Biotechnology/Genomics Lab – Spectrophotometry Instructions

Lab Goals, Part I: quantify your dilution series of oligos using a spectrophotometer. We will use the Nanodrop 1000 UV for this and the NanoFluor for comparison (see link for example on how it works).

This spectrophotometer measures as little as 1.5 ul volume.

Preparation: There is a very entertaining video here: http://www.nanodrop.com/nd-1000-nanodrop-it-like-its-hot.html . You will obtain absorbance readings at 260nm, 280 nm and 340nm for each of the samples. After you have measured the absorbance of each sample, wipe off the surface where the sample is placed using Kimwipe and water and then 70% ethanol. Don't try to save the 1- ul of sample, this contaminates the rest. Save the remainder of the solutions as you will run gels with some of them.

I. Spectrophotometry: Nanodrop 1000, NanoFluor300

Step by Step: Collect a 10 or 20 ul Pipetteman and a box of the correct tips and a box of Kimwipes. Take along a 1.5 ml (Eppendorf) tube of water and one of 3X SSC, your reference buffer.

Turn on the instrument, bring up the software interface

Apply water to the reference spot

Apply buffer to the reading spot – record the value

Apply 1.5ul of the sample to the reading spot, record the value.

Recap and save the remaining sample, we will run gels with this material

Wipe off the sample spot with water and a Kimwipe, apply next sample.

Collect the data from each instrument and determine the concentration of each of the samples.

Note that when the solution is too concentrated the instrument may not give a reading – it may appear to be 'zero' instead of infinity.

Note that air bubbles can create problems – you should repeat each reading at least once, and if you get a very 'off' value, repeat the measurement yet again.

Determine the molar and mass concentrations of the DNA – you will need to know this for the gels.

Beers Law states that $A_\lambda = \varepsilon_\lambda cl$, you will need the extinction coefficient

The Nanodrop should give you the approximate mass (it converts for the path length, and you can provide the information that this is single-stranded DNA so that it picks an approximate of the extinction coefficient for single-stranded DNA that is 50 nt in length). Since you know the length, and

the nucleotide content is about equal for all 4 nucleotides you should be able to calculate the molar concentration as well.

What can you say about the precision of your dilutions?

What changes when there is a Cy3 attached to the oligonucleotide?

II. Electrophoresis

The next step is to run gels (agarose and acrylamide) to verify the length and concentration of the products, using electrophoretic separation.

Per lane, you should load about 1-15ng of DNA per band that you expect to see after staining, if you are using a fluorescent dye like ethidium bromide or SYBR Green. In polyacrylamide SYBR Green is much more efficient than ethidium bromide (which is quenched). You can load up to a 10ul volume in a well (you will need to add some loading dye to make the solution dense so it does not float away, we have a stock of 4X loading dye that includes glycerol and some tracking dyes).

A. Acrylamide Gels: a 50 nt oligo will be well resolved on a 20% native gel, although single and double-stranded DNA does not migrate in exactly the same way, so the double-stranded size standards will not correlate exactly in migration. You can heat-denature and snap-cool the size standards to make them single stranded if you want to have a more accurate assessment of size.
   a. Prepare the apparatus: take two glass plates, 3 spacers, one comb and 9 clips.
      i. Make a sandwich of the plates, with the spacers along 3 edges and the comb on top as the fourth edge. The spacers should butt up against each other as closely as possible. Clip the spacers into place (not the comb).
      ii. Pull up a small amount of melted 1% agarose in water ('Sealing agarose' – in the water bath), in a Pasteur pipette attached to a green pipette pump. Run a bead of this along the two edges and bottom of the sandwich (where the spacers are) to seal along the outside edges so the gel solution will not leak out.
   b. Prepare the gel solution. We have a 30% stock of acrylamide in water, and will run this in 1X TBE. Each gel sandwich requires about 8ml, so if you make 10ml you will have plenty. In a 15 ml Falcon tube add (with serological pipettes):
      i. 10ml (20%) = x ml (30%) so use 6.6 ml of the 30% acrylamide stock. [Pipette goes in biohazard bag]
      ii. 1 ml of 10X TBE stock
      iii. Add 2.4 ml of water, swirl to mix.
      iv. Using a 100ul micropipetter, add 6ul per ml of 10% Ammonium persulfate (APS) = 60ul [Tip goes in biohazard bag.]
      v. Using a 20ul micropipetter, add 0.5ul per ml of TEMED = 5ul [Tip goes in biohazard bag.]
      vi. Swirl quickly and thoroughly to mix.
      vii. Using a green pump and a Pasteur pipette, pull up some of the solution and pipette it inside the sandwich, down one side. Tilting slightly – this is to avoid air bubbles. Fill the sandwich to the top of the teeth in the comb, then

push down the comb slightly– you want as little contact with the air as possible, oxygen inhibits polymerization. [Pipette goes in biohazard bag. If you spill, wipe up with Kimwipes and dispose in BioHazard bag, along with your gloves.]

