Purification of pARA-R from an Overnight Culture

INTRODUCTION

Genes of interest to biologists can be found in either genomic DNA or in a plasmid. To study the DNA of the gene, the DNA must be isolated from the thousands of other kinds of molecules in the cell. Some of these molecules include lipids, proteins, and other nucleic acids such as RNA.

The purpose of this laboratory is to introduce one method commonly used to extract and purify plasmid DNA. This protocol isolates the recombinant plasmid, pARA-R, used to transform *E. coli* in Laboratory 5. This is a necessary step that leads to the final "proof" that transformation was the result of the pARA-R plasmid construct.

First, the transformed cells, from an overnight culture, are suspended in a Glucose/Tris/EDTA (GTE) buffer. GTE functions as a pH buffer, destabilizes the plasmalemma, and binds divalent cations, which are common cofactors for DNA degrading nucleases which maybe present. Cells are then lysed using a sodium dodecyl sulfate/sodium hydroxide solution, SDS/NaOH. SDS is a detergent that breaks down the plasma membrane, and NaOH creates an alkaline solution, which denatures chromosomal DNA. The covalently closed circular nature of a plasmid helps it to resist irreversible denaturation at this pH.

Cell debris and genomic DNA are then precipitated from solution using a solution of potassium acetate/acetic acid, KOAc. Because the bacterium's genomic DNA is attached to its plasma membrane, it will precipitate along with the cell debris. KOAc neutralizes the NaOH allowing the plasmid helix to renature. This will leave the plasmid DNA in the supernatant. Isopropanol is used to precipitate the plasmid DNA from the supernatant leaving the proteins in solution. Finally, a 70% ethanol solution is used to wash the plasmid DNA.

MATERIALS

Reagents and Cultures

- Overnight *E. coli* cultures (R and W)
- GTE buffer
- SDS/NaOH
- KOAc
- Isopropanol
- Ethanol (70%)
- Crushed ice
- TE buffer, pH 8,0

Supplies and Equipment

- P-20 micropipette with tips
- P-1000 micropipette with tips
- 1.5-mL microfuge tubes
- Beaker with disinfectant
- Speed Vac (or hair dyer)
- Test tube rack
- Marker
- Microcentrifuge
- Vortex apparatus

METHODS

Preparing cells for lysis

1. Obtain the two culture tubes containing transformed *E. coli* prepared during the previous lab. Remember the cells have been grown in a broth containing ampicillin. Finger vortex the tubes to re-suspend the cells.

2. Label two 1.5-mL microfuge tubes with your group identification and culture designations (R and W); transfer 1000 μL of each *E. coli* culture into the appropriate microfuge tube.

3. Close the caps and place the microfuge tubes in a balanced configuration in the microcentrifuge. Spin for **30 seconds** (@12,000xg) to pellet the cells. A cream-colored cell pellet will be visible at the bottom of each tube.

4. Without disturbing the cell pellet, discard the liquid *supernatant* from both tubes by dumping it into the beaker containing disinfectant. Use a Kimwipe to wick away any remaining LB/amp broth. *Do not* allow the Kimwipe to touch the cell pellet. Discard the Kimwipe in the disinfectant.

5. Add 100 μL of ice-cold GTE buffer to the tubes with cell pellets, cap the tubes and resuspend by dragging the tubes across the plastic tube rack several times. Continue to resuspend the cells until no visible clumps of cells remain.

Lysing the cells to extract plasmid DNA

1. Add 200 μL of SDS/NaOH solution to each tube, cap each tube, and mix by inverting four times.

2. Return the two microfuge tubes containing the lysed cells to ice for **five minutes**. The suspension will become clear as the cells are lysed.

3. Add 150 μL of ice-cold KOAc solution to *each* tube, close caps and mix solutions by rapidly inverting tubes four times. A white precipitate will form. The potassium acetate precipitates the SDS from solution, along with proteins and lipids. Genomic DNA is trapped in this precipitate but the plasmid DNA remains in the supernatant.

4. Return the two microfuge tubes to the ice for **five minutes**.

5. Place the tubes in the microcentrifuge and spin for **five minutes** (@12,000xg). Cell debris will appear at the bottom of each tube.

6. Transfer 200 μL of the **supernatant** from *each* tube to two clean 1.5-mL microfuge tubes labeled with your group number. ***Avoid transferring any of the precipitate***. Discard the old microfuge tubes, containing cell debris, into the disinfectant. At this point, you will have two microfuge tubes each containing 200 μL of supernatant. Suspended in this liquid is plasmid DNA.

Separating DNA from proteins

1. Before proceeding to step number 2; be certain the microcentrifuge is available since it will be needed *immediately* following step 2. It may be necessary to wait for a few other groups before going on so that a number of groups can use the microcentrifuge together. In the mean time, be certain that your tubes are clearly marked so that you will be able to recover them following centrifugation.

