GOAL
The goal of this lab is to give experience in one technique for extracting chromosomal DNA from plant tissue and in determination of chromosomal DNA quality and quantity.

OBJECTIVES
After completion, the student should be able to:
1. List and explain the steps of chromosomal DNA isolation.
2. Determine the quality and purity of chromosomal DNA using gel electrophoresis and UV spectrophotometry.
3. Calculate DNA concentration and from agarose gel electrophoresis results
4. Calculate DNA concentration from UV absorbance results.
5. Use laboratory protocol reference books and the Internet to locate information for use in the laboratory.

TIMELINE
This lab will take 1 laboratory period to:
1. Determine DNA quality, concentration and purity using agarose gel electrophoresis
2. Determine DNA concentration and purity using UV absorbance

BACKGROUND
Nucleotide Structure
The building block or monomer of nucleic acid is the nucleotide. Nucleotides consist of three parts: (1) a five-carbon sugar, (2) a nitrogenous base, and (3) a phosphate group. There are two kinds of nucleic acids: DNA and RNA. The monomers of DNA and RNA vary slightly. RNA contains the five-carbon sugar ribose; DNA contains the five carbon sugar deoxyribose, which has one less oxygen. See Figure 1, below and nucleotide models in the lab. Note that the carbons in the sugar molecules are designated with numbers 1’-5’ (pronounced 1 prime to 5 prime) to distinguish them from the carbons in the nitrogenous bases. Also, the 5’ carbon in the sugar is not part of the ring structure, but sticks out above it.

![Figure 1. A. DNA Nucleotide; B. RNA Nucleotide](image-url)
The nitrogenous bases of DNA include adenine (A), thymine (T), guanine (G), and cytosine (C). Cytosine and thymine are single-ring structures called pyrimidines; adenine and guanine are double-ring structures called purines (see Figure 2). RNA contains the same nitrogenous bases as DNA except for thymine. Uracil replaces thymine in RNA. Uracil, like thymine, is a single-ring molecule. These nitrogenous bases are able to hydrogen bond with their complement, another base that forms the same number of hydrogen bonds. A purine will always hydrogen bond with a pyrimidine. Cytosine and Guanine are complementary, forming three hydrogen bonds between them. Adenine and thymine (uracil in RNA) are complementary, forming two hydrogen bonds between them. This hydrogen bonding between complementary bases is referred to as base pairing. One set of nucleotides hydrogen bonded together at their complementary bases is referred to as a base pair. See Figure 2 and models in the lab.

DNA Structure and Function
DNA is a double stranded molecule made up of two polymers of nucleotides. The nucleotides of each polymer are bonded together at the sugar of one nucleotide and the phosphate of the next. The two polymers are joined in the center by hydrogen bonds between the nitrogenous bases. DNA has the shape of a ladder, with the sides of the ladder composed of alternating phosphates and sugars while the rungs of the ladder are the hydrogen-bonded bases. The ladder is twisted so that it resembles a spiral staircase and therefore, is called a double helix.

DNA carries the master blueprint for heredity in the form of a code. Within a gene that codes for a protein, sets of three nucleotides code for a particular amino acid. The 64 different three-nucleotide combinations of the four nucleotides of DNA (A,T,G,C) are called the genetic code. The sequence of 3-nucleotide codons in the DNA serves as the blueprint for the primary structure (the amino acid sequence) of a protein.
Chromosomal DNA Extraction
The technique for chromosomal DNA extraction depends on the type of cell or tissue being used. Since plant cells have a fibrous cell wall consisting of cellulose and pectin, plant tissue must be ground, macerated and sometimes heated to 65°C to rupture the cells releasing the DNA. With animal and gram positive bacterial cells, enzymes such as Protease K, lysozyme or papain (a protein-cleaving enzyme derived from papaya and certain other plants) are used. More extreme measures must be taken to remove the thick peptidoglycan layer from gram positive bacteria. One technique is to vortex the cells with glass beads and a detergent such as sodium dodecyl sulfate (SDS). Gram negative cells, such as E. coli, can be lysed using SDS without vortex mixing. The SDS dissolves the phospholipid bilayer of cell membranes and also the nuclear membrane in eukaryotic cells. To precipitate the DNA, a salt such as ammonium, sodium or potassium acetate is added and then alcohol is added (ethanol or isopropanol, depending upon the protocol). The salt must be added first because DNA will not precipitate in alcohol otherwise. Since cold enhances the precipitation of DNA, -20°C alcohol can be used and the DNA/alcohol mixture can be incubated for a short time (5-20 minutes) at -20°C. Pure DNA is clear and nearly transparent, but in many extractions, it appears as a cloudy mucous-like mass of strands floating in the alcohol. Protein gives DNA this milky, opaque appearance. With plant DNA extraction (especially from fruit), there can also be polysaccharide contamination, visible as a clear jelly-like mass. Both protein and polysaccharide contamination must be removed because each inhibits the action of enzymes, the molecular tools of biotechnology. If floating white flakes appear in the alcohol, the DNA was sheered into small pieces and is not usable for further genetic manipulation. The DNA precipitate is then centrifuged to separate it from the alcohol. The DNA pellet is resuspended in Tris buffer containing Ethylenediamine tetraacetic acid (EDTA). The EDTA chelates (binds) divalent cations that are cofactors necessary for Dnases, the enzymes that degrade DNA. DNA resuspended in Tris-EDTA should be stored short term at -20°C or long term at -80°C.

