

DNA EXTRACTION AND QUANTIFICATION

Lab 1: DNA Extraction

Goal for this lab:

Extract DNA from various leafy vegetables.

Introduction

In this lab, we will extract DNA from common salad ingredients. The DNA we obtain today can be used for many different experiments, so this is an important activity!

In any DNA purification (or extraction) protocol, there are three important considerations:

1. We must break up the tissues and cells to release the DNA.
2. We must prevent the DNA from being degraded by cellular enzymes during the purification.
3. We must get rid of cellular components that are not desired—in this case, proteins, lipids, carbohydrates (cell walls), and RNA.

Break up tissues and cells.

We will cause tissue and cell disruption in three ways. The first is mechanical grinding. We will use a mortar and pestle to grind frozen leaf tissue into a fine powder. Freezing (in liquid nitrogen) makes the leaf tissue brittle and easy to grind. This breaks up the extracellular matrix in which the cells reside and allows better access of our chemicals to the cells themselves. The second method of disruption is chemical. The DNA extraction buffer that we use contains a high concentration of a detergent, sodium dodecyl sulfate (SDS). You will recall that detergents have both hydrophobic and hydrophilic moieties, and thus will bind both lipid and water. This detergent will solubilize the lipid bilayers in the cells, so that the plasma membrane and also the internal membranes are dissolved. In addition, we use the metal chelating compound ethylene diamine tetra-acetic acid (EDTA), which tightly binds calcium (Ca^{++}) and magnesium (Mg^{++}) effectively locking it away from cellular components. In the absence of Ca^{++} and Mg^{++} , proteins denature and membranes lose their integrity. The last disruption is high temperature. This encourages the detergent and EDTA to work efficiently and hastens cellular disintegration. This high temperature step is critical for efficient cellular disruption and release of DNA.

Prevent DNA degradation.

We prevent DNA degradation by inhibiting the enzymes (deoxyribonucleases, or 'DNases') that degrade DNA. Conveniently, DNases require Mg^{++} as a co-factor, so, by chelating the Mg^{++} with EDTA, we inhibit the activity of DNases. Prior to the addition of EDTA, we control DNase activity using low temperature (freezing in liquid nitrogen). If we were to break up the cells at room temperature, cellular DNases released during grinding would immediately start to degrade the DNA. Thus, it is imperative to get the EDTA containing DNA extraction buffer well mixed with the ground tissue before the temperature is raised.

Get rid of undesirable molecules.

We will use two methods to accomplish this. Both rely on differential solubility of specific macromolecules in particular solutions. Proteins and complex carbohydrates (such as those found in cell walls) are insoluble in solutions containing high concentrations of potassium

acetate (KOAc). By adding KOAc, we force the proteins and carbohydrates to precipitate (become solid and fall out of solution) while the nucleic acids will remain in solution. Nucleic acids are insoluble in solutions containing high levels of sodium plus the alcohols ethanol (EtOH) or isopropanol (ISOP), while lipids are quite soluble in these conditions. Proteins and carbohydrates are somewhat soluble in alcohol. By adding alcohols to a salty solution, we can force the nucleic acids to precipitate, while leaving the lipids, and the remaining carbohydrates and proteins, in solution. The main problem with alcohol precipitation of nucleic acids is that the sodium precipitates along with the nucleic acids. This is undesirable, and so we always rinse nucleic acid pellets with 70% EtOH (containing no salts) to remove the sodium from the pellets prior to dissolving the nucleic acids in the next solvent.

Materials:

Reagent	conc		pH	also known as
DEB	100 mM	NaCl	8.0	Sodium chloride
(DNA Extraction Buffer)	50 mM	Tris		trishydroxymethylaminomethane
	25 mM	EDTA		Ethylene diamine tetra-acetic acid
	1 %	SDS		Sodium dodecyl sulfate
	10 mM	BME		β -mercaptoethanol
T ₁₀ E ₅	10 mM	Tris	8.0	
	5 mM	EDTA		
T ₁₀ E ₁	10 mM	Tris	8.0	
	1 mM	EDTA		
EtOH	95 %		-	Ethanol
EtOH	70 %		-	Ethanol
ISOP	100 %		-	Isopropanol
KOAc	5 M			Potassium acetate (CH ₃ COOK)
NaOAc	3 M		5.2	Sodium acetate (CH ₃ COONa)
LN ₂				Liquid nitrogen

Mortar and pestle

Miracloth and a funnel

Paper and tape

A choice of edible leaves

Method:

Before you begin, read through the directions and get everything out that you will need so you don't have to fumble for it in the middle of some critical step in the extraction procedure.

