Isolating plasmid DNA using Alkaline Lysis and Spin Columns

a) Introduction

Bacteria are unicellular prokaryotes. Recall that a prokaryotic cell is a small primitive cell type with no nucleus. Each prokaryotic cell has only a single chromosome, which is circular in shape and contains all the genes necessary for basic survival and reproduction of the cell. Many bacteria, however, contain additional genes located on small circular pieces of DNA called plasmids. Plasmids usually do not contain genes that are used in the normal life processes of the bacteria. Instead, plasmids often contain genes that give the bacteria a survival advantage in unusual circumstances, such as antibiotic resistance genes that allow the bacteria to survive when antibiotics are present. As another example, some plasmids contain genes that give the bacteria the ability to metabolize certain metals that would otherwise be toxic to the bacteria.

Plasmids replicate independently of the bacterial chromosome and can exist in multiple copies in a single bacterial cell. In many bacteria species, plasmids are naturally transferred from one bacterium to another through a process called conjugation. Many plasmids have been isolated and analyzed in recent years.

Thanks to advances in recombinant DNA technology, some naturally occurring plasmids have been redesigned by scientists for use in cloning (making many copies of) gene sequences of interest, whether or not those genes are from bacteria or from other species (such as human beings). A gene of interest can be inserted into a plasmid, and then the “recombinant” plasmid (the bacterial plasmid combined with DNA from a non-bacteria source) is put into a bacterial cell. The bacteria are then grown in the laboratory by putting them into a nutrient-rich liquid in a warm environment (this is called making a bacterial “culture”). Every time the bacterium divides, it makes copies of the plasmid (including the recombinant gene that the plasmid contains). Because bacteria can reproduce very rapidly, in a few hours the bacterial culture contains a very large amount of the bacteria that contain the recombinant plasmid.

The final step is to isolate the plasmid from the bacterial culture. This step involves lysis (breaking open) of the bacteria cells and then separating the plasmid DNA from the chromosomal DNA and from all of the other molecules inside the cell. When the plasmid has been isolated, the scientist now has large amounts of the gene of interest that can be used for study and analysis of the gene. The isolated plasmid DNA can be analyzed for purity and quality using a technique called electrophoresis.

Making copies of a piece of DNA (such as by the plasmid method described above) is called “cloning” the piece of DNA. Molecules like plasmids that are used for making copies of DNA are called “cloning vectors” or sometimes just “vectors”. Plasmids used as cloning vectors are typically small, so they are easy to manipulate. They contain an origin of replication sequence that allows them to replicate in any bacteria. They also contain a gene for resistance to some type of antibiotic. This serves as a “selectable marker,” which is anything that allows the scientist to determine which bacteria contain the plasmid and which do not. For example, if the plasmid has an antibiotic resistance gene, then only bacteria that have the plasmid can grow in a bacterial culture that contains the antibiotic. Any bacteria that do not have the plasmid will die in the presence of the antibiotic.

Although some plasmids are used simply as DNA cloning vectors (to make copies of the gene of interest), other plasmids are designed to allow the bacteria to express the gene of interest. This allows scientists to purify large amounts of a protein that might be difficult to purify from the species that the protein naturally occurs in. For example, human growth hormone is a protein given to children with growth problems. Large amounts of it are now produced inexpensively by bacteria containing the human growth hormone gene, whereas in the past human growth hormone had to be isolated a great expense from human cadavers.

The plasmid we will work with contains a gene for a protein called GFP (green fluorescent protein). The GFP gene comes from jellyfish, but it has been placed into the plasmid by genetic engineering techniques. We are using this as a model system to study aspects of recombinant DNA technology and protein expression. Briefly, we will isolate the plasmid from the bacteria then cut up the plasmid with restriction enzymes as a way of analyzing the size of the GFP gene.

The experiment will be performed in a series of steps, the first being to lyse open the bacteria and purify the plasmid. Much is known about the chemical properties of DNA and other cellular components, so there are several well-established procedures for isolating and purifying plasmid DNA. The following procedure uses a method of isolating plasmid from bacteria called the alkaline lysis method, but our method also includes the use of devices called spin columns to help purify the plasmid DNA.

b) Procedure

1) Everyone in the group should put on gloves. Obtain the following items:

- Pipet stand
- p200 micropipet and disposable tips
- p1000 micropipet and disposable tips
- Three clean empty microcentrifuge test tubes
- Microcentrifuge test tube containing Plasmid Resuspension Buffer B1 (the buffer in this tube should be pink)
- Microcentrifuge test tube containing Plasmid Lysis Buffer B2 (the buffer in this tube should be blue)
- Microcentrifuge test tube containing Plasmid Neutralization Buffer B3 (the buffer in this tube should be yellow)
- Microcentrifuge test tube containing Plasmid Wash Buffer 1
- Microcentrifuge test tube containing Plasmid Wash Buffer 2
- Microcentrifuge test tube containing Plasmid Elution Buffer
- Spin column
- Plastic biohazardous waste bag for all solid and liquid wastes
- Marking pen

