Bi 1X, Spring 2011

Week 2

- **Isolate DNA from Caltech Pond**

Your Mission:

In today's lab, each of you will collect a wet soil sample from one of the Caltech ponds where different organisms and bacteria live. Then using a specialized extraction kit, you can isolate the genomic DNA of these microbes for the 16s sequencing that will proceed throughout Bi 1X. At the end you will measure the purity of the extracted DNA using a NanoDrop spectrophotometer.

I. Sample Prep:

Materials:

- 50mL conical tube
- Centrifuge
- Weighing paper
- Balance
- Spatula
- PowerBead Tube
- Microcentrifuge tubes
- Filter tips

Protocol:

1. Using a 50mL conical tube and gloves, take a wet soil sample from the pond. You will need at least 0.25g of soil at the end of the procedure. **Be sure to record in your notebook which pond you selected from, where in the pond and any characteristics about the location you think are important.**
2. Centrifuge the tube for 5min at 4,000 × ***rpm*** to spin down all the soil, organism and bacteria to the bottom. Find a partner and make sure your tubes have equal mass to ***balance*** the centrifuge. A TA will help you load and start the centrifuge.
3. Remove the supernatant (the upper, non-pelleted liquid phase) by decanting into the sink, leaving just the pellet. (A muddy pellet is ok.)
4. Leave the conical tube with the pellet on the side for a moment. Take a PowerBead Tube (black) and transfer the beads and bead solution into another sterile 1.5mL microcentrifuge tube by decanting. Store the beads and solution at room temperature.

   The PowerBead Tube contains a bead matrix that will help physically lyse your sample as well as a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids (contaminants that occur in environmental samples) and (c) protect nucleic acids from degradation.

5. Take the ***empty*** PowerBead Tube and the soil pellet to the weighing area. Add 0.25±0.01g of your wet soil sample to the PowerBead Tube with a spatula. Make sure to **tare** the balance with the weighing paper before measurement.

6. Gently tap the PowerBead Tube on the bench to move the soil to the bottom. Centrifuge at room temperature for 30sec at 10,000 × ***g***.
7. Remove as much **liquid** as possible with ***filter*** tips.
8. Add beads and bead solution back from the microcentrifuge tube to the PowerBead Tube by decanting. Make sure to transfer all of the solution using ***filter*** tips to the PowerBead Tube.

II. DNA Extraction:

Materials (per person):

- PowerBead tube containing your sample
- Extraction solution set – C1, C2, C3, C4, C5, C6
- Four 2 ml collection tubes
- Spin filter in collection tube
- Filter tips

Protocol:

1. **Gently** vortex to mix (vortex on ~50% power for 3 – 4sec).

   Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the PowerBead Solution.

2. Add 60 µl of **Solution C1** and invert the tube several times or vortex briefly.

   Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms.

3. Secure PowerBead Tubes horizontally in the **Vortex Adapter tube holder** (ask your TA for help). Vortex at maximum speed for 10 minutes.

   The vortexing step is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1-2 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open.

4. Centrifuge tubes at 10,000 x ***g*** in a microcentrifuge for 30 seconds at room temperature. **CAUTION: Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Be sure NOT to exceed 10,000 x *g* or tubes may break.**

   This step will pellet the lysis beads and cell debris to allow them to be separated from your sample.

5. Transfer **all** of the supernatant to a clean 2 ml Collection Tube.

   Expect between 400 to 500 µl of supernatant at this step. The exact recovered volume depends on the absorbancy of your starting material and is not critical for the procedure to

be effective. The supernatant may be dark in appearance and still contain some environmental debris. Subsequent steps in the protocol will remove these contaminants.

6. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

   Solution C2 contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications, like PCR.

7. Centrifuge the tubes at room temperature for 1 minute at 10,000 x ***g***.

8. **Avoiding the pellet**, transfer **up to** 600 µl of supernatant to a clean 2 ml Collection Tube.

   The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

9. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.

   Solution C3 is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins.

10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x ***g***.

11. **Avoiding the pellet**, transfer **up to** 750 µl of supernatant to a clean 2 ml Collection Tube.

    The pellet at this point contains additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

12. Add 1.2ml of **Solution C4** to the supernatant (**be careful solution doesn't exceed rim of tube**) and vortex for 5 seconds.

    Solution C4 is a high concentration salt solution. DNA binds tightly to silica at high salt concentrations, and this buffer will adjust the DNA solution salt concentrations to allow binding of DNA (but not non-DNA organic and inorganic material that may still be present at low levels) to the Spin Filters.

13. Load approximately 675 µl of your sample onto a Spin Filter and centrifuge at 10,000 x ***g*** for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x ***g*** for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x ***g*** for 1 minute at room temperature; discard the flow through. **Note: A total of three loads for each sample processed are required**.

DNA is selectively bound to the silica membrane in the Spin Filter device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

14. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x ***g***.

    Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

15. Discard the flow through from the 2 ml Collection tube.

    This flow through fraction is just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

16. Centrifuge the **empty** Spin Filter at room temperature for 1 minute at 10,000 x ***g***.

    This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

17. Carefully place Spin Filter in a clean 2 ml Collection Tube. **Avoid splashing any Solution C5 onto the Spin Filter.**

    It is important to avoid any traces of the ethanol-based wash solution.

18. Add 70 µl of **Solution C6** to the **center** of the white filter membrane. **Be careful not to poke the membrane with your pipette tip!**

    Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6, which lacks salt.