Chromosomal DNA Cleanup
Good quality DNA is not only intact but is also clean – free of salt, proteins and polysaccharides that inhibit DNA-modifying enzymes. To remove proteins, an affinity gel can be employed to either bind the DNA while the protein is washed away or bind the protein while the DNA is washed off and collected. Equilibrated phenol (saturated with 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA to increase the phenol’s pH to 7.8 ± 0.2, which is required for DNA extractions), chloroform or a combination of these two organic solvents, are used to wash the DNA. The non-polar portions of the proteins are soluble in organic solvents so they are dragged down and out of the aqueous DNA solution as the dense solvents sink to the bottom of the tube. The hydrophilic parts of the proteins get dragged down and out of the aqueous solution and end up in a whitish layer, or interface, floating on top of the organic phase at the bottom. A small amount of isoamyl alcohol can be used to separate the two phases more efficiently, making it easier to draw off the aqueous DNA layer at the top. Phase Lock Gel™ can also be used to separate the aqueous phase for easy removal. This is a proprietary product that eliminates protein contamination by migrating during centrifugation to form a tight seal trapping the interface material and organic phase below the aqueous phase. The aqueous phase is then simply decanted or pipetted off the top and transferred to another tube (Murphy and Hellwig).
Polysaccharides are precipitated with ethanol at a concentration lower (0.35 volumes) than that used to precipitate DNA (2 volumes). The salt concentration of the aqueous phase is critical here – no more than 0.25 M or the excess salt will cause the polysaccharides to remain in solution (Michaels, John, Amasino). Any remaining salt is removed by washing the pellet of DNA with isopropanol or ethanol. RNA will also be present, but does not interfere with the action of DNA-modifying enzymes used in genetic engineering. RNA can give an inflated DNA concentration, so it is often removed by incubation with RNase, an enzyme that degrades RNA, prior to taking UV absorbance readings. There are commercial DNA clean-up kits available (Qiagen, Zymo Research, Sigma-Aldrich) that work well to remove contamination.

**Determination of DNA Quality and Quantity**

After the extraction procedure, the DNA is checked to verify that it is intact and clean of cellular contaminants. Determination of the quality and concentration can be accomplished in two ways: gel electrophoresis and UV spectrophotometry.

**Gel Electrophoresis**

DNA can be diluted and run on an agarose gel. By running a molecular weight marker of known concentration, the extracted DNA concentration can be determined. The appearance of the DNA on the gel can also reveal if it is clean and intact. Agarose is a derivative of agar, a polysaccharide derived from algae. DNA fragments, including molecular weight markers, are often heated to 65°C prior to electrophoresis to straighten any loops formed along the length of the molecules so that migration through the gel is uniform. In solution, ionization of the phosphate groups along the backbone of the DNA results in many negative charges on the molecule. Once loaded into the gel, an electric current is applied and the negatively charged molecules of DNA move through the gel toward the positive electrode. The gel is exposed to ethidium bromide, a flat molecule that intercalates, or slides between, the stacked base pairs of the DNA. Ethidium bromide fluoresces orange in UV light, making it possible to visualize the DNA (Figure 3).