viii. You will be able to tell when polymerization has occurred by the difference in the diffraction of light around the wells of the comb.

c. A test for complete polymerization: pull some of the solution up into the Pasteur pipette, let it sit – when it is polymerized your gel should be as well.
d. Remove the comb (Wiggle gently to pull edges away from the polymer). Unseat the clips along the bottom and remove the bottom spacer.
e. Remove the clips along the sides and slide the gel into the gel box. Attach with two clips, one on each side. Seal into the top chamber using the 1% melted agarose again.
f. Add 1X TBE buffer (you will need ~600 ml per gel rig) to the top and bottom buffer reservoirs.
g. Load samples, (pre-measure – final volume should include using 1/4 volume of the 4X loading buffer- the wells will hold ~12 ul if well-formed). See sample prep in note n below for more details. Don't forget to reserve the end lanes for size standards, use about 10ng per band – generally this means that you load around 0.25ug total, depending on the ladder.
h. Attach the electrodes (red at the bottom) to the apparatus and power supply, set on constant voltage at 120V, electrophorese for 2 hours, or until the lower blue dye is at the bottom of the gel.
i. Turn off the power supply. Remove the electrodes from the apparatus. Pour the buffer down the sink. Remove the gel sandwich from the apparatus. Remove the side spacers. With the sandwich laid flat, use a spatula or razor blade to pry apart the glass plates, being careful not to tear the gel. Make a notch in the lower right-hand corner of the gel (retains orientation). Place in a small glass staining tray. Add 50 ml SYBR Green I stain (in 50 ml of water put 5ul of dye) for 5 minutes, covered, with gentle agitation.
j. Rinse in ddH2O for 15 seconds.
k. Place on transilluminator in Gel Doc.
l. Take picture (play around with the aperture, time, high versus low wavelength of UV and other parameters, until you get the contrast you want).
m. The Gel Doc has software that allows you to size the products. Find out the size of the reference bands by checking the product description online.Find out the mass per band as well. Enter the band sizes and masses in the software, then determine how many bands, of what size, and what amounts were present.

n. Sample prep: remove the amount of sample that will give 3-5ng of DNA in 8ul or less (you can dilute too concentrated samples, but cannot do anything with the over-dilute ones), add 1/4 volume of 4X loading dye, mix, pipette into the wells using a gel-loading tip. It can be hard to make sure the tip is between the plates, you may want to practice just loading 1X loading buffer until you get it right. It can be flushed out of the well, so you don't lose space by doing this. Mising the sample and loading buffer can be done in tubes but is usually easier on a piece of Parafilm, since it will bead up there. Generally I make whatever dilution I will need (using 1X TBE buffer), place that on the Parafilm, add the 1/4 volume of loading buffer,

pipette up and down a few times to mix them as I am picking the sample up with the micropipetter to put in the well.

B. Agarose gel electrophoresis is not as sensitive and cannot give the same precision about the length, but is much easier to set up and run. Try for 10-15 ng per band.

   a. Setting up, pouring and running a gel is kind of shown here, although a narrative would have been much more useful than the music. http://www.molecularstation.com/science-videos/video/18/agarose-gel-electrophoresis-method/

   b. The minigels we are using require 30-50 ml of solution. To make the gel, you will need to weigh out some agarose. For a 50-mer we will run a 2.5% agarose gel. Measure 1.25g for a 50 ml final volume. To make the solution 0.5X in TBE, you will need to dilute in some of the 10X buffer, in a 1:20 ratio, or 2.5ml per 50 ml total. 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 glove, glasses and to point the neck of the flask away from your face. If you hold the flask up to the light it should look perfectly clear. We are going to make double-layer gels, so two teams will work together.