SAFETY: LN₂ is very very cold, -320° F (-196° C), and will severely damage tissue that it contacts! Use extreme caution while handling to avoid freezing injuries.

1. Pre-cool your mortar and pestle by pouring some liquid nitrogen (LN₂) into the mortar and letting the pestle sit in it. Allow the LN₂ to evaporate.

2. Now, add some more LN₂, and ~0.25-0.5 g of leaves (about 1/3 spinach leaf for example). **Do not let the tissue thaw** once it is frozen. Grind slowly at first, so you won't slop the LN₂ and leaves everywhere. Then, when the LN₂ has mostly evaporated (but while the tissue is still frozen) grind hard to get the tissue into a fine powder. The more you grind, the more you'll get. You can carefully add additional LN₂ to keep your tissue frozen if necessary.
3. Transfer the powder into a 15 mL ROUND BOTTOMED plastic tube which contains 6 mL of DNA extraction buffer (DEB). (A paper cone made from a sheet of printer paper will make this much easier.) **The amount of tissue used should fit easily on top of the extraction buffer in your tube. Do not overload the tubes!** Overloading with too much tissue will result in poor quality DNA.
4. **Use a spatula to shove the powder down into the tube and mix well**, then snap the cover down to the second stop and **shake vigorously** to mix. Wait (mixing occasionally) until the mixture has completely thawed before going on to the next step.
5. When your sample is ready, transfer the tube to a 65°C water bath for 10 minutes.
6. Place the tube on ICE and add 2.5 mL 5M KOAc. Mix well by inverting the tube repeatedly. Incubate ON ICE for 20 minutes.
7. Centrifuge 5000 rpm 10 minutes at 4°C.
8. Remove your tube from the centrifuge and notice the very large pellet consisting of tissue debris and precipitated protein. REMEMBER, you are trying to GET RID of the pellet in this step!
9. Transfer the supernatant (the liquid on top of the pellet) through Miracloth filter into a fresh round-bottomed 15 mL tube containing 6 mL of 100% isopropanol. Mix well by inverting the tube gently but repeatedly. **Precipitating nucleic acids may be clearly visible at this point.**
10. Spin in the centrifuge at 5000 rpm for 10 minutes at 4°C.
11. Remove your tube from the centrifuge. REMEMBER, this time, YOU WANT YOUR PELLETT—the pellet is the nucleic acids—including your DNA. Pour off the supernate.
12. Rinse your pellet carefully with 2-3 mL 70% EtOH. Aspirate as much liquid as humanly possible, then let the pellet air dry for a few minutes. Getting rid of the EtOH is essential before proceeding to the next step.
13. Add 450 µL T₁₀E₅ to your pellet. It is important to get your DNA pellets to dissolve completely into the T₁₀E₅. We still use a high concentration of EDTA at this step, because not all the proteins are gone yet, so nucleases could still attack our DNA. Transfer the liquid into a labeled 1.5 mL microtube.
14. Add 50 µL 3M NaOAc pH = 5.2. Mix. Add 1 mL 95% EtOH. Mix gently by inversion until phases are completely mixed. Fluffy whitish clouds of DNA should almost fill the tube. If you do not see DNA (or can't lift it out of the tube in the next step), consult an instructor. You may have to spin it down and rinse it with ethanol, and let it air dry before going on.
15. Use a 1 mL pipette tip like a spatula to lift out the nucleic acid fluff and transfer it into a clean, dry, labeled 1.5 mL microtube. Add 1 mL of 70% EtOH to rinse. If it cannot be scooped, spin it down, rinse with ethanol and air dry before going on.