![Figure 3. Bands of chromosomal DNA previously incubated with ethidium bromide fluoresce in UV light at 302 nm. RNA can be seen at the bottom of some lanes (arrows). Lane 8 = Molecular weight marker DNA](image)

The concentration of DNA can be estimated by running it on an agarose gel. It is best to dilute the DNA 1/10 and 1/100 and run both dilutions on the gel with a molecular weight marker (MWM) of known concentration. The DNA concentration is determined by comparing it to one band in the MWM that most resembles it in brightness. See Figure 4 for a sample calculation.
The appearance of the DNA on the gel can also reveal if it is clean and intact. If the DNA is intact, it will appear as a distinct band on the gel. If it is degraded, it will appear as a smear of thousands of small fragments. If the DNA is contaminated with protein, there will be a bright band of DNA at the bottom of the well and along the migration path from the wells where the slower moving protein trapped DNA. If the well is over loaded with DNA that is too concentrated, the band will have a jagged smear above it. See Figure 5.

**Figure 5.** Lane 1, molecular weight marker DNA; Lane 2, degraded DNA; Lane 3, intact chromosomal DNA; Lane 4, overloaded well. Below, Lanes 7-10, DNA is contaminated with protein, some DNA is degraded, and overloading is evident in lanes 7-9. RNA is seen at arrow.

**UV Spectrophotometry**

The second method of determining the quality and concentration of DNA is by UV spectrophotometry. A spectrophotometer is a laboratory instrument that measures the intensity of light passing through a solution and compares this to the amount of light entering the solution. The major parts of a spectrophotometer are given in Figure 6.

**SPECTROMETER** – emits light of specific wavelengths on a sample tube

**PHOTOMETER** – measures the light transmitted through the tube. It includes a photoelectric cell that is sensitive to the emitted wavelengths of light and a galvanometer that records the electrical potentials from the photoelectric cell.
Light is made up of radiant energy waves and is part of the electromagnetic spectrum. The wave size (distance between the peaks) can be measured in nanometers ($10^{-9}$ meters) or in Angstroms ($10^{-10}$ meter). Shorter wavelengths have greater energy and longer wavelengths have less energy. Visible light is between 400 and 800 nanometers and ultraviolet is between 180 and 400 nm (Figure 7). Chemicals absorb different wavelengths of light due to their chemical structure.

**Figure 6. Basic Components of a Spectrophotometer**

**Figure 7. The Electromagnetic Spectrum**
Preparing Samples for UV Absorbency Readings

When determining DNA concentration by UV spectrophotometry, it is important to follow standard guidelines given in *Current Protocols in Molecular Biology* by Ausubel et. al. or *Molecular Cloning: A Laboratory Manual* by Sambrook, et al. Water should not be used to dilute the DNA because the pH must be precisely 7.4, and the pH of water can vary. Therefore, TNE buffer is used when results must be extremely accurate. The temperature of the samples is also a variable that must be controlled because as temperature increases, the absorbance reading increases. Therefore, DNA samples and 1x TNE should be at room temperature. Only matching quartz cuvettes or cuvettes designed exclusively for UV spectrophotometry should be used as other types of cuvettes do not allow the UV wavelengths to pass through the sample correctly, giving an inaccurate reading. In addition, the cuvettes and solutions must be clean and free of particulates that can cause light scattering and give an inaccurate reading. **A reading of 1x TNE buffer only at 325 nm should give an absorbance of \( \leq 0.01 \). If it is higher, the cuvette may be dirty or the TNE buffer may have particulates, requiring it to be made fresh and filtered.**

Notice if the protocol is asking for an absorbance or transmittance reading and set the spectrophotometer accordingly. In this lab, we are using absorbance of UV light at a wavelength of 260 nm to determine double-stranded DNA concentration. Fifty micrograms of double stranded DNA in one milliliter of buffer has an absorbance of 1.0 at this wavelength, but if there are other nucleic acids in the sample, the reading will be incorrect. This is because both single stranded DNA and RNA absorb UV light at this wavelength.

