BIO440 Genetics Lab  Isolation of Recombinant Plasmids

Objective:

- To continue your introduction to DNA purification methods and the handling of DNA

Introduction: background information on plasmids and plasmid purification.

Plasmids are covalently closed, extrachromosomal pieces of DNA in bacterial cells. Typically, natural plasmids contain genes that are not essential for the survival of the bacteria under all conditions. Some of the genes encoded on plasmids include: antibiotic or heavy metal resistance, antibiotic production, nitrogen fixation, pigment production, catabolism of various carbon sources (i.e. naphthalene, benzene, sucrose, urea), tumorigenesis (i.e. Agrobacterium), and virulence factors (i.e. capsule formation). All plasmids also contain genes that ensure their own replication; in some cases, these genes may provide for several copies of the plasmid to be made with each round of DNA replication (i.e. multi-copy plasmids), and in other instances, only a single copy is made and replicated (single, or low copy number plasmids).

Most high copy number plasmids are significantly smaller than the bacterial genome (1-20 kilobase-pairs (kb) versus ~ 5000 kb in the E. coli genome). It is this size difference which enables us to effectively isolate plasmid DNA free of chromosomal DNA. Most of the low copy number plasmids are much larger (50-500 kb), and these are much more difficult to purify away from genomic DNA.

Plasmids are also valuable tools that we use in gene cloning. Plasmids can be used to produce a large amount of a particular nucleic acid sequence, which can then be used for DNA sequencing or other genetic studies. For a typical cloning project, you would cut a plasmid vector with a restriction endonuclease, then insert a DNA fragment (produced with the same restriction endonuclease) into the cut site and religate the plasmid. The new plasmid, called a recombinant plasmid, would be transformed into competent bacterial cells, and each copy of the recombinant plasmid that was made would contain a copy of the cloned DNA fragment. Alternatively, you might use the T/A cloning approach, which is useful for cloning PCR products--we'll do that later this semester.

If you plan your cloning carefully, you can also express a protein encoded on the cloned DNA fragment. For example, in this experiment we will use a plasmid called pLUX. pLUX was constructed using the cloning vector pGEM. A map of pGEM will be handed out in class. To make pLUX, genomic DNA was first isolated from the bioluminescent bacterium Vibrio fischeri. Then, this DNA was digested with the restriction enzyme Sal I, and the digested DNA was ligated into Sal I - cut pGEM. The plasmid pLux contains a large Sal I fragment from V. fischeri that contains the genes for the production of the enzyme Luciferase. Luciferase converts chemical energy into light energy, and thus this plasmid confers the ability to bioluminesce. E. coli carrying this plasmid glow in the dark.
Once you have grown up a culture containing your recombinant plasmid, you then need to purify the plasmid away from all of the other components of the bacterial cell -- membranes, cell walls, ribosomes, etc, and especially RNA and chromosomal DNA. There are several different approaches to plasmid purification. We are starting with a less commonly used procedure in order to help you to understand the chemistry behind the various steps. This approach is simple and (almost) always works well at isolating plasmids from proteins (including DNA-degrading nucleases), RNA and chromosomal DNA. Later in the semester we will use a simpler, silica-gel-based approach to isolate recombinant plasmids that you have constructed. The following paragraphs describe the chemistry of this plasmid purification scheme.

After growing the cells and concentrating the cells in a microcentrifuge tube, the first step is to effect cell lysis -- i.e. burst 'em open. We will resuspend the cells with a buffer containing Tris and EDTA. Remember that EDTA binds divalent cations (which is why we used it to resuspend DNA the first week of lab -- many nucleases require those divalent cations to function). Divalent cations such as Ca++ and Mg++ are also involved in stabilizing the membrane of E. coli, and thus the inclusion of EDTA helps to destabilize the bacterial membrane. We then add a solution containing NaOH and the detergent SDS (sodium dodecyl sulfate). The detergent helps to solubilize the membranes, and causes the cells to lyse (an obvious, physical change, accompanied by protein precipitation and viscosity increase).

