constructed, you must sort out the two orientations and obtain cultures of each. As you will learn, it is difficult and time-consuming to produce large amounts of highly purified plasmid DNA. However, there are a number of procedures for isolating small amounts of plasmid relatively quickly from a large number of cultures.

The technique that you will employ here is the *alkaline lysis* method. In this procedure you first use lysozyme to gently strip away the peptidoglycan in the cell wall to produce spheroplasts, which are then lysed by a combination of SDS and NaOH. SDS dissolves the lipopolysaccharides in the cell wall and NaOH produces an osmotic shock to burst the remaining membrane. When the cell lyses, the chromosome is fragmented into linear pieces, but the plasmid remains intact and circular. In addition to lysing the cell, the NaOH also denatures the DNA. The linear chromosome fragments separate, but the circular, supercoiled plasmid strands remain tightly bound. When the lysate is neutralized by acetate, the chromosomal fragments try to reanneal and become hopelessly entangled with one another and with the cell debris to form an insoluble pellet. The plasmid, however, because of the close proximity of the strands, instantly reanneals and remains in solution. The lysate is then centrifuged and the chromosomal DNA is pelleted and removed, leaving a small but relatively pure plasmid sample. Although we will use a different technique for the large-scale prep, the alkaline lysis method could easily be scaled up for this process.

Materials

GET buffer
lysozyme solution (40 mg/ml)
0.5 M NaOH
10% sodium dodecyl sulfate (SDS)
potassium acetate
phenol/chloroform
ice-cold 95% ethanol
RNase solution (10 mg/ml)

**Procedure for
First Day**

1. Recover the broth cultures that you inoculated at the end of Experiment 3. Transfer 1.5 ml of each culture into a microfuge tube and centrifuge for 2 minutes. Carefully pipette off the supernatant.
2. Add 90 μ l of *ice-cold* GET buffer and 10 μ l of lysozyme solution to each pellet. Vortex to resuspend.
3. Allow the tubes to stand at room temperature for 5 minutes. During this time, the lysozyme will strip away the peptidoglycan layer of the cell wall. The glucose in the GET will create an isotonic environment, which will prevent the spheroplasts from rupturing.
4. Prepare a fresh NaOH + SDS solution as follows:

2.0 ml 0.5 M NaOH
0.5 ml 10% SDS
2.5 ml water

Add 200 μ l of this solution to the spheroplasts, mix by rapidly inverting the tubes several times (*do not vortex*). Place on ice for 5 minutes. The combination of alkali and detergent will dissolve the cell membranes and cause the cells to lyse. Your samples will become very viscous.

THIS IS A CRITICAL STEP! DO NOT ALLOW DENATURATION TO OCCUR FOR MUCH LONGER THAN FIVE MINUTES OR THE PLASMID WILL NOT REANNEAL EFFICIENTLY!

5. Add 150 μl of *ice-cold* potassium acetate (pH4.8). Mix *very gently* by vortexing the tubes *upside down* and store on ice for 5 minutes. The acidic potassium acetate will change the sample from alkaline to neutral pH and the DNA's will attempt to reanneal. You will see a fluffy white precipitate. Centrifuge for 5 minutes.
6. Transfer each supernatant to a fresh tube and add an equal volume (450 μl) of phenol/chloroform (note: *phenol/chloroform is extremely caustic*). Rock gently to mix, and centrifuge for 2 minutes. This will partition the proteins into the lower phenol phase and the nucleic acids in the upper aqueous phase.
7. Carefully pipette off the top layer:
 - Set your pipette to a volume slightly larger than the amount of your sample (e.g. 400 μl).
 - Carefully draw up your entire sample plus some of the phenol chloroform. Locate the boundary between the phenol/chloroform and aqueous layers.
 - Carefully pipette back into the tube to just past the boundary layer.
 - Transfer the remainder of the pipette to a fresh tube.

If all was done carefully, your sample will contain mostly plasmid DNA and RNA. Sometimes, however, residual proteins from the lysate carry over and can interfere with subsequent restriction digestions. This can usually be cleared up by a second/phenol chloroform extraction.

It is prudent to simply repeat steps 6 and 7 as a matter of course before going to step 8.
8. Add two volumes of ice-cold 95% ethanol to each sample and allow to stand on ice for 10 minutes to precipitate the nucleic acids. Centrifuge for 5 minutes. It is not necessary to add NaCl here as you did during the BAP treatment in Experiment 3 because the acetate used in the neutralization step serves the same purpose.

9. Carefully pour off the ethanol (you may see a white pellet) and allow the residual to evaporate.
10. Prepare a TE + RNase (20 µg/ml) solution and resuspend each of your pellets in 50 µl. If the RNA in the plasmid preps is not removed, it will be visible on your gel and will interfere with your ability to analyze the results of restriction digestions.

In a normal lab situation, the plasmid extraction would be done in the morning and there would then be time to analyze the results on a gel in the afternoon. However, the plasmid will keep and this is a convenient breaking point. Save your plasmid preparations until next time.

**Procedure for
Second Day**

1. Transfer 10 µl of sample from each plasmid prep to a fresh microfuge and set up a 20 µl *Hind*III digestion. Since you are using 10 µl of DNA, you will compensate by adding 5 µl less water. To speed up your manipulations, you can prepare a cocktail of buffer, water, and enzyme in one large lot and dispense 10 µl of the mix to each of your plasmid samples. You should include an 11th sample, pUC9, as a control.
2. Prepare a second set of samples as uncut controls. Transfer 5 µl to a microfuge tube and add 15 µl of water.
3. Pour a 1% “piggy-back” gel.
4. Add 5 µl of tracking dye to your samples and load onto the gel. The cut samples should go on the top row of the gel and your uncut samples should go on the bottom. With the cut samples, include 1 kb ladder and 1 *Hind*III DNA molecular weight standards. Run the gel for approximately one hour at 120 V.
5. Stain your gel with ethidium bromide and take a photograph.
6. A complete digest of your chimeric plasmids will give you two bands. If you see more, then you have a partial digestion caused, most likely by inhibitory contaminants in your plasmid preps. However, you should still be able to identify the two bands resulting from a complete digestion. Those plasmids in which the *Hind*III sites are distal shall be designated pRIT4501. Those in which the sites are proximal shall be designated pRIT4502.

GET Buffer

50 mM glucose
10 mM EDTA
25 mM Tris pH8.0

Potassium Acetate pH 4.8

| | |
|----------------------------|---------|
| 5 M Potassium Acetate..... | 60.0 ml |
| glacial acetic acid..... | 11.5 ml |
| water..... | 28.5 ml |