Meticulous technique in measuring the DNA is extremely important for accurate results. Use a small bore tip (the smallest tip available in the lab) and be sure to pre-rinse the tip with the DNA sample by pipetting it up and down a few times first. There should be no liquid on the outside of the tip, so wipe it with a Kim wipe before adding the DNA to the in the cuvette. The amount of buffer is determined by the design of the cuvette. With most quartz cuvettes, 2 ml of buffer must be added or the light path will be above, rather than through, the buffer. Add 1-2 μl of the DNA sample per ml of 1x TNE buffer and mix before inserting the clean cuvette into the spectrophotometer. Take several readings of each sample and average to calculate the concentration and be sure to use the dilution factor. For example, if the samples are diluted 1 μL into 1 mL three times and the absorbance readings were 0.0158, 0.0161, and 0.0180 the average is 0.0166 and the concentration of the DNA is 830 μg/ml \((0.0166 \times 50 \text{ ug/ml} \times 1000 \text{ d.f.})\). If the spectrophotometer has a program that does the calculation for you, take 3 readings and average the three calculated DNA concentrations. If readings fluctuate greatly from one reading to the next, the DNA concentration is probably too low to give accurate readings with the spectrophotometer you are using. Try adding more of the DNA sample to the cuvette (10 – 20 μL instead of 1 – 2 μL per mL buffer) and take the readings again.

**Equation for calculation of DNA concentration:** \( A_{260} \times 50 \mu g/ml \times \text{dilution factor} \)
Protein in a sample can be detected at $A_{280}$. The relative amount of protein contamination for a DNA sample can be determined by calculating the $A_{260}/A_{280}$ ratio. Ratios of 1.8 to 1.9 indicate that the DNA is highly purified. If the ratio is even slightly lower than 1.8, there is substantial protein contamination in the sample. Since both DNA and RNA absorb at $A_{260}$, if there is RNA in the sample, the calculated concentration of double stranded DNA will be inaccurately high. Running a sample on an agarose gel separates DNA from RNA ([Figures 3 & 5]) and can be used to determine if RNA is present. Contaminates such as EDTA ($\geq 10$ mM) and ethidium bromide can interfere with absorbance readings. Consult *Current Protocols in Molecular Biology* by Ausubel, et. al. for directions to correct for these contaminants, if they are suspected.

**A Reminder about High-speed Centrifugation**

When loading a centrifuge, the placement of samples must be balanced according to position (both within a swinging bucket and across the rotor) and the weight and density must be the same in the samples and the blanks. Refer to the centrifuge manufacturer’s guide for balance placement and for the maximum amount of variation in weight and density permitted. You may also need to make a conversion in speed, since protocols sometimes specify a Sorval rotor, which is not available in all laboratories. Review the topic of centrifugation in your reference book *Basic Laboratory Methods for Biotechnology* by Seidman and Moore for more information.

**LABORATORY OVERVIEW**

The quality, purity and concentration of DNA samples will be determined by agarose gel electrophoresis and UV spectrophotometry.

**SAFETY GUIDELINES**

Refer to the Material Safety Data Sheets (MSDS) by checking Sigma-Aldrich or other chemical websites; MSDS are also available in the lab prep area.

**Ethidium bromide** is a strong mutagen. Gloves must always be worn when handling gels or buffers containing this chemical.

**Boiling agarose** can cause burns. Wear hot gloves when removing agarose from hot plate or microwave oven.

The **electric current** in a gel electrophoresis chamber is extremely dangerous. Never remove a lid or touch the buffer once the power is turned on. Make sure the counter where the gel is being run is dry.

**UV light**, used to illuminate the DNA stained with ethidium bromide, is dangerous. Eye protection must be worn.
MATERIALS for 0.8% Gel Casting
Powdered agarose
50X Concentrated TAE Electrophoresis Buffer (dilute to 1X working concentration)
Horizontal gel electrophoresis boxes – one/team
Electronic balance, weigh-boats and spatulas
Microwave oven and hot gloves
Electronic pipette pumps
Thumb-operated pipette pumps
Serological pipettes – 10 ml
100 ml graduated cylinder - one/team
250 ml flasks - one/team
Dishpan in sink for dirty glassware
Scissors – one/team
Sharpie marking pens

MATERIALS for Electrophoresis and UV Spectrophotometry
Uncut Lambda DNA to use as genomic standard
3 samples, 50 μL each, of DNA in 1.5 mL microcentrifuge tubes
High mass DNA ladder
6x or 10x Gel Loading Solution
Practice Gel Loading Solution
50X TAE electrophoresis buffer (dilute to 1X working concentration)
1x TNE Buffer – two 50 mL bottles
2 Beckman UV Spectrophotometers and quartz cuvettes
Kim wipes