19. Centrifuge at room temperature for 30 seconds at 10,000 x ***g***.

20. Discard the Spin Filter. The DNA in the tube is now pure and ready for any downstream application. Label your tubes clearly and place them **on ice**. **Treat the tubes gently, and avoid shaking excessively, which may shear the unprotected DNA.**

III. Spectrophotometry of DNA

Spectrophotometry is a process by which the ability of a sample to absorb light at different wavelengths can be examined quantitatively. Spectrophotometry can be used to measure the concentration and purity of a sample of DNA. You will analyze your purified DNA using a NanoDrop ND-2000 spectrophotometer. It is a cuvette-free device that can scan very small volumes of sample.

Quantification

DNA absorbs light primarily at 260 nm; spectrophotometric measurements of DNA at this wavelength can be used to determine its concentration, which varies directly with absorbance. It has been established experimentally that a DNA solution measured to have an OD_{260} of 1.0 in a standard 1 cm path length cuvette has a concentration of 50ng/µL. Multiplication of the OD_{260} of a DNA sample by this factor of 50ng/µL will result in its concentration. The NanoDrop mimics a 1 cm path length when supplying its results.

Purity check

Common contaminants in DNA samples are proteins, carbohydrates, solvents, and chelators. Because these tend to absorb at wavelengths other than 260 nm, their presence in a sample of DNA can be monitored. Protein, for example, absorbs at 280 nm. Therefore, the "260/280 ratio" (ratio of absorbance at 260 nm to that at 280 nm) is an important indicator of protein contamination. Pure DNA generally has a 260/280 ratio of 1.8. Solvents, ion chelators, humic acids (environmental contaminants) and certain carbohydrates will absorb at 230 nm. The "260/230 ratio" is therefore an additional measure of DNA purity. Pure DNA will have a 260/230 ratio of around 1.8-2.2.

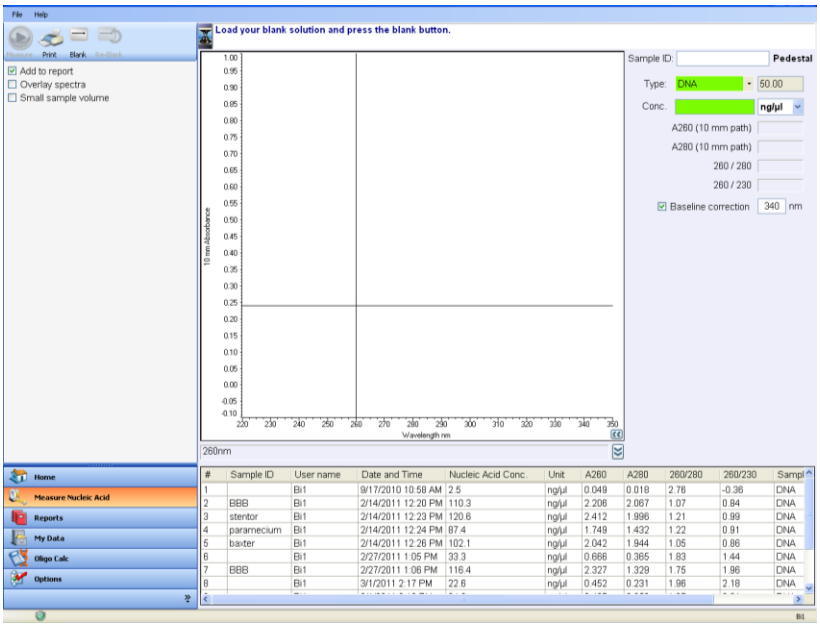
Using the Nanodrop ND-2000

If the instrument is already initialized, skip to step 4.

1. Start the Nanodrop ND-2000 software from the Start menu or by double clicking its icon on the desktop.

2. Select the **Nucleic Acid** application from the main menu. If the wavelength verification window appears, ensure the arm is down and click **OK**.

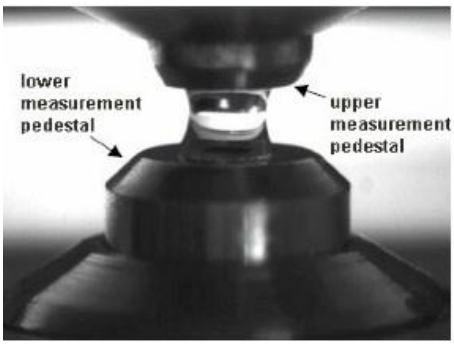
3. Select **Add to report** to automatically include all measurements in the current report. After initialization, the main nucleic acid measurement screen will appear (shown on the next page). For DNA readings, none of the settings need to be adjusted. Type in a sample ID.



4. *Perform a blank measurement*: Clean the pedestals by first raising the sample arm to the upright position and then wiping each pedestal severals times with a KimWipe (as shown).

Pipette 2 µl of "blank" buffer (in today's case, you would use 2 µl of plain **Solution C6**) onto the measurement pedestal (as shown).

Gently lower the arm to the downward position and click the **Blank** button. During the measurement, the device will form a sample column between the two pedestals and adjust automatically for an optimal path length (0.05 mm – 1 mm).



5. *Perform a sample measurement*: Raise the sample arm and clean off the pedestals with the KimWipe. Pipette a 2 µl sample of your DNA sample onto the measurement pedestal. Lower the sample arm and click **Measure**. Your measurement will be displayed.

6. Print your measurement by clicking **Print**. Save a copy of this in your lab notebook.

7. Clean off the pedestals with the KimWipe and the instrument is ready to measure the next sample.

References

1. Mo Bio Labs PowerSoil DNA Isolation kit Instruction manual www.mobio.com
2. Thermo Scientific NanoDrop 2000 Spectrophotometer v1.0 User Manual www.nanodrop.com