2) Write LC (for "lysed cells") and your group's name on one of the empty test tubes. Use a pipet on the front desk to fill this test tube to the top with liquid bacteria culture. Put your filled LC tube into the centrifuge. Be sure to note the space number where you placed your tube. As always, be sure that there is a tube of equal weight opposite your tube in the centrifuge and that the lid is on the rotor before centrifuging. Centrifuge the LC test tube at maximum speed for 30 seconds to pellet the bacterial cells.

3) When the centrifuge has stopped, remove your tube and dump out the supernatant into the biohazard waste bag at your desk. Dump the tube out several times and hard! The pellet of bacteria will not fall out.

Refill the LC tube with bacterial culture from the front desk and re-centrifuge the tube again for 30 seconds. Again, dump out the supernatant into your biohazard bag.

Refill the LC tube with bacterial culture from the front desk yet again, and re-centrifuge the tube again for 30 seconds. Again, dump out the supernatant into your biohazard bag.

Place the tube back into the centrifuge again (being sure again that your tube is opposite another tube and that you note your space number) and centrifuge again at maximum speed for 10 seconds. After this, use your p200 micropipette (set at 200 ul) to pipet out as much as of the supernatant as possible. Try not to pipette out any of the pellet of bacterial cells at the bottom of your tube. Discard the pipet tip into the biohazard waste bag.

4) After putting a new tip on your p200, add 200 ul of Plasmid Resuspension Buffer B1 (pink) to the pellet using the p200. Resuspend the pellet by gently pipetting up and down many times while digging at the pellet with the pipette tip as you pipet up and down. Keep resuspending the pellet until the bacterial cell solution is completely homogenous (no chunks). When done resuspending the cells, discard the pipet tip into the biohazard waste bag.

Resuspension Buffer B1 is an isotonic buffer. In other words, it prevents the bacterial cells from being lysed open by osmotic water gain.

5) After putting a new tip on your p200, add 200 ul Plasmid Lysis Buffer B2 (blue) to the LC test tube. After adding Plasmid Lysis Buffer B2 to the LC tube, close the cap on the tube and then mix by inverting the LC tube SLOWLY and GENTLY 6 times in 30 seconds. If the solution is mixed correctly it will become dark transparent pink and viscous.

6) Put a tip on your p1000 and add 400 ul of Plasmid Neutralization Buffer B3 (yellow) to the LC test tube. After adding Plasmid Neutralization Buffer B3 to the LC tube, close the cap on the tube and then mix by inverting the tube SLOWLY and GENTLY 6 times. If the solution is mixed correctly it will become yellow and you will see a precipitate (solid material). When done mixing, incubate the tube at room temperature for 2 minutes.
The Plasmid Lysis Buffer that you added in step 5 lysed open the bacteria by disrupting their cell membranes. The lysis buffer contains SDS (a detergent that dissolves cell membranes) and NaOH (a base that detaches fatty acids from the glycerol in the membrane phospholipids).

The Plasmid Neutralization Buffer that you added in step 6 contains potassium acetate. The potassium acetate neutralized the NaOH base that you added in the previous step. The potassium acetate also precipitated most of the large bacterial molecules (such as proteins, membrane lipids, the cell wall, and the chromosome). The potassium acetate does not precipitate the plasmid or other small molecules. Therefore, after you added the Plasmid Neutralization Buffer, the plasmid was in the supernatant (the liquid) not in the precipitate (the solids) that you saw floating in the test tube.

The Plasmid Neutralization Buffer also contains the enzyme RNAse A, which degrades most of the RNA from the bacteria.

7) Centrifuge your LC tube for 5 minutes at maximum speed to pellet the precipitated large molecules.

8) Write your group's name on the spin column. When you have retrieved your LC test tube from the centrifuge, use the p1000 to carefully transfer the upper 700 ul of the supernatant (which contains the plasmid released from the bacteria) to the spin column. **Be sure to not transfer any of the pellet** (which contains precipitated proteins, lipids, and other cellular debris from the bacteria). It is better to leave some of the supernatant behind than to transfer any of the pellet. If there is any precipitate floating on the top of the supernatant, also

avoid transferring any of it. Throw away the LC test tube with the pelleted debris into your biohazard waste bag.