MATERIALS THAT SHOULD BE AVAILABLE OR KEPT IN THE LAB
[10 mg/mL] ethidium bromide solution (stored at 4°C)
Microwave oven and hot gloves
Electric pipette pumps
Thumb-operated pipette pumps
Serological pipettes
Kim wipes
Lab diapers
Box of plastic wrap
Sharpie markers
Sterile 1.5 ml microcentrifuge tubes
Microcentrifuge tube trays
Microcentrifuges
Personal microcentrifuges
Micropipetters and tips (2.5 μl and P-1000)
Vortex mixers – 4 per lab
Ice buckets and crushed ice
Horizontal gel rigs
Labeling tape
Electrophoresis grade agarose

Electric balance, weigh paper/boats, spatulas, clean-up brush
250 ml Erlenmeyer flasks
Beakers and bottles of various volumes
Various sized Corning orange capped bottles
25, 100, 500 & 1000 ml graduated cylinders
Microwave oven and mitts
Molecular grade water
UV transilluminator, camera, film, face shields, spatulas, plastic dishes
Carboy of dH₂O
Disposal for Ethidium Bromide contaminated items
Dishpan for dirty glassware
RECIPES

10x TNE Buffer stock solution:
- 100 mM Tris base (Tris [hydroxymethyl] aminomethane)
- 10 mM EDTA
- 2.0 M NaCl
Adjust pH to 7.4 using concentrated HCl and a pH meter. Dilute to 1x working concentration using dH₂O.

50x Concentrated TAE Electrophoresis Buffer (40 mM Tris-acetate, 2 mM EDTA)
Add the following to dH₂O to give a final volume of 1 liter
- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA (pH 8)

PROCEDURE
Note: Write all information on the stock solutions used in your lab notebook. If you open a commercial solution for the first time, write the date and initial the label, also.

Part I: Prep
Set up an ice bucket with crushed ice.

1X TAE ELECTROPHORESIS BUFFER
Note: One liter of 1X TAE will be enough for 3 teams.

Dilute 50x TAE Electrophoresis Buffer with dH₂O to yield 1 liter of 1x TAE Electrophoresis Buffer. Verify your calculations with the instructor first. Label the bottle per eGMP: What the solution is, its concentration, lot number of the 50X stock used, the date, your team number, the name of this lab exercise, and Biotech I.

Part II. Casting an Agarose Gel
Agarose gels can be poured a few days in advance of their use and kept at 4°C with running buffer over them in the gel chamber. If it will be longer than three days until use, do not add running buffer but place the gel in a zipper-type plastic bag and store at 4°C. (Bacteria capable of producing enzymes that degrade DNA can grow in agarose gels.) A gel should be removed from the cold and brought to room temperature just prior to the time it is needed.

1. Weigh out 0.4 grams of agarose and place in a 250 ml flask. (GLP: Clean the balance when finished!) Add 50 ml 1X TAE Electrophoresis Buffer and microwave until completely melted, 1 - 2 minutes. Check the volume after heating and add more dH₂O to return the volume to 50 ml, if necessary. This will be enough agarose for one gel.
2. Set up the electrophoresis chamber using a **10-well comb** according to your instructor’s directions. If the rig needs to be cleaned before using, DO NOT dry it with paper towels, which leave paper fragments that float in the molten agarose and in the running buffer, creating spots on your gel photo.

3. When you can hold your hand on the bottom of the flask of heated agarose for 30 seconds, it is cool enough to pour. Pour the slightly cooled melted agarose into the casting tray. Insert the comb about 1 centimeter from the end that will have the negative electrode attached. (To make this determination, fit the lid on the gel box and connect the electrodes to the power supply.)

4. Allow the gel to cool and solidify (15 – 20 minutes). Do not disturb the gel during this time.

5. When the agarose has solidified, gently remove the comb by pulling it straight upwards in one motion. If the gel is to be stored, carefully place it into a small zipper bag and store it at 4°C until the next lab session. Clean the rig and air dry, upside down.

