LABORATORY 3. PREPARATION OF PLASMID DNA

Today we will be isolating the plasmid DNA from the 3ml cultures that resulted from the colony selection after ligation and transformation. We will quantify this DNA using the nanodrop. In the next class, we will use restriction digests to confirm that the ligation produced the product we desired.

Exercise 1. Preparation of plasmid DNA.

There are a number of techniques for isolating plasmid DNA. Most labs have adopted one of the spin column kits on the market. These are fast and reliable. For DNA purification, we will use anion-exchange resin/ spin column technique available through Qiagen (Santa Clarita, CA). It is based on the alkaline lysis method of isolation of plasmid DNA. In step 1 you pellet your bacteria, remove the media, and resuspend the pellet in resuspension buffer. Next the bacteria are lysed by addition of an alkaline lysis buffer. The lysis time and buffer allows for the maximum release of plasmid DNA without release of chromosomal DNA. In the following step, the reaction mixture is neutralized and the adjusted to a high salt concentration. The high salt causes denatured proteins, chromosomal DNA, cellular debris and double stranded DNA to precipitate while the smaller plasmid DNA renatures and remains in solution. Follow the attached protocol for isolation.

Protocol: Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of *E. coli* in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 44.

Note: All protocol steps should be carried out at room temperature.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α™ do not require this additional wash step.

8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

Exercise 2. Quantifying DNA Using a Nanodrop spectrophotometer (modified the handouts from Dr. Paul T. Imhoff, University of Delaware, Dr. George Watts, University of Arizona and phagesdb.org)

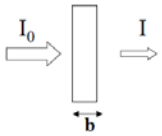
BACKGROUND

Nucleic acids absorb light at a wavelength of **260 nm**. If a 260 nm light source shines on a sample, the amount of light that passes through the sample can be measured, and the amount of light absorbed by the sample can be inferred. For double-stranded DNA, an **Optical Density** (OD) of 1 at 260 nm correlates to a DNA concentration of 50 ng/µl, so DNA concentration can be easily calculated from OD measurements.

These measurements were traditionally taken with standard spectrophotometers, but we now use a tabletop spec called a NanoDrop that requires only 1 µl of a sample for quantification. The principle of action is the same, but the practical usage is much easier.

Principle

Beer Lamberts Law



$$A = \varepsilon b c$$

A → absorbance (-)

ε → molar absorbtivity with units of L mol^{-1} cm^{-1}

b → path length of the sample (cuvette)

c → Concentration of the compound in solution, expressed in mol L^{-1}

The Beer-Lambert law draws a direct correlation between absorbance and concentration. While nucleic acids absorb at many wavelengths, they have a peak absorbance of UV light at 260 nm because of the aromatic base moieties within their structure. Thus, the amount of light absorbed in the 260 nm region can be used to determine the concentration of DNA in solution by applying the Beer-Lambert law. However, the Beer-Lambert equation is only linear for absorbances between 0.1 and 1.0. This translates to concentrations between 10.0 ng/uL and 3700 ng/uL when using the Nanodrop ND-1000. Samples outside of this range should be dried-down or diluted to produce more accurate spectrophotometry results.

PROCEDURE

1. Open the NanoDrop software on the computer by double-clicking the “ND-1000” icon that looks a bit like an hourglass.
2. Initialize the NanoDrop.
    a. Click on the “Nucleic Acid” button in the NanoDrop software. This will bring up a dialog box. DO NOT click “Okay” until you’ve added water.
    b. Add 1 µl of purified water to the lower pedestal, then lower the upper arm.
    c. Click “Okay” on the computer and wait ~20 seconds while the NanoDrop initializes.
    d. When it’s done, lift the upper arm and dry the pedestal with a wipe.

3. Blank the NanoDrop.
    a. Add 2 µl of the buffer your sample is in. If you resuspended a DNA pellet using TE, for example, blank now with TE.
    b. Lower the upper arm of the NanoDrop and click the "Blank" button on the software.
    c. Wait ~20 seconds for the blank measurement to be made.
    d. When it's done, lift the upper arm and dry the pedestal with a wipe.
4. Measure your sample.
    a. Add 1 µl of your sample to the lower pedestal, then lower the upper arm.
    b. In the "Sample ID" box, type in the name of your sample.
    c. Click the "Measure" button on the software and wait ~20 seconds for measurement.
    d. When it's done, lift the upper arm and dry the pedestal.
5. Collect your data.
    a. Write down any measurements you're interested in. You can move the cursor to check the absorbance number at various wavelengths.
    b. Click the "Print Screen" button to print the complete spectrum, if desired.
    c. When finished making all measurements, click "Print Report" to get a table of all data.
6. Clean the pedestal.
    a. Add 3 µl of purified water to the lower pedestal, then lower the arm.
    b. Wait 30-60 seconds.
    c. Lift the upper arm and use a wipe both the upper and lower pedestals.

Notes –

<u>Absorbance at 260 nm</u>

Nucleic acids absorb UV light at 260 nm due to the aromatic base moieties within their structure. Purines (thymine, cytosine and uracil) and pyrimidines (adenine and guanine) both have peak absorbances at 260 nm, thus making it the standard for quantitating nucleic acid samples.

<u>Absorbance at 280 nm</u>

The 280 nm absorbance is measured because this is typically where proteins and phenolic compounds have a strong absorbance. Aromatic amino acid side chains (tryptophan, phenylalanine, tyrosine and histidine) within proteins are responsible for this absorbance. Similarly, the aromaticity of phenol groups of organic compounds absorbs strongly near 280 nm.

<u>Absorbance at 230 nm</u>

Many organic compounds have strong absorbances at around 225 nm. In addition to phenol, TRIzol, and chaotropic salts, the peptide bonds in proteins absorb light between 200 and 230 nm.

<u>A260/280 ratio</u>

The A260/280 ratio is generally used to determine protein contamination of a nucleic acid sample. The aromatic proteins have a strong UV absorbance at 280 nm. For pure RNA and DNA, A260/280 ratios should be somewhere around 2.1 and 1.8, respectively. A lower ratio indicates that the sample is protein-contaminated. The presence of protein

contamination may have an effect on downstream applications that use the nucleic acid samples.

A260/230 ratio

The The A260/230 ratio indicates the presence of organic contaminants, such as (but not limited to): phenol, TRIzol, chaotropic salts and other aromatic compounds. Samples with 260/230 ratios below 1.8 are considered to have a significant amount of these contaminants that will interfere with downstream applications. This is especially true for reverse transcription. In a pure sample, the A260/230 should be close to 2.0