2. Add 200 μL of isopropanol to each tube of supernatant, close caps and mix *vigorously* by rapidly inverting the tubes four or five times or briefly vortexing. Let each tube stand *at room temperature for only **two minutes***. Isopropanol precipitates DNA but will precipitate proteins if given enough time. So the longer it takes to get the tubes into the microcentrifuge, the more protein contamination will result

3. Place the two tubes in the microcentrifuge and spin for **five minutes** (@ 14,000xg). Align the tubes so that the *hinge* of each tube is on the side furthest away from the center of the rotor head. Oriented this way, the DNA will pellet on the hinge-side of the tube.

4. From both tubes, *carefully* pour-off the supernatant, saving the DNA pellets that should be located near the bottom and on the hinge side of each tube. Invert each tube and gently tap each tube over a Kimwipe to drain any residual isopropanol from the tubes.

5. Add 300 μL of 70% ethanol to each tube; close the caps and *finger* vortex to wash the pelleted DNA. The water in the ethanol solution removes some of the remaining salts and SDS from the precipitate.

6. Place each tube in the microcentrifuge (hinge out) and spin for about **three minutes** (@ 14,000xg).

7. Discard the supernatant from both tubes *being careful not to disturb the DNA pellet*. Invert each tube and gently tap each tube over a Kimwipe to drain any residual ethanol from the tubes.

8. Use the speed vac to dry the DNA pellet. Your instructor will explain how to use this instrument. Alternatively, you can use a small hair dyer to *gently* evaporate the ethanol. Do not, however, aim the hair dryer directly into the microfuge tube as this could blow the plasmid pellet out of the tube.

Resuspending DNA for restriction analysis

1. Once the ethanol has been evaporated from the microfuge tube, add 15 μL of TE buffer to *each* tube, and resuspend the DNA by finger vortexing or flicking the tube with your finger. Check carefully to see that all of the DNA has been redissolved and that no DNA remains on sides of the tubes. Vortex if necessary. TE buffers the solution and protects the DNA from degradation by DNases.

2. You will analyze the DNA isolated from each colony separately. By analyzing them separately you will be able to tell if the bacterial cell was transformed with a recombinant plasmid or if there was a double transformation with both original plasmids. Be sure each tube is labeled with your group number and "R" or "W" to indicate the two colonies.

Store the DNA/TE solution at -20°C if restriction analysis must wait for the next laboratory session.

CONCLUSIONS

There are several protocols that can be used to extract and purify DNA. This protocol is often referred to as a "miniprep" since the number of cells required and the DNA yield are small. The procedure, however, uses reagents that are inexpensive and relatively non-toxic. There are commercial products that can greatly reduce the time required for both "mini-" and "maxipreps." Most of these, however, are based on some modification of this miniprep protocol.

Although one could conclude that transformation had taken place by examining bacteria growing on a selective medium containing ampicillin, final "proof" generally requires that the plasmid be extracted, purified and subjected to restriction analysis. The plasmid DNA that was extracted during this protocol will be used for restriction analysis during the next laboratory.

1. In order to purify plasmid DNA, it is necessary to separate the plasmid from the other biological molecules in the cell, including the genomic DNA of *E. coli*. During what steps, in this protocol, was the plasmid DNA separated from each of the following:

   Proteins

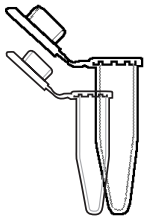
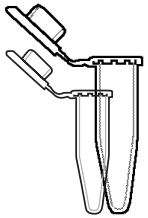
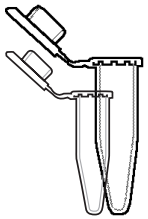
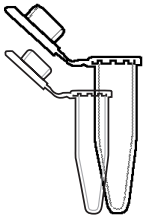
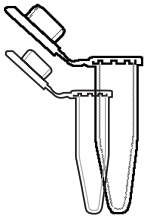
   Lipids

2. Fill out the worksheet on the next page to help complete your analysis.

3. Would you expect the final DNA/TE solution to be free of other biological molecules?

Miniprep Flow Chart

For each of the tubes, draw in a pellet, if there should be one, and list the contents of the pellet and supernatant wherever appropriate.

Add 1 mL of your E. coli culture to each of your tubes. Centrifuge for 30 seconds.	Add 100 µL of GTE. Add 200 µL of SDS/NaOH. Add 150 µL of KOAc. Centrifuge five minutes.	Transfer 200 µL of Supernatant to a clean Microfuge tube. Add 200 µL of isopropanol. Centrifuge five minutes.	Discard supernatant. Add 300 µL of ethanol. Centrifuge three minutes.	Discard ethanol. Dry pellet. Add 15 µL of TE.
				
Contents:				
Pellet	Pellet	Pellet	Pellet	Pellet
Supernatant	Supernatant	Supernatant	Supernatant	Supernatant