### Part III. Electrophoresis of DNA to Determine Quality and Concentration

Each team will need the following supplies:
- Practice gel loading dye/solution
- 6x or 10x gel loading dye/solution
- Horizontal electrophoresis unit
- Power supply, to be shared with another team
- Standard Genomic DNA of known concentration
- 3 samples of genomic DNA of unknown concentration, quality and purity
- High Mass molecular weight DNA Ladder
- Heating block at 65°C for 1.5 ml tubes
- 0.8% agarose gel cast previously
- 1x TAE Electrophoresis Buffer
- [10 mg/ml] ethidium bromide stock solution, 1 tube/class

1. Place the gel into the electrophoresis unit. Place the unit next to the power supply that will be used and cover the gel with 1x Electrophoresis Buffer to a depth of at least 5 mm so that the wells are completely submerged. (Note: if the buffer is too shallow, the gel may melt; if it is too deep, the gel will run more slowly).

2. If you have not loaded an agarose gel before, and need practice, use some of the sample gel loading dye. Your instructor can demonstrate the correct way to load a gel. There is no need to put the pipette tip into the well when delivering the sample. The gel loading dye added to each sample is denser than the
electrophoresis buffer and will carry the DNA down into the well when delivered directly above it.

3. Transfer 20 μL of each DNA sample to its own new and labeled 1.5 mL tube. Set the remainder aside to use for UV absorbance readings. Repeat for the DNA + protein sample and set the remainder aside.

4. Prepare the 20 μL DNA samples for loading by adding 3 μL 6x or 10x gel loading dye to each. Gently pipette up and down or spin the tubes briefly in a microcentrifuge (pulse spin) to mix.

5. Consult the product guide as to whether the molecular weight marker DNA must be heated before loading onto the agarose gel. If so, heat in a 65°C heating block for 2 minutes or according to the product guide. Pulse spin the tubes to remove condensation of liquid from the sides and cap.

6. Immediately load 20 μl of the molecular weight marker DNA and the three unknown DNA samples into separate wells of the gel.

7. Add 25 μL of [10 mg/mL] ethidium bromide stock to the buffer. (Note: since the total buffer is about 250 mL, the final concentration of ethidium bromide is 1.0 μg/mL.)

8. Place the cover on the electrophoresis unit, making sure to have the negative electrode at the well end of the gel.

9. Insert the electrode cords into the proper input of the power supply. Set the power supply on ‘low’ and turn it on. Set the voltage at 90-95 volts. Check to make sure bubbles are forming in the buffer on the platinum electrodes along the ends of the gel box. Watch the gel closely for 2 – 5 minutes to make sure that the dye is migrating in the correct direction.

10. Allow the tracking dye to migrate about 5 centimeters from the wells so that the molecular weight marker fragments separate sufficiently.

11. Turn off the power supply, unplug it, and disconnect the electrodes. Remove the lid from the gel box. Remove the gel tray to a plastic container and transfer it to the transilluminator.

12. All those observing the gel must use a UV face shields to protect their eyes and skin. If your transilluminator has more than one setting, make sure it is on the 302 nm wavelength. Camera systems in biotechnology labs vary. Take the photo as directed by your instructor. The photo should be included in the results section of your lab notebook.
13. After photo documentation of your results, dispose of the gel and buffer and anything contaminated with ethidium bromide according to your instructor’s directions. (*NOTE: Information on the proper disposal of ethidium bromide is on the Blackboard website Course Documents for the class.*)

14. Clean the gel rig and its parts by running in tap water, then rinse with dH₂O, and air dry (gel rig upside down) on top of a paper towel. Do NOT dry the inside of the rig. Fragments from a paper towel can remain in the rig and interfere in a future gel electrophoresis experiment. There is also a risk that you will damage the platinum electrodes in the bottom of the gel’s buffer chambers.

**Part IV. Determination of DNA Purity and Concentration by UV Absorbance**

NOTES: Before turning the spectrophotometer on, make sure that the sample chamber door is closed. *Leaving the spec open while the machine is warming up can cause damage to the instrument.* Quartz cuvettes are fragile. Handle them with extra caution and care. They are sold in sets, all cut from the same piece of quartz and are therefore extremely expensive. Always use quartz cuvettes from the same matching set. Place the cuvette into the spectrophotometer correctly, with the arrow on the cuvette top pointing in the direction of the light path.

With the sample chamber door closed, turn on the spectrophotometer and allow it to calibrate and warm up (see note, above). Set up a table in your notebook for the data you will be recording (example, below.)