      i. Take a 250 ml Erlenmeyer flask.
      ii. Wearing gloves, take out a weigh boat or paper and tare it.
      iii. Measure out 1.25 gm of agarose. If you weigh out too much do not return it to the reagent bottle, but put it in a 50 ml Falcon tube and label that. We can use it for sealing acrylamide gels, so it is not wasted.
      iv. Add 2.5 ml of 10X TBE buffer, using a 10 ml plastic pipette and the green pump.
      v. Add 47.5 ml of sterile water, slowly, swirling constantly. Let sit 1-2 min.
      vi. Swirl, then place in the microwave for 30 sec on maximum power.
      vii. Wearing a protective glove, swirl the solution, pointing the neck of the flask away from your face.
      viii. Repeat until the solution is clear – it will be boiling, but you don't want it to boil over.
      ix. Cover the neck of the flask and let the mixture cool at room temperature for 20 minutes or so, swirling occasionally – if you might take longer put it in the water bath so it will stay liquid while you do other things.
      x. Pour into the apparatus that has the ends sealed and a comb in place. Let stand about 30 minutes. If not running, wrap in plastic wrap or a Baggie, and store at 4C.

   c. The apparatus: We are pouring a horizontal, or flat-bed gel, so we are making a thin layer of agarose across the surface. We have to keep it from pouring out the ends of the mold. Some makers provide rubber gaskets, but tape actually works pretty well.

i. Wearing gloves, tear off a piece of tape long enough to more than reach across the ends of the mold. Attach so that the sides and bottom appear to be well sealed, repeat for the other end.
ii. You need a place to put the samples – this is made by creating wells in the gel material, with a device called a comb. Insert the each of the two combs toward the top end of each half of the mold.
iii. When the agarose is ready, pour it into the prepared mold – you need a thin continuous layer, but you don't want it to be very thick – it should not take all of the agarose you prepared.
iv. Let the agarose harden – this should take about 20 minutes, depending on how hot it was. You will be able to tell that it is ready when it becomes translucent rather than clear. Remove the come and the tape.
v. Place the mold in the electrophoresis chamber. Pour 0.5X TBE buffer into the chamber – you will probably need ~300 ml, so use the 1L graduated cylinder, add 50 ml of concentrated buffer, fill to the 1L line with buffer, cover with Parafilm, cover that with your hand (in a glove), and invert several times to mix.
vi. Now you are ready to load the gel.
vii. To each sample add 1/3 (of its volume) of 4X blue gel-loading dye (so final is 1X of the total), mix by pipetting up and down several times. You should load 15-50 ng per band, and generally wells hold 15-20 ul, but this depends on the comb used and thickness of the gel, so you can measure this before proceeding if you are not sure.
viii. Loading requires a steady hand- you need to layer the sample in the bottom of each well without stabbing the pipette tip through the agarose that forms the container nor by floating the sample over the top of the gel. Practice is the only answer!
ix. One lane on each side will be size standards that we will give you, note what they are since you will use these as calibration standards with the documentation software.
x. Put the lid on the apparatus, plug the electrodes into the power supply (red should be at the bottom, the direction the samples are heading towards), turn on constant voltage, set at 75V. You will run this until the lower of the blue dyes is about 75% of the way down each half of the gel. This will probably take a couple of hours, so one of you will have to come back to turn off the gel and stain it (5 minutes) and take a picture (5 minutes).
xi. When the dye front is where you want it, make a 1:10,000 dilution of SYBR Green I in water (make 200 ml of solution, so 20ul of SYBR Green into 200 ml of water will be fine). Mix, put into a staining tray with the gel (you may want to cut the gel in half). Cover with foil to protect the dye from light, rock gently for 5 minutes.

xii. Place the gel on the GelDoc transilluminator 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, email it to yourself and your partner so that you can print it out and put it in your notebook.

Materials needed
Agarose
10X TBE
30% acrylamide stock
Flasks
TEMED
10% APS
10mg/ml ethidium bromide
1% agarose in water, sealing agarose
Water
SYBR Green
Loading dye
Agarose: Gel casting trays, tape, combs, tanks
Acrylamide: plates, spacers, combs, clips, tanks
Questions to consider:

1. What type of resolution are you able to achieve on each type of gel (the size standards will be good for this)
2. What type of sensitivity can you achieve on each type of gel (using several in your titration series will help with this)
3. Are the unknowns a single product? Which gel gives you the most certainty in answering tyhis question?
4. Use the software on the GelDoc to set up a sizing curve and to estimate the quantity present./ How does this compare to your spec readings?