16. Use a pipette tip to transfer the nucleic acid fluff into a clean dry labeled 1.5 mL microtube.
17. Spin briefly in the microfuge—15 seconds is plenty of time. Remove as much EtOH as humanly possible. If necessary, you may try drying the sides of the tube with a sterile swab.
18. Gently dissolve* the pellets in 100 μ L T₁₀E₁. We use a low concentration of EDTA now, because our nucleic acid preparation is fairly pure—protein contamination is minimal now. However, we keep some EDTA around ‘just in case’. If we had too much EDTA in our final solution, we could never do subsequent enzymatic manipulation of our DNA. Let the pellets dissolve COMPLETELY-- leaving LABELED tubes **in the refrigerator overnight if necessary**. Store at -20°C once it is **completely** dissolved.
19. If time permits, measure the concentration of your DNA by the Nanodrop, as explained beginning on page 59.
20. Label your tube carefully with your group initials, the date, and gDNA (genomic DNA). Put it in a rack in a freezer box with your group name on it in label tape.
21. Write in your lab notebook what you did, either “exactly as in manual”, or “as in manual, except that ...” Give the date, and what label you put on the tube. Later on in the semester, when you will need this DNA again, all this will help you remember (or figure out) what’s in the tube you just put in the freezer.

Clean-up:

Alcohols are safe to go down the drain *only when they are less than 10%*. *Run plenty of water while discarding alcohols so that this dilution will be achieved.*

Save your reagents. If you have to make DNA again later, you’ll be glad to have them. You will use your T₁₀E₁ again.

Put the tubes that contained any β -mercaptoethanol in the marked bucket in the hood.

Put plant debris and used potting soil in the bucket labeled “Compost”.

Dispose of glass pipettes in the glass trash box.

Set your mortar and pestle to soak as directed by your instructor.

* If it doesn’t go into solution, add more T₁₀E₁ and heat to 65°C for a few minutes. Do not “rack” more than once.

Lab 2: DNA Quantification

Goal for this lab:

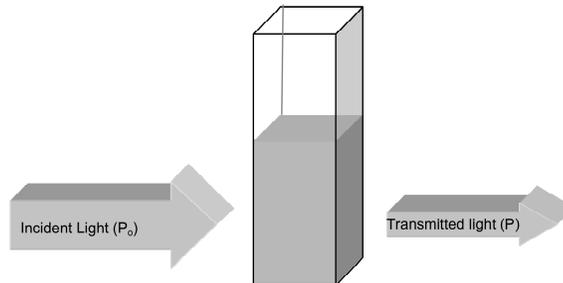
Quantify plant DNA by two methods.

Introduction:

Reliable measurement of DNA concentration is important for many applications in molecular biology. DNA quantification is generally performed by spectrophotometric measurement of the absorption at 260 nm, or by agarose gel analysis. In this lab, you will compare the two methods for quantifying the genomic DNA that you prepared during our last meeting.

DNA quantification by molecular absorbance spectroscopy.

DNA concentration can be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer using a UV-transparent cuvette (usually quartz). In molecular absorbance spectroscopy, a beam of ultraviolet or visible light (P_o) is directed through a sample. Some of the light may be transmitted through the sample (P). Light that was not transmitted through the sample was absorbed. Transmittance (T) is defined as the ratio of P/P_o . Absorbance (A) is defined as $-\log(T)$. The light that was absorbed is what we care about: the absorbance at 260 nm, or A_{260} .



Unfortunately, spectrophotometric measurement does not differentiate between the two nucleic acids commonly found in a cell (DNA and RNA). The OD₂₆₀ measurement thus tells you something about the total amount of nucleic acid in a preparation, but can't let you measure the concentration of DNA in a preparation that also contains RNA. RNA contamination of a DNA preparation can lead to overestimation of DNA concentration, if spectroscopy is the only method used to determine concentration.

DNA quantification by agarose gel electrophoresis

Quantification of DNA can also be done using agarose gel analysis. This method is only effective with small amounts of DNA, since high concentrations of DNA cannot be accurately measured this way. As little as 5 ng DNA can be detected by agarose gel electrophoresis with SYBR-Safe staining. In this method, the DNA sample is run on an agarose gel alongside known amounts of DNA standards. The amount of sample DNA loaded can be estimated by comparison of the band intensity with the standards using a scanner or imaging system.

