Lab 4: DNA concentration and length – this will take 1-2 days

Introduction: We need to determine *how much* DNA we purified, because most chemical reactions require balancing the reactants – that is, we only add a certain amount of DNA to the other components or all of it won't react. We also need to show about *how long* the pieces are, on average, because if the DNA is too short then our PCR and Restriction Digestion assays won't work. Because we ground up the DNA, we broke the chromosomes, chloroplasts and mitochondria into rather small parts. They start out hundreds of thousands to millions of bases long, but usually when we hand-grind the final length is 15,000-20,000bp. This length is fine for our assays, but if we failed to inactivate the enzymes that degrade the DNA then the pieces will be *much* smaller, so we need to check. Also, if we did not get rid of all the RNA it will interfere with our calculations of reagents. The RNA does not inhibit our assays but because it looks like DNA to the spectrophotometer it will give us too high a measurement for how much DNA we purified. RNA from cells is much shorter than the DNA (hundreds to a few thousands of nucleotides) so if we visualize the length of the DNA we will also see a smear of shorter pieces that is RNA. This length visualization is done using gel electrophoresis and a fluorescent dye that slides between the bases of the DNA and glows (fluoresces) under UV light. The dye does not glow very much if it is not inserted in the DNA helix so we will only see glowing bands in the gel where we have DNA.

And finally, in terms of quality control, we may have co-purified some small organic molecules that will inhibit the enzymes used in the polymerase chain reaction (PCR) or restriction digestion reactions we are using to genetically type the DNA. We will do a PCR test using primers that are specific to chloroplast DNA sequences – there are hundreds of copies of the chloroplast DNA in most leaf cells, so we don't have to use very much of the sample to carry out this test. These primers make a very short fragment so it does not take very long to do the test – the product is also displayed by gel electrophoresis.

Goals: understand how to use spectrophotometry to quantify the amount of DNA we purified, gel electrophoresis to quantify the length of the DNA we have (and indirectly double-check that concentration), and the polymerase chain reaction to make sure we have not co-purified small molecules that will inhibit the reactions we want to carry out for molecular characterization of the samples.

Part 1: DNA concentration from NanoDrop Spectrophotometry

1. Take the samples to the Nanodrop 1000 spectrophotometer. You should find next to the spectrophotometer : a 10ul micropipetter, 10ul tips, the resuspension buffer (we used 1X TE buffer) for blanking (correcting for buffer contributions to the absorbance), Kimwipes, a squirt bottle of water and a waste box as well as your samples.

    a. Start the Nanodrop software on the laptop (there is an icon on the desktop of each computer)

    b. Select the correct module – this will be Nucleic Acid, and the Sample type will be DNA-50 or dsDNA (double-stranded).

    c. For the first use of the day, the instrument will need to be initialized – the arm is placed in the down position (gently) and the instrument will let you know when it is ready. It is checking that the distance the light travels (the light path) is OK.

    d. *Blank* the instrument by placing 2ul of the correct buffer on the reading surface and gently putting down then arm, then clicking on 'Blank' (top left corner of screen). The instrument will tell you when it is ready.

    e. Read a sample: move the arm up, blot off the buffer with a Kimwipe and place 2ul of the sample solution on the reading surface. Put the arm down, and click the 'Measure' button.

    f. You should get a spectrum of absorbance at the listed wavelengths below as well as ratios between then for 260nm, 280 nm, 230nm, and an estimated mass concentration. Note that the concentration uses an average molecular weight for nucleotides. Write down the ng/ul and the A_{260}/A_{280} ratio values in your notebook.

        i. Repeat the measurement at least once unless you are extremely limited in sample volume. If the two measurements are more than 10% different repeat the measurement again.

        ii. Things that may cause problems:

            1. When the solution is *too concentrated* the instrument may not give a reading – it may appear to be 'zero' instead of infinity. If your solution is very 'sticky' it may be very concentrated, so make a 1:10 dilution (10ul sample +90ul water, vortex, quick-spin) and then try reading the diluted sample on the spectrophotometer.

            2. Air bubbles can create problems, blot off the sample and try again.

3. If your repeated readings are significantly different from each other, then try a 1:10 dilution to see if the readings stabilize.

4. Once you have 2 readings that are within 10% of each other, take the average and use this as the concentration of the sample (write it on the side of the tube). If you had to make a dilution don't forget to take that into account.

