

Lab 5 – Plasmid Purification and Quantification

Background:

In the laboratory, scientists can modify plasmids and use them to carry genes of interest into host bacteria. Once inside the bacteria the plasmids multiply as the bacteria divide. The plasmids can be isolated from the host bacteria using various techniques, such as the “plasmid miniprep procedure.” The miniprep procedure takes advantage of the small, coiled nature of the plasmids to separate them from the larger, linear chromosomal DNA.

Purpose:

To purify bacterial plasmid DNA using homemade reagents and learn to quantitate and analyze the purity of the purified DNA.

Procedure:

1. (This step has already been done for you.) Grow the plasmid-containing bacteria culture overnight in LB media. *LB media contains nutrients for bacterial growth.*
2. Remove 1.5 mL of the culture and spin down cells in microfuge.
3. Discard the supernatant and completely resuspend the cells in 100 μ L of ice-cold GTE buffer. *The buffer is kept cold to inhibit nucleases released during the procedure. GTE buffer contains tris to maintain proper pH, EDTA to bind divalent cations in the lipid bilayer to weaken the membrane, and glucose to prevent cells from clumping.*
4. Add 200 μ L of SDS/NaOH and gently mix. *The bacterial cells are lysed by the SDS, which solubilizes the membrane lipids and cellular proteins. The base denatures both the chromosomal and plasmid DNA. The strands of circular plasmid DNA remain intertwined. The suspension is mixed gently to avoid shearing or breaking the chromosomal DNA.*
5. Set on ice for 10 minutes. *This “resting step” allows time for the SDS/NaOH to contact all the cells and the process in Step 4 to go to completion.*
6. Add 150 μ L of cold potassium acetate/glacial acetic acid and mix gently. A white precipitate will appear. *The acetic acid neutralizes the NaOH added in Step 4, allowing the DNA to renature. The potassium acetate causes the SDS to precipitate, along with associated proteins and lipids. The large chromosomal DNA strands renature partially into a tangled web that precipitates along with the SDS complex. In contrast, the small, coiled renatured plasmids remain suspended. In this step, therefore, they are separated from chromosomal DNA, many proteins, and the detergent.*
7. Centrifuge the mixture; save the supernatant. *The discarded precipitate contains cellular material, linear DNA, protein, and detergent, whereas the supernatant contains the suspended plasmids.*
8. Transfer 400 μ L of the supernatant to a clean eppendorf tube, add an equal volume of isopropanol, and mix. *Isopropanol, like ethanol, precipitates DNA. Isopropanol is used here because a smaller volume of isopropanol is required than ethanol. The alcohol quickly precipitates DNA and more slowly precipitates proteins. **This step needs to be done quickly.***
9. Centrifuge and save the pellet. *The alcohol-precipitated plasmid DNA is in the pellet.*
10. Wash the pellet in 200 μ L of 100% ethanol. *Washing means to slowly pellet the ethanol up and down a few times to try to rinse the pellet. Although isopropanol precipitated the DNA in the previous step, an ethanol wash helps remove extra salts and other unwanted contamination.*
11. Centrifuge, dump out the ethanol, and turn the eppendorf tube upside down on a paper towel to dry, saving the pellet. *A white pellet may or may not appear when dry on the bottom of the tube. Do not proceed to the next step until the tube is dry.*
12. Resuspend the pellet in 15 μ L of tris/EDTA buffer (TE). *TE is often used for short storage of DNA. Tris maintains the pH and EDTA binds divalent cations to inhibit nuclease activity.*

Measuring Plasmid DNA Concentration

1. Turn on the spectrophotometer and wait for it to boot up.
2. Press the “λ” key
3. Enter “1” for the number of wavelengths.
4. Enter 260nm for the wavelength to measure.
5. Enter “NO” when asked if you want to subtract the background. The spectrophotometer is now ready to read a sample.
6. Pipette 500µL of diH₂O into the cuvette and press “Read Blank”
7. Remove water from the cuvette
8. Make 100 µL of a 1:10 dilution of the purified plasmid DNA in an autoclaved eppendorff tube.
9. Mix by inverting the tube several times.
10. Transfer the dilution to a clean cuvette
11. Place cuvette with sample in the spectrophotometer and press “Read Sample”
12. Record the absorbance
13. Repeat steps 11 and 12 two more times.
14. Obtain a separate cuvette with water in it.
15. Reset the spectrophotometer to measure at 280nm and measure the absorbance of you sample
16. Dispose of all tubes used and any remaining purified DNA in the trash.

Data: You will have three absorbance readings at 260 nm and one reading at 280nm. Calculate the DNA concentration for each absorbance reading.

How to Determine Plasmid Yield and Purity Using UV Absorbance Measurements

DNA yield can be determined spectrophotometrically by measuring the absorbance of a dilution of the purified plasmid at 260 nm. The normal calculation for dsDNA is $1 A_{260} = 50 \mu\text{g/ml}$, for a 1 cm path length cuvette. Assuming a plasmid yield of 1 mg resuspended in 1 ml, the expected absorbance of an undiluted sample will be approximately $A_{260} = 20$. For optimal accuracy, the target absorbance for UV spectrophotometer readings should be between 0.1–0.5. Therefore, the sample plasmid DNA should be diluted from 1:40 to 1:200 in deionized water prior to measuring UV absorbance. The recorded A_{260} should be multiplied by the dilution and the extinction coefficient ($50 \mu\text{g/ml}/1.0 A_{260}$) to obtain the concentration of plasmid DNA in $\mu\text{g/ml}$. Note that the results of A_{260} readings are accurate only in the absence of significant contamination by RNA and other UV-absorbing materials. Critical samples should also be analyzed by agarose gel analysis to confirm that the UV absorbing material is, in fact, plasmid and that the absorbance reading is consistent with the yield observed on an agarose gel. Additional information about purity can be obtained by reading the absorbance at 280 nm and determining the A_{260}/A_{280} ratio. Nucleic acids have an average absorbance maximum of approximately 260 nm, whereas proteins (assuming a normal distribution of aromatic residues) have an average absorbance maximum of 280 nm. Pure DNA and typical SpinPrep plasmid isolates have A_{260}/A_{280} ratio of 1.75–1.95. Preparations contaminated with protein have significantly lower ratios of 1.3–1.5, while higher ratios (greater than or equal to 2.0) may indicate the presence of significant levels of RNA.

Conclusion:

Calculate the average DNA concentration that you measured.

Calculate the purity of your plasmid sample.

Is your purity good? Why do you think so?

Background and protocol were taken from:

Seidman, L. A., & Moore, C. J. (2000). *Basic Laboratory Methods for Biotechnology - Textbook and Laboratory Reference*. Upper Saddle River, New Jersey: Prentice-Hall. Pages 500-501.