Lab: DNA Quality Control

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 it is too small then our PCR and Restriction Digestion assays won't work. Because we ground up the DNA, we broke the chromosomes into rather small parts. They start out hundreds of millions of bases long, but usually when we hand-grind the final length is 15-20,000bp. This length is fine for our assays, but if we failed to inactivate the enzymes that degrade the DNA it will be much smaller so we need to check. Also, if we did not get rid of all the RNA we will see a long 'smear' of smaller fragments. The RNA does not interfere with our assays but because it looks like DNA to the spectrophotometer it will give us an incorrect measurement for how much DNA we purified. We visualize the length of the DNA 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 PCR reactions we are using to genetically type the DNA, so we will do a PCR test using primers that pick up chloroplast DNA – 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. This is detailed in Lab 5: Chloroplast PCR test and Agarose Gel.

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 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 all wavelength and ratios of readings at 260nm, 280 nm, 230nm, and an estimated mass concentration. Note that the concentration uses an average mw 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 the 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', make a 1:10 dilution (10ul sample +90ul water, vortex, quick-spin) and then try reading the diluted sample.

      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 repeat the measurement yet again and try a 1:10 dilution to see if the readings stabilize.

Agarose Gel Electrophoresis

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 get to the bottom fastest.

Nucleic acids are negatively charged, so gels are run from black (negative) to red (positive) - also called the anode and cathode. Gels allow size to be estimated if the correct size standards are included.
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 molecule may be degraded) – this is also called their integrity.

If you put a control molecule on, whose size and mass you know, you can use that to estimate the size and mass of your unknown molecule(s). Usually you have to use a stain of some kind in order to visualize the molecules. We usually use fluorescent dyes that intercalate into the DNA bases and fluoresce under ultraviolet (UV) irradiation.

Your genomic DNA should be ~10kb in length. Thus we want to run a fairly low-percentage gel, and use DNA markers that go from 1kb to 10 kb or more. On the gel run ~0.5-1ug of your genomic DNA estimated from your Nanodrop readings. If you incorporate ethidium bromide in the gel you can check the migration of the DNA on a trans-illuminator and return it to the electrophoresis chamber to run longer if necessary. Generally you need 15-20 ng per band to visualize it well, but since we have a wide size range we need to load a fairly large amount. The minigels we are using require ~40 ml of solution (a thinner gel is better, but too thin means very small well volume for sample loading).

Select your gel tray 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). The mini gels require ~40ml of gel solution, the midi-gels about 70 ml and the large-format gels about 130ml. Make sure you have a comb that fits the tray, and that you can find the tanks for the particular tray as well (we have a lot of mixed rigs and they are not all compatible).

1% agarose gel with ethidium Bromide

Note: (*wear gloves, eye protection and lab coats and dispose of the gel in the chemical hood – let it try out and then place in 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. Pour a 1.0% agarose gel.
   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 until the solution is perfectly clear – it will be bubbling, but you don't want it to boil over.
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 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.
    3. If you pour it when it is too hot it will crack the gel tray. (BAD)
ix. Add 10ul of the 10ug/ml *ethidium bromide* and swirl to mix. *Dispose of the tip in Biohazard waste*.

3. Pour ~40 ml into the prepared apparatus.
    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 it is less likely to tear.
    2. Remove the comb.
    3. Place the gel in its casting tray in the electrophoresis chamber.
    4. 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 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.
    5. Pour 1X TBE buffer into the electrophoresis tank, until you just barely cover the gel.
        a. You will need ~300 ml
        b. Pour any remaining back into the carboy.

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.
   i. Samples
      1. 2ul of Bionexus Hi-Low ladder, 2 ul of water
      2. Sample 1: 6-10ul of gDNA sample containing 0.5-1ug, 3ul of 4X Gel Loading Buffer (GLB)
      3. Sample 2: 6-10ul of gDNA sample containing 0.5-1ug, 3ul of 4X Gel Loading Buffer (GLB)
      4. 2ul of Bionexus Hi-Low ladder, 2 ul of water

   ii. 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 blue dyes is about 75% of the way down.
      2. This takes 2-3 hours, so you will have to come back to turn off the gel and take a picture on the GelDoc (5 minutes).

b. Wearing gloves, place the gel on the trans-illuminator surface. Close the door, turn on the UV light. Look through the observation window. You should see glowing bands in the gel – if you do, start the imaging software and take a picture. If you do not, make a note in your notebook that the gel failed. If you see bands, save the image and we will print out a copy for you and your partner for your notebook (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).
   i. Dispose of the gel in the labeled box in the chemical hood - we will let the gels dry out and then put them in the waste to be autoclaved.
   ii. Spray the transilluminator surface with 70% ethanol and then wipe the surface dry with a Kimwipe.
   iii. Make sure the transilluminator position is 'off' before leaving.

c. Wearing gloves, using a funnel, put a 1L reagent bottle in the sink, pour the used electrophoresis buffer into the bottle, cap it. Label this as '1X TBE +EtBr, used' and put the date. The buffer can be re-used 4-5 times.
   i. Rinse the funnel, casting tray and the gel rig in Nanopure water.

To make sure the DNA does not contain enzyme inhibitors, carry out Lab5: Chloroplast PCR test