Part 2: DNA Length from Agarose Gel Electrophoresis

Background

Gels are used for quality control of biomolecules, particularly nucleic acids and proteins. Molecules have a net charge (positive or negative) so they will migrate towards the opposite charge in an electric field. If you have a gel (sort of like a water-filled sponge) the molecules have to work their way through the pores in the gel – the bigger the molecule, the harder it is to fit through the pores and the slower they are. Smaller molecules work their way through more quickly. To make the molecules move through the gel, a force has to be applied – in electrophoresis we use an electric field to impose that force.

Nucleic acids are negatively charged, so in the electric field they will move towards the positive end of the field. Thus gels are set up with one edge having a negative potential and the other a positive potential, There are two poles on the electrophoresis device, one is black (negative) and the other is red (positive) - also called the anode and cathode.

Gels also allow you to tell if the molecules are pure (is there only one band?) and if they are intact (if there are smaller products the sample may be degraded) – this is also called their integrity.

As mentioned in the introduction, the size of the pores in the gel limits how fast different length scan move under the charged field, so the molecules separate by length – if you include some size standards you can estimate the length of your sample.

Usually you have to use a stain of some kind in order to visualize the molecules. We use fluorescent dyes that slide between the stacked nucleotides ('intercalate' into the DNA bases) and then fluoresce under ultraviolet (UV) irradiation.

Your genomic DNA should be ~15kb (15,000) in length. Thus we want to run a fairly low-percentage gel (= big pores in the gel), and use DNA markers that go from 1kb to 15kb (or more). To see the DNA we need at least ~0.5-1ug of your genomic DNA if most of it is a common size. This will be estimated from your Nanodrop spectrophotometer readings.

When putting the sample on a gel, you will have to mix it with a Loading Dye, which has some glycerol in it to make your DNA solution heavier than the electrophoresis buffer, so it sinks down into the well of the gel. If you incorporate ethidium bromide (a fluorescent dye) in the gel you can check the migration of the DNA on an ultra-violet light transmitting illuminator. Be sure that there is a shield in place between your face/eyes and the UV radiation!! You can check the gel at various times and return it to the electrophoresis chamber to run longer if necessary. Generally you need 15-20 ng per band of DNA to visualize it well with eithidium bromide, but since we have a wide size range we need to load a fairly large amount.

Technical Notes

The minigels we are using require ~40 ml of gel solution (a thinner gel is better, but too thin means very small well volume for sample loading) and the midi-gels about 70 ml.

Select your gel tray and comb depending on the number of samples: you will want 1 lane per sample and 2 lanes for the DNA size standards (sometimes called DNA ladders or markers). Make sure your comb fits your gel casting tray before you start. Make sure the casting tray fits into the electrophoresis tank before you start. We have a lot of mixed rigs and not all of the pieces are compatible.

1% agarose gel with ethidium Bromide

Note: (*wear gloves, eye protection and lab coats and dispose of the gel in the tray in the chemical hood – we will let it dry out and then place it in the BioHazard waste*).

1. Prepare the gel tray by putting the dams on the ends (or taping up the two open ends if dams are not available).
   i. Make sure the casting tray and comb are clean – do a final rinse with distilled water and wipe dry with a Kimwipe.
   ii. Fit dams on each end of the casting tray
   iii. Place on a flat surface and insert a comb near the top (this will form wells).
2. Make and pour a 1.0% agarose gel solution.
   i. Weigh out 1.0gm of agarose into a weigh boat (don't forget to tare it) and transfer to a 250 ml Erlenmeyer flask
   ii. Measure 10ml of 10X buffer and add to the flask, swirling to avoid lumps in the powder
   iii. Measure 90ml of ddH2O and add to the flask, swirl again.
   iv. In order to hydrate the agarose so it goes into solution you have to heat it – for this we use the microwave, and because the agarose is granular, you do have to worry about superheating – when you remove the flask to swirl the solution be sure to wear a protective orange glove, glasses and to point the neck of the flask away from your face.
   v. Swirl, then place in the microwave for 30 sec on maximum power.
   vi. Wearing a protective glove, swirl the solution, pointing the neck of the flask away from your face.
   vii. Repeat 30 second heating/mixing steps until the solution is perfectly clear – it will be bubbling, but you don't want it to boil over. Hold it up to the light to make sure you don't see any specks of undissolved material.
   viii. Cover the neck of the flask with foil, and let the mixture cool to about 60C – this usually takes ~ 20 minutes or so- swirling occasionally
      1. You can put it in the 65C water bath so it will stay liquid while you do other things but it will slow down the cooling.
      2. You can swirl it under cool water in the sink to speed up the cooling (about 1 minute).
      3. If you pour it when it is too hot it will crack the gel tray. (BAD)
   ix. Once it is cool, add 10ul of the 10ug/ml *ethidium bromide* and swirl to mix.
      1. *Dispose of the tip in Biohazard waste*.
3. Pour ~40 ml into the prepared apparatus (not quite half).
   1. Cover the lid of the Erlenmeyer flask with Parafilm and label it ('1.0% agarose in 1X TBE with EtBr')– you will be able to use this later.
4. Let stand 20- 30 minutes. The gel is set when it is no longer clear – it will look like fogged glass and the casting tray will be cool to the touch.