We will then exploit several differences between the plasmid DNA and chromosomal DNA in order to separate the two. We earlier discussed the fact that increases in pH cause double-stranded-DNA (DS DNA) to separate into single-stranded DNA strands (SS DNA). The NaOH included in the lysis buffer will increase the pH and cause the chromosomal DNA to denature. Because the plasmid DNA exists as a small, circular, highly supercoiled molecule, it will not denature as rapidly as the chromosomal DNA. We will then rapidly drop the pH (and increase the cation concentration) of the solution by the addition of potassium acetate. The chromosomal DNA will not be able to correctly re-form its original base-pairing structure, and will precipitate out of solution. The plasmid DNA, however, can renature, and will stay in solution. We can then remove the precipitated chromosomal DNA by centrifuging the solution, and the plasmid DNA will remain in solution.

The plasmid DNA is now in a solution that contains some soluble proteins and a high concentration of inorganic salts. Typically at this step, the DNA is purified either by a silica gel purification step or by extracting proteins in phenol and chloroform. Phenol is an organic solvent that will cause the proteins to denature and precipitate. This organic extraction is typically followed by an ethanol precipitation in order to get rid of salts and concentrate the DNA. In this situation, we would have to pellet our precipitated DNA by centrifugation, rather than spool it onto a glass rod, since the concentration will be considerably lower than our salmon sperm DNA solutions. The silica gel purification relies on the fact that DNA will bind to silica gel under conditions of high salt concentration, and then un-bind under conditions of low salt concentration. The plasmid DNA will bind to the membrane, but the proteins and salts will go through the membrane.
and be discarded. RNA would bind the membrane, too, but the RNA-degrading enzyme RNase A is usually included in the potassium acetate solution. After binding the DNA to the column, you wash the column with ethanol to remove any traces of salts, and then elute your DNA from the silica gel column with a small volume of dilute TE buffer.

**Plasmid Isolation - Protocol**

I have started an overnight cultures of an *E. coli* strain that is carrying the pGEM-pLUX plasmid in LB + Ampicillin broth. **Each student (i.e, not each group) should prepare a plasmid as detailed in the protocol below.** Before beginning your plasmid isolation, determine what the concentration of bacterial cells in the culture is by the use of dilutions and spread plates, and spectrophotometry. The concentration will probably be about 10^8 cells/ ml.

**A. Harvesting the cells-**

1. Transfer 1.5 ml of the overnight culture into a sterile microfuge tube.
2. Spin at max. speed for one minute in the microfuge. Make sure to put tubes into microfuge such that the cap hinge faces outward.
3. Remove all the supernatant using the P1000 (and P200) pipettes - be sure that you do not disrupt the pellet at the bottom of the tube. Typically, the majority of supernatant is withdrawn with the larger pipetman, and then the final supernatant is withdrawn using the smaller -tipped pipetman.
4. Transfer another 1.5 ml of the overnight culture into the same tube containing the pellet and repeat steps 2 and 3. put tube on ice. (Check with me to see if the pellet is big enough, or if you will have to use more culture).
5. Add 1 ml of ice-cold TE (10 mM Tris/1 mM EDTA) to your cell pellet. Vortex the cells to resuspend the pellet completely, and then centrifuge to re-pellet the cells. Remove the supernatant, and then repeat this wash step an additional time, using another 1 ml of ice-cold TE. **You now have a pellet from a total of 3 ml of culture.**

**B. Lysis by alkali**

1. Resuspend the bacterial pellet (obtained from step A5 above) in 100 µl ice cold solution I (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0.). You will need to vortex vigorously. Your pellet must be completely resuspended - i.e. the solution must be completely homogeneous with no visible cell chunks. Put tube on ice.

2. Add 200 µl of freshly prepared solution II (0.2N NaOH, and 1% SDS). Close cap tightly and invert rapidly 5 times - make sure the entire contents of the tube has come into contact with solution II. **DO NOT VORTEX!!!!**

Immediately put tubes on ice. Incubate on ice for 3 min. This should effect lysis of the cell solution--the viscosity of the liquid should increase, and a large amount of white precipitate or floc should be present.
C. Renaturation and recovery.