**DATA TABLE: UV Absorbance by DNA Samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>wavelength (nm)</th>
<th>[μg/mL] Reading 1</th>
<th>[μg/mL] Reading 2</th>
<th>[μg/mL] Reading 3</th>
<th>Average</th>
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</thead>
<tbody>
<tr>
<td>1x TNE buffer</td>
<td>325</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>260</td>
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<td></td>
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<tr>
<td>DNA Sample 2</td>
<td>260</td>
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<td></td>
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<tr>
<td>DNA Sample 2</td>
<td>260/280</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Sample 3</td>
<td>260/280</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A. PREPARING THE SAMPLES

1. Make sure you have the following supplies before beginning:

- DNA samples WITHOUT gel loading dye
- Matching quartz cuvettes
- Lab labeling tape
- Sharpie marker
- 1x TNE buffer
- P-1000 automatic pipetter & tips
- P-0.5 – 2.5 ml automatic pipetter and tips
- Beaker for used tips
- Kim wipes
- Cuvette tray

This lab requires extremely accurate pipetting. Carefully follow these procedures:
- Use a small bore tip for measuring the DNA (the smallest tip available in the lab).
- Keep the pipetter upright to prevent any air bubbles from forming in the 1X TNE or in the DNA sucked into the tips.
- Pre-rinse the tips with the DNA sample by pipetting up and down a few times first.
- Wipe the tip with a Kim wipe before adding the DNA to the 1x TNE buffer in the cuvette.
2. Place the cuvettes into the cuvette tray and then add **exactly** 2.0 ml of 1x TNE buffer to each.

3. Add 2-10 μL of Sample 1 chromosomal DNA to each of the cuvettes. *(NOTE: You will handle one sample at a time, take three readings of that sample, then wash the cuvettes, and go to the next sample. The Blank does not need to be prepared fresh each time.)*

4. Add cuvette lid and mix well by inverting.

5. Clean the outside of each cuvette by wiping with a Kim wipe and replace into the tray. From this point on, do not touch the sides of the cuvette where the light will pass (find the arrow on the top – this indicates the direction of the light path.)

6. Information on how to use the Beckman 500 UV spectrophotometer are given below, but take readings as directed by your instructor.

**B. SETTING THE NUCLEIC ACID PROGRAM on the BECKMAN 500 SPEC**

![Diagram of Beckman 500 spectrophotometer keyboard]

- 1. Soft Keys
- 2. Exit
- 3. Scroll Keys
- 4. Enter Data
- 5. Clear Entry
- 6. Decimal Point and Instrument Setup
- 7. Numeric Pad
- 8. Printing or Download Display
- 9. Data Recall
- 10. Data Storage
1. The Main Menu screen will be displayed on the monitor after turning on the spec.

2. Press the soft key pointing to **NUCLEIC ACID** at the bottom of the Main Menu.

3. The Parameter Setup screen then appears:

4. Press the down scroll key (arrow) on the right side of the key pad until Warburg-Christian is highlighted.

5. Press **ENTER** and the Nucleic Acid Warburg-Christian screen will appear. The ‘UV’ in the upper right corner of the screen will blink while the UV lamp warms up. **DO NOT OPEN THE LID OF THE SPEC WHILE THE LIGHT IS WARMING.**
The Warburg-Christian method is designed to give the most accurate calculation of DNA concentration. The calculations are performed by the spec using $\lambda$ 260.0 nm and constants calculated by Warburg and Christian. A reading at $\lambda$ 320 is also automatically performed and any correction for background absorbance by the buffer and cuvette are included in the calculation.

6. Press the soft key below **DIL X: 1.0000**. Type in the dilution factor of the DNA in the 1X TNE buffer. For example, if you added 2 μL DNA to 1 mL 1X TNE, the dilution factor is 500 ($2 \mu L / 1000 \mu L = 1/500$; inverse = 500). Press **ENTER**.

**C. TAKING DNA CONCENTRATION READINGS**

1. Insert the BLANK cuvette into the spectrophotometer so that the arrow on the cuvette is aligned with the direction of the light pathway.

2. Press the soft key below **BLANK**. The reading should be zero. **READ** will now appear as one of the options on the screen.

3. Remove the blank cuvette and place the first DNA sample into the holder, arrow pointing to the right. Press the soft key below **READ**. Record the calculated DNA concentration.