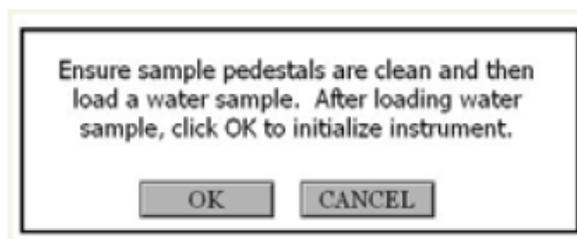
Quantification using agarose gel electrophoresis is also extremely useful when DNA samples are contaminated with RNA, since RNA is separated from DNA in agarose gels by virtue of the difference in length (size) of an average genomic DNA fragment versus the average size of a mRNA, tRNA or rRNA molecule. RNA is small: tRNA is only ~100 nucleotides (nt) long, and mRNA averages ~1.5 kilobases (kb), depending on the size of the encoded protein.

Genomic DNA is theoretically the size of a whole chromosome (1000s of kb), although breakage during handling causes the size of an extracted DNA sample to be smaller (~34-50 kb). By analyzing DNA that has been *physically* separated from RNA on agarose gels, the concentration of DNA can be measured in the absence of contaminating RNA.

Method 1: Spectrophotometric quantitation via Nanodrop^{*}

Module Startup

When the software starts, you should see this message:



For best results, ensure measurement pedestal surfaces are clean, load a water sample onto the lower measurement pedestal and then click 'OK'. The message "Initializing Spectrometer- please wait" will appear. When this message disappears, the instrument will be ready for use. All data taken will automatically be logged in the appropriate archive file.

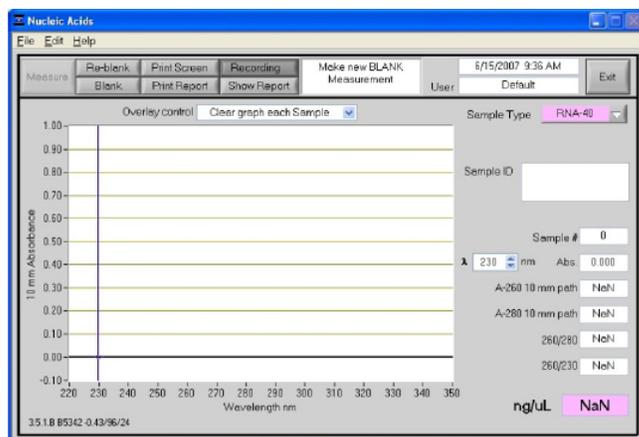
Measure (F1)

Each time a software module is opened (initiated), the Measure button is inactive as noted by its "grayed-out" appearance. A blank must first be measured before the Measure button will become active.

The Measure button is used to initiate the measurement sequence for all samples (non-blanks). It is actuated by depressing the F1 key or clicking the Measure button. The entire measurement cycle takes approximately 10 seconds.

Blank (F3)

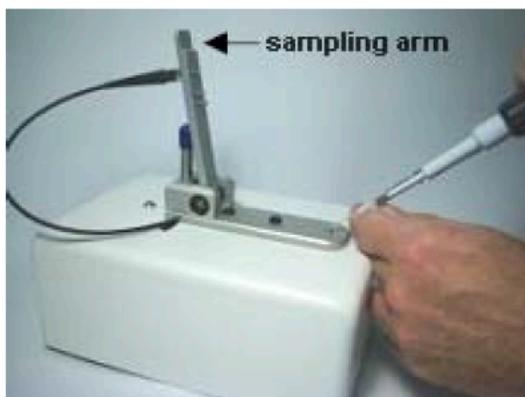
Before making a sample measurement, a blank must be measured and stored (see "Blanking and Absorbance Calculations" on page 62 for more details on absorbance calculations). After making an initial blank measurement, a straight line will appear on the screen; subsequent blanks will clear any sample spectrum and display a straight line, as shown in the following image:



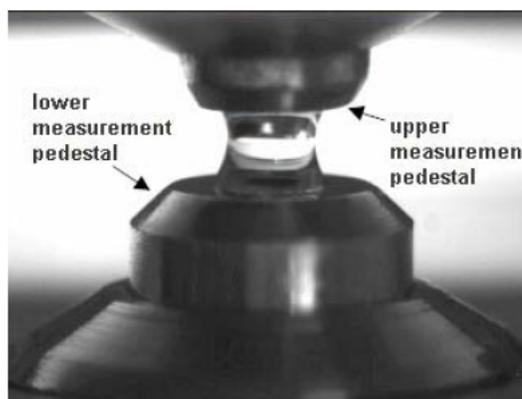
^{*} Information and instructions taken from Thermo Scientific's NanoDrop 1000 Spectrophotometer V3.8 User's Manual, <http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf>, accessed 1/14/13.