1. Add 150 µl of ice-cold solution III (3 M potassium 5 M acetate solution pH 4.8-5.2). Close cap tightly and invert 5 times - make sure the entire contents of the tube has come into contact with solution III. DO NOT VORTEX!!!!. Store tube on ice for 10 minutes.

2.) Centrifuge for 5 minutes and immediately put tube on ice. After centrifugation, you should see white material alongside the outside of the tube below the hinge. This is the pellet of cell debris, chromosomal DNA, polysaccharide, etc. Your plasmid DNA is in the supernatant.

3.) Using your p200 pipetman set at 150 µl, carefully transfer supernatant to a fresh tube (typically 3 x 150 µl). METICULOUSLY AVOID all white particulate matter (precipitated proteins/genomic DNA). If you accidentally disturb pellet - you should probably repeat the centrifugation. There is a large excess of plasmid DNA present-getting all of the supernatant is of much less concern than avoiding carryover of cell debris. Typically at this step, I purify the DNA by extracting proteins in phenol and chloroform. We are going to omit that this time, although we will be doing phenol-chloroform extraction steps next week when we purify bacterial genomic DNA.

4.) Precipitate the double stranded plasmid DNA with 2 volumes of ice cold 100% ethanol - and mix by inverting or vortexing. (i.e if you have ~ 450 µl, then add 900 µl ethanol.

5.) Centrifuge at max. speed for 5 minutes. Make sure to put tubes into microfuge such that the cap hinge faces outward.

6.) While avoiding the very small (clear white) pellet - carefully remove all supernatant from the tube. Invert the tube onto a piece of paper towel - to let all residual ethanol wick out of the tube onto the paper.

7.) Wash the pellet with 1 ml of 70% ethanol - invert tube a couple times and repeat steps 5 and 6.

8.) Let tubes sit open in sterile hood for ~ 10 minutes to allow the ethanol to evaporate, and then resuspend the nearly invisible pellet in 30 µl TE buffer (10 mM Tris, 1 mM EDTA pH ~8.0).

E. Plasmid Isolation - Determining the concentration of your isolated plasmid using spectrophotometry. Take 10 µl of your isolated plasmid, add it to 240 µl of TE, and then measure A260 and A260/A280 of your diluted plasmid. Record your results in the ‘Observations and analyses’ section.
**Plasmid Isolation: Observations and Analyses**

**Determination of cell concentration**

Part 1. Concentration of bacterial culture, estimated using spectrophotometry. Calculate the initial concentration of the bacterial culture, based on the Abs at 600 nm. 

\[ C_i = C_f / D \]  
Remember: 1 OD600nm = \( \sim 8 \times 10^8 \) cells / ml.

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<tr>
<th>Plate</th>
<th>Dilution</th>
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<th>( C_f ) (cells / ml in dilution)</th>
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**Calculations**

**Plasmid Isolation**

1. Why was it necessary to grow your cells in the presence of ampicillin for the plasmid isolation protocol? Keep in mind that this culture was grown long after the plasmid had been constructed.
2. What is the role of each of the following in plasmid isolations?
   EDTA
   SDS
   NaOH
   potassium acetate
   ethanol

3. Calculate the initial concentration of your undiluted DNA stock. Be sure to incorporate the dilution that you used in your calculation. Include units. Remember 1 OD260 = 50 µg/ml DNA.

OD 260nm of diluted plasmid DNA _____________________

Ratio: OD 260 / 280 _________________________

Concentration of isolated plasmid DNA (include units) _________________________

What was the total mass of plasmid that you recovered? (include units)

4. Based on your determinations in number 3, and your calculations of bacterial cell abundance, estimate the mass of plasmid DNA/bacterial cell. Using this value, estimate the number of plasmid molecules/bacterial cell. You will need to convert mass of plasmid into # of plasmid molecules in order to do this. The values from question 2 of your observations and analyses from your DNA spooling/ethanol precipitation lab will be helpful with this. Finally, interpret your answer -- do you think that this accurately represents the number of plasmids/cell?