4. Take one reading of each of the three samples, using the blank each time and average the three calculated DNA concentrations.
PART V. DETERMINATION OF $A_{260}/A_{280}$

Protein in a sample can be detected at $A_{280}$. The relative amount of protein contamination for a DNA sample can be determined by calculating the $A_{260}/A_{280}$ ratio. Ratios of 1.8 to 1.9 indicate that the DNA is highly purified, and totally pure DNA gives a ratio of 2.0. If the ratio is even slightly lower than 1.8, there is substantial protein contamination in the sample.

1. Return to the Main Menu.

2. Press the soft key pointing to **NUCLEIC ACID** at the bottom of the Main Menu.

3. The Parameter Setup screen then appears:

4. Select the first option, 260/280 Ratio. Press **ENTER**.
5. Insert the BLANK cuvette into the spectrophotometer so that the arrow on the cuvette is aligned with the direction of the light pathway.

6. Press the soft key below BLANK. The reading should be zero. READ will now appear as one of the options on the screen.

7. Remove the blank cuvette and place the first DNA sample into the holder, arrow pointing to the right. Press the soft key below READ. Record the calculated 260/280 ratio.

5. Take three readings of each of the unknown DNA samples, using the blank each time and average the three calculated 260/280 readings.

DATA ANALYSIS
Use your electrophoresis and UV spectrophotometer results, along with background information, to determine the following about the genomic DNA that you tested:

- Was the DNA sample intact or degraded?
- How clean was the DNA, i.e., was there much protein present? Was RNA present?
- Calculate the concentration of all three DNA samples. What was the concentration of the DNA in μg/μl as estimated by gel electrophoresis? As determined by UV spectrophotometry?
- In your conclusion, explain any discrepancy between the DNA concentrations you estimated from the gel photo and the concentrations as determined by UV spectrophotometry. Discuss the implications of your findings and comparisons.

QUESTIONS
1. Search the literature (same source as above, or the Internet) and find other methods for isolation of DNA from plants. Name two major differences that you found in other methods for extraction of DNA from plants as compared to that used in this lab.
2. Name four contaminants of plant chromosomal DNA and explain how each can be removed during DNA extraction.
3. Explain what is meant by excellent quality DNA. Why is it necessary for extracted DNA to be of excellent quality?
4. Explain why the concentration of a sample of DNA determined by UV absorbance may differ greatly from that determined by gel electrophoresis of the same sample.
5. Complete the DNA Concentration Practice problem on page 2-21.
6. Determine the concentration of DNA in lane 8 in the electrophoresis results if 10 µl of the DNA was loaded on the gel and it was a 1/100 dilution of the original DNA sample. The molecular weight marker band at the arrow contains 20 ng of DNA. Assume that it is twice as bright as the band of DNA in lane 8. Give your answer in µg/µl. (Note: Even if the photo below is not the best, you are given enough information in this question to do this calculation.)

6. Do the practice problem on the next page and turn it in at the beginning of the next class.

Sources:

http://staff.jccc.net/pdecell/evolution/mutations/mutation.html, 2005

http://www.science-projects.com/VAST-DNA.htm, 2005


DNA CONCENTRATION CALCULATION

PRACTICE PROBLEM

Lane 1: 1/10 dilution of chromosomal DNA – 10 μL were loaded
Lane 2: undiluted chromosomal DNA – 1 μL was loaded.
Lane 3: undiluted chromosomal DNA – 10 μL were loaded
Lane 4: Stratagene KB Ladder loaded so that the total DNA loaded = 500 ng, as per the photo, right, above.
Lane 5: Empty.

1. Calculate the concentration of the chromosomal DNA using these data. Put your answer in ng/μL.

2. The A₂₆₀ reading was 0.014 when you diluted it 10μL in 1 mL. Calculate the concentration (in ng/μL) of the sample of DNA using this information.

3. The UV spec 260/280 reading was 1.0. What does this indicate about this sample of chromosomal DNA?

4. Do the two calculations give the same concentration results? How would you explain a discrepancy?
KEY
DNA CONCENTRATION CALCULATION
PRACTICE PROBLEM

1. \((20 \text{ ng}/10 \mu\text{L}) \times (100) = 200 \text{ ng}/\mu\text{L} = 200 \text{ mg}/\text{mL}\)

2. \((0.0156) \times (50 \text{ mg}/\text{mL}) \times (400) = 312 \text{ mg}/\text{mL}\)

3. No; looking at the gel photo, there appears to be some RNA in the sample.

4. The sample is relatively protein-free.