

Isolation, Spectrophotometric Analysis and Melting of Onion DNA

A. Spectrophotometric Analysis of Nucleic Acids

Nucleic acids and proteins absorb light in the ultraviolet region of the electromagnetic spectrum. As such UV-spectrophotometry can be used to quickly qualify and to some extent quantify the quality and purity of a DNA (as low as 2 $\mu\text{g}/\text{mL}$ DNA) extraction and its contaminants. Proteins have two absorbance peaks in the UV region, one due to electronic transitions in the peptide backbone which absorb at 190-210 nm and another at 280 nm due to absorption by the aromatic amino acids tyrosine, tryptophan and phenylalanine. Purines found in nucleic acids have an absorbance maximum slightly below 260 nm, while pyrimidines have a maximum slightly above 260 nm. Thus, although it is common to hear that the absorbance peak of nucleic acids is 260 nm, in reality, the absorbance maxima of different fragments of DNA vary depending on the base composition. While the molar absorptivity of proteins at 260 nm is very small, both proteins and nucleic acids absorb radiation at 280 nm (including RNA). Therefore, if nucleic acids and proteins are mixed in the same sample, their spectra are additive in this region and therefore convoluted. It is also well known that a pure DNA sample with an $\text{Abs}_{260} = 1$ has an approximate concentration of about 50 $\mu\text{g}/\text{mL}$ and that this value depends on the mole % of G + C in the DNA. We will ignore this variation.

As normally isolated in the laboratory DNA exists as a ordered duplex of DNA strands, RNA and contaminating protein. The goal of this experiment is to isolate, measure and characterize the purity of a DNA sample isolated from onion (or pea) and to assess the temperature, T_m , at which DNA transitions from an ordered duplex to a disrupted strands of DNA that have lost some hydrogen bonds (called thermal melting). The "melting" temperature can be ascertained from the measurement of absorbance data collected as a function of temperature. A plot of the the normalized absorbance vs temperature yields a sigmoidal curve from which the T_m can be measured. This lab consists has several working objectives. They include:

1. to isolate and crudely purify DNA from onion (or pea);
2. to measure the absorbance at 260 nm to quantify DNA;
3. Measure the absorbance at 280 nm to estimate protein contamination;
4. to measure the absorbance at 230 nm and 320 nm to understand what contaminants are present;
5. to calculate $\frac{A_{260}}{A_{280}}$ ratios to estimate the purity of a DNA preparation;
6. Produce UV absorbance spectra of DNA as function of DNA sample temperature;
7. Calculate the melting temperature of the sample, T_m .

B. Extraction of DNA from White Onion

DNA is found in the nucleus of membrane-bound cells. The membranes are composed of phospholipid and proteins in composition. The cell membranes must be lysed in order to release the DNA. A common onion will be diced into small pieces and added to a buffer to which sodium chloride and a solubilizing detergent is added. The detergent, common sodium lauryl sulfate, will break down and effectively emulsify the lipid and protein components of the cell and disrupts the polar interactions that hold the cell membrane together. The detergent then forms complexes with these lipids and proteins, causing them to precipitate out of solution. NaCl salt shields the negatively charged phosphate ends of the DNA which allows these ends to come closer so that the DNA can precipitate out easily using a ice cold layer of 95% ethyl alcohol solution. The EDTA chelates any magnesium which is needed for DNase activity (undesirable). The DNA is then spooled out of the ethanol layer, washed several times and characterized using UV-Vis Spectrophotometry.

Materials

- Lysis Buffer: 50 mM sodium citrate + 50 mM EDTA (acetate or TRIS buffer) (pH = 7.6-8.0)
- Solid NaCl (add 3-5 grams per 100 mL of extraction buffer)
- 10 ml 20% w/w Sodium dodecyl sulfate (or