For the most consistent results, it is best to begin any measurement session with a blanking cycle. This will assure the user that the instrument is working properly and that the pedestal is clean. Follow the steps below to perform a blanking cycle:

1. With the sampling arm open, load a 1 μL blank sample (the buffer, solvent, or carrier liquid used with your samples) onto the lower measurement pedestal. Make sure there are no bubbles!



2. Close the sampling arm. The sample column is automatically drawn between the upper and lower measurement pedestals. Click the “Blank” button (F3)



3. When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000-fold in concentration. See the Nanodrop website (www.nanodrop.com) for performance data on sample carryover.

Wiping the sample from both the upper and lower pedestals (as shown to the right) upon completion of each sample measurement is usually sufficient to prevent sample carryover and avoid residue buildup. Although generally not necessary, 2 μL water aliquots can be used to clean the measurement surfaces after particularly high concentration samples to ensure no residual sample is retained on either pedestal.



4. Analyze an aliquot of the blanking solution as though it were a sample. This is done using the “Measure” button (F1). The result should be a spectrum with a relatively flat baseline. Wipe the blank from both measurement and pedestal surfaces and repeat the process until the spectrum is flat.
5. Analyze 1 μL of your sample with the “Measure” button. Do a second measurement to be sure, following the steps above.

Blanking and Absorbance Calculations

When the NanoDrop 1000 Spectrophotometer is “blanked”, a spectrum is taken of a reference material (blank) and stored in memory as an array of light intensities by wavelength. When a measurement of a sample is taken, the intensity of light that has transmitted through the sample is recorded. The sample intensities along with the blank.

intensities are used to calculate the sample absorbance according to the following equation:

$$\text{Absorbance} = -\log \frac{\text{Intensity sample}}{\text{Intensity blank}}$$

Thus, the measured light intensity of both the sample and of the blank are required to calculate the absorbance at a given wavelength.

Concentration Calculation (Beer’s Law)

General

The Beer-Lambert equation is used to correlate the calculated absorbance with concentration:

$$A = E \times b \times c$$

Where

A is the absorbance represented in absorbance units (A),

E is the wavelength-dependent molar absorptivity coefficient (or extinction coefficient) with units of liter/mol-cm,

b is the path length in cm, and

c is the analyte concentration in moles/liter or molarity (M).

Nucleic Acids

For nucleic acid quantification, the Beer-Lambert equation is modified to use an extinction coefficient with units of ngcm/mL . Using this extinction coefficient gives a manipulated equation:

$$c = A \times e \times b$$

Where

c is the nucleic acid concentration in $\text{ng}\mu\text{L}$

A is the absorbance in AU

e is the wavelength-dependent extinction coefficient in $\text{ng}\times\text{cm}\mu\text{L}$

b is the path length in cm

The generally accepted extinction coefficients for nucleic acids are:

Double-stranded DNA: 50 ng×cm μ L

Single-stranded DNA: 33 ng×cm μ L

RNA: 40 ng×cm μ L

For the NanoDrop 1000 Spectrophotometer, path lengths of 1.0 mm and 0.2 mm are used compared to a standard spectrophotometer using a 10.0 mm path. Thus, the NanoDrop 1000 Spectrophotometer is capable of measuring samples that are 50 times more concentrated than can be measured in a standard spectrophotometer.

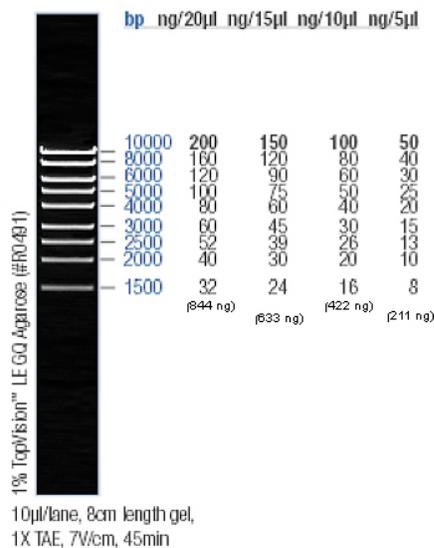
Note: Absorbance data shown in archive files are represented as displayed on the software screen. For Nucleic Acid, Protein A280 and Proteins and Labels modules, data are normalized to a 1.0 cm (10.0 mm) path. For MicroArray, UVVis, Protein BCA, Protein Bradford, Protein Lowry and Cell Culture modules the data are normalized to a 0.1 cm (1.0 mm) path. For high absorbance UV-Vis samples, data are normalized to a 0.1 mm path.