1. If not running the same day, wrap in plastic wrap or a Baggie, label, and store at 4C.

Note: while waiting for the gel to set you can make the 1X TBE running buffer (step 4 below) if there is none already prepared in the carboy and prepare the samples for loading on the gel.

5. To set up the gel for electrophoresis
    1. Remove the tape or dams from the edges
        a. Note: if you use a spatula to hold the end of the gel nearest to the comb away from the dam then the gel is less likely to tear when you pull away the dam.
    2. Remove the comb.
    3. Place the gel in its casting tray in the electrophoresis chamber.
        ii. *Make sure the wells are on the 'black' end so the samples will run towards the 'red' end.*
    1. We have provided a carboy (10 liter container with a spigot) of 1X TBE electrophoresis buffer.
        iii. If needed: make 1L of 1X TBE running buffer by adding 100ml of 10X TBE to a graduated cylinder and filling to the 1L line with 18 Mega-Ohm (Ω) Nanopure water from the carboy. Stretch a piece of Parafilm over the end, then use your palm to form a pressure seal – invert 5-6 times to mix.
    1. Pour 1X TBE buffer into the electrophoresis tank, until you *just barely cover the gel*.
        a. You will need ~300 ml of buffer for the minigels.
        b. Pour any remaining buffer back into the carboy or stock bottle.

6. Now you are ready to load the gel – these wells generally hold 15-20ul but this depends on the thickness of the gel. You don't want the sample to overflow the well, so you might want to check your maximum volume with some water with gel loading buffer (GLB) in it (you can rinse it out once you are sure of the volume). Make sure the wells are at the end of the rig with the black electrode so samples run towards the red electrode. Regular micropipetter tips will work fine for loading samples, but you will need a steady hand since you need to layer the sample into the bottom of each well without stabbing through it.
    a. Samples
        i. 5ul of DNA size standard (ladderA)
        ii. Sample 1: 6-10ul of gDNA sample containing 0.5-1ug, 5ul of 4X Gel Loading Buffer (GLB)
        iii. Sample 2: 6-10ul of gDNA sample containing 0.5-1ug, 5ul of 4X Gel Loading Buffer (GLB)
        iv. 5ul of DNA size standard (ladderA)

v. Put the lid on the apparatus, plug the electrodes into the power supply (red electrode on the lid should be at the bottom, the direction the samples are heading towards),
    1. Turn on constant voltage, set at 50V. You will run this until the lower of the two blue dyes that are in the Loading Dye is about 75% of the way down the length of the gel.
    2. This takes 2-3 hours

vi. We can check the progress on the UV transilluminator (make sure a shield is in place) – described below.

vii. We can document this by taking a digital image with an instrument in Dr. Weller's lab called the GelDoc, which you will use at UNCC.

b. Checking DNA progress through gel: wearing gloves, place the gel on the trans-illuminator surface. Close the shield, turn on the UV light. (If you are using the GelDoc there is a door that is closed, and you can look through the observation window).

i. You should see glowing bands in the gel – if you do, start the imaging software and take a picture, or make a sketch if no digital imaging device is available.

ii. If you do not see ANY bands (even the ladder), make a note in your notebook that the gel failed.

iii. If you see only see DNA ladder bands, you probably did not load enough sample – note this also.

iv. We will take gels run at Olympic to the UNCC lab to create a digital image for your notebook and print out a copy for you and your partner
    1. Note: we will reverse the image to save ink so your print will have dark bands on a white background instead of bright bands on a dark background.

v. Turn off the transilluminator and remove the safety shield.

vi. Dispose of the gel in the labeled Baggie in the hood - we will take this to the chemical hood at UNCC and let the gels dry out and then put them in the BioHazard waste to be autoclaved.

vii. Spray the transilluminator surface with 70% ethanol and then wipe the surface dry with a Kimwipe.

c. Wearing gloves, using a funnel, pour the used electrophoresis buffer back into the 1X TBE carboy. The buffer can be re-used 4-5 times.

i. Rinse the funnel, casting tray and the gel rig in Nanopure water.