The Spin Column column contains a silica matrix that binds DNA tightly. There is a natural attraction between DNA and silica. Non-DNA molecules (RNA, proteins, and other bacterial molecules) also bind to the silica but these molecules bind less tightly to the silica than the plasmid DNA.

8) Write WC (for "wash collection") and your group's name on a clean test tube. Insert the spin column into the WC tube. Centrifuge the spin column/WC tube for 1 minute. After taking the spin column/WC tube out of the centrifuge, pour out the flow-through liquid into your biohazard waste bag.

The plasmid DNA and certain other molecules that are attracted to the silica matrix are still in the spin column. The flow-through liquid contains the bacterial molecules that did not bind strongly to the silica matrix in the spin column.

9) After discarding the flow-through from your WC tube, re-insert the the spin column into the empty WC tube. Pipet 200 ul of Plasmid Wash Buffer 1 into the spin column. Then centrifuge the spin column/WC tube for 1 minute. After taking the spin column/WC tube out of the centrifuge, pour out the flow-through liquid into your biohazard waste bag.

The plasmid DNA is still bound to the silica matrix in the column. Plasmid Wash Buffer 1 removed some of the RNA, proteins, and other non-DNA molecules that were also attached to the silica matrix in the column.

10) After discarding the flow-through from your WC tube, re-insert the spin column into the empty WC tube. Pipet 400 ul of Plasmid Wash Buffer 2 into the spin column. Then centrifuge the spin column/WC tube for 1 minute. After taking the spin column/WC tube out of the centrifuge, pour out the flow-through liquid into your biohazard waste bag.

Plasmid Wash Buffer 2 (similarly to Plasmid Wash Buffer 1) removed certain non-DNA molecules from the silica in the column. Plasmid Wash Buffer 2 contains alcohol. Recall from the genomic isolation lab that alcohol precipitates DNA, but alcohol does not precipitate most other molecules. As a consequence, essentially all of the non-DNA molecules (such as salts and other small molecules) attached to the silica matrix were dissolved by the Plasmid Wash Buffer 2 and were therefore removed from the silica matrix by the buffer. The precipitated plasmid DNA (which remains attached to the silica matrix) is therefore purified by Plasmid Wash Buffer 2.

11) Discard the WC tube (including the flow-through that it contains) into your biohazard waste bag. Obtain a clean test tube and write Plasmid and your group's name on the clean test tube.

12) Insert the spin column into the Plasmid tube. Pipet 40 ul of DNA Elution Buffer into the spin column. Be sure that the 40 ul completely covers the silica matrix inside the column. Incubate the spin column/Plasmid tube for one minute.

During the one minute incubation, the Elution Buffer will detach the plasmid DNA from the silica in the column. The Elution buffer contains substances that protect the DNA from being degraded by enzymes that destroy DNA.

13) Centrifuge the spin column/Plasmid tube for 1 minute. Take the spin column/test tube out of the centrifuge. Discard the spin column.

The Plasmid tube should now conatin the purified plasmid dissolved in the 40 ul of buffer. Give this test tube to your instructor. Your plamsid DNA will be stored in a freezer until you analyze it by digestion with restriction enzymes in a future laboratory period.

c) Review questions

1) What is the defining difference between a prokaryotic cell and a eukaryotic cell?

2) What are two differences between the chromosome(s) of prokaryotes and eukaryotes?

3) What is a plasmid? In what ways are they similar and different to the prokaryote's chromosome?
4) Define the following:

Bacterial culture:

Cloning a piece of DNA:

Cloning vector:

RNase enzyme:

5) What is the role of SDS in plasmid isolation?

6) What are the products of the reaction between NaOH and membrane lipids?

7) After the bacterial cells were lysed in step 5, the test tube contained a mixture of all the molecules from inside the bacteria, including large molecules such as the bacterial chromosome, proteins, and cell wall material, as well as small molecules such as the plasmid. In step 6, you added a neutralization buffer. This buffer separated the large molecules of the bacteria from the smaller molecules. Briefly describe how this separation was accomplished (hint: The large and small molecules were still in the same test tube, but they were in different phases (solid versus liquid) within the test tube).

8) What is the name of the substance inside the spin column that binds the plasmid?
9) Plasmid Wash Buffer 2 contains large amounts of ethyl alcohol. Briefly explain how the alcohol helped in purifying the plasmid from contaminating salts and other small molecules that were also bound to the silica in the spin column.

10) The final purified plasmid was eluted (removed by dissolving) from the spin column using an elution buffer. We could have also eluted the plasmid from the column using plain water. What is the advantage of dissolving plasmid DNA in the Elution buffer rather than dissolving the plasmid DNA in water?