Method 2: Quantification using agarose gels.

Materials:

Reagent	conc		pH	also known as
DB	10	mM	Tris	7.5 Dilution Buffer
1× TAE	40	mM	Tris	8.0 Running Buffer
	20	mM	Acetic acid	(dispense it yourself from the carboy at the back of the room)
	1	mM	EDTA	
Agarose gel	0.9	%	Agarose	
			SYBR-safe	DNA binding dye
RNase	0.9	%	Agarose	
Agarose gel			SYBR-Safe	
	1.0	μ g/mL	RNase	
Loading dye	30	%	glycerol	
	0.3	%	Bromphenol blue	
	0.3	%	Xylene cyanol	

Fermentas #SM0393 MassRuler High Range DNA Ladder (See below.)



SAFETY: *SYBR safe is a weak mutagen and a possible carcinogen, so must be handled carefully. WEAR GLOVES when handling gels and electrophoresis buffers to protect your skin. Discard your gloves when you've stopped handling your gel. AVOID DRIPS so that other people don't accidentally come into contact with SYBR-safe. The best way to avoid drips is to use a Kimwipe to wipe off your gel tray before moving around with it.*

Using agarose gel electrophoresis, you can simultaneously assess both the **yield** and **quality** of your DNA isolations by visualizing the DNA on 0.9% agarose gels containing SYBR-Safe. SYBR-safe is a dye that can intercalate between the bases of DNA in a manner that is essentially proportional to the amount of nucleic acid present. Because there is some double strandedness in most RNA preparations, SYBR-safe will also stain RNA. When exposed to ultraviolet light (250-350 nm), SYBR-safe fluoresces yellow, which is then visualized by photography. The DNA concentration from your samples can be estimated by comparing the SYBR-safe fluorescence intensity to the intensities observed for standards of known DNA concentration run on the same gel.

Running a gel

(Your instructor will demonstrate setting up the gel apparatus.) Prepare two 0.9% agarose gels. *The gel contains SYBR-Safe, so wear gloves.* Get two 50-mL tubes of 0.9% agarose from the 65°C water bath. Make sure that one tube contains RNase, and the other does not. Set up the gel molds with a comb in place to form the wells. Use label tape to mark which one contains RNase.

Pour your RNase-free gel first, to prevent contaminating your gloves, etc. with RNase and spreading it to other things in your workspace.

Get approximately 250 mL of running buffer (1× TAE) from the carboy for each gel.

When your gels have set, transfer the gel *in its little plastic tray* to the electrophoresis apparatus, and add running buffer. Avoid cross-contaminating RNase from one gel apparatus to the other.

DNA is negatively charged, and so will migrate toward the positive electrode. Make sure you have the wells at the correct (NEGATIVE) end. (An easy way to remember is that gels run in alphabetical order: from black to red.) Remove the comb very gently and carefully, pulling straight up.

While your gel is setting up, you can prepare your samples.

Dilute your DNA

Make four dilutions of the DNA sample you prepared at our last meeting^{*}: 1/20, 1/50, and 1/100. Make the dilutions as follows: Place the requisite volume of dilution buffer into a labeled 1.5 mL microtube, add the appropriate volume of DNA stock solution, mix, then spin the liquid to the bottom of the tube.

^{*} If you had to dilute your sample to get the DNA into solution, and your volume is >500 µL, you may need much less dilution: 1/2, 1/5, 1/10. Check with your instructor.

Dilution	DNA stock solution	Dilution buffer
1/5	40 μL	160 μL
1/20	10 μL	190 μL
1/50	4 μL	196 μL
1/100	2 μL	198 μL

Save these samples on ice. Do not discard. You will use them again later today.

Remember, you are running two gels, so prepare two of each of your samples. Into labeled 1.5 mL microtubes, place 5 μL of your diluted sample (1/5, 1/20, 1/50 or 1/100). To each, add 1 μL loading dye. This dye serves two purposes: it contains glycerol, which is heavy, and will make your solution sink under the electrophoresis buffer in the gel wells. It also contains two dyes (xylene cyanol (XC) and bromophenol blue (BPB)), which migrate at different speeds in the gel, and allow you to track your gel's progress visually while it runs.

Load your gels. You will include two lanes of 'standards': one lane with 2.5 μL (105.5 ng) of MassRuler—the DNA standard of known molecular weights and masses—and a second lane with 5 μL (211 ng) of the same standard. (See previous page.) Then load each of your diluted samples into one of the four adjacent lanes. Record the order in which the samples were loaded in your notebook.

Run the gel at 100 volts for 45 minutes.

Photographing your gel

Take the tray with your gel out of the electrophoresis apparatus and place the tray on a paper towel to catch excess buffer.

Take your gel (on its tray) over to the gel doc system.

Place the gel (without the tray) on the UV box, and close the door.

TAKE OFF YOUR GLOVES—NO GLOVES ON COMPUTERS, EVER.

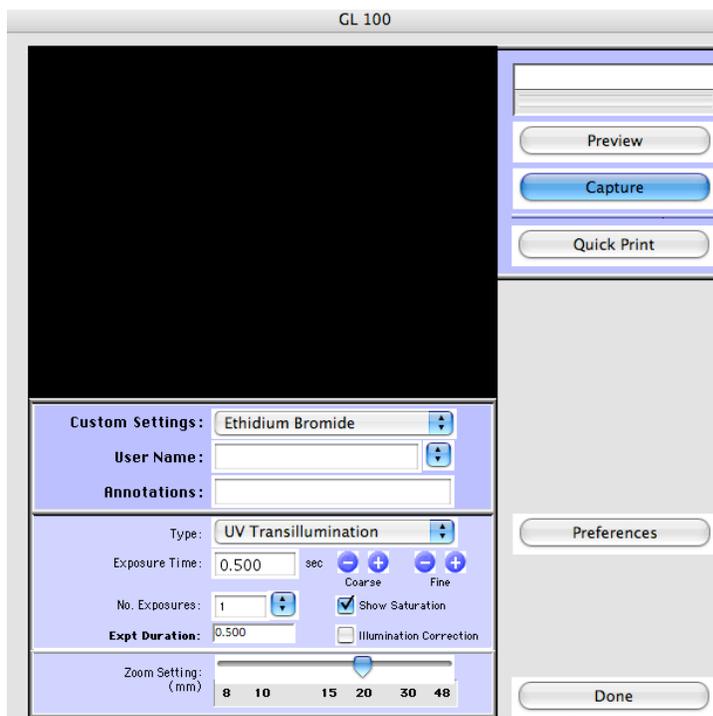
Open the Kodak software (the big red K on a yellow background in the dock at the bottom of the screen).

Back in the Kodak software, go to the "File" menu, and "Select Digital Camera". In the dialog box that follows, select the GL100/GL200 camera, and click OK.

Look in the extreme upper right corner of the screen. You should see a button saying 'Capture GL 100'. HOWEVER, more likely, you will see a button saying, 'Capture GL2200'. If you do, go back to the file menu, select digital camera again, and select GL100/GL200 camera, and click OK. This time, you will be asked whether you want a GL100 (you do) or a GL200. Choose the GL100.

Look in the extreme upper right corner again. Now it should correctly say, 'Capture GL100.' Click the button.

Now you see a dialog box for the camera (See below).



Choose 'Preview' to view your gel.

If the gel is not visible, make sure that the UV light is on. If the gel is still not visible, use the 'IOXperts Camera Control' program (in the dock, to the right of the Kodak icon—see below) to adjust the gain on the camera.



When your image is to your liking, click on the 'Capture' button (in the Kodak MI software). Your image will open.

Save your image to the desktop. Remember to save the image you take to your USB drive and/or to your UDrive space. Do note that files saved from the Kodak software will be '.bip' files, which can be read ONLY by the Kodak software. This is enough for right now, but later you want to be sure to save the image in other formats for the purposes of presentation in lab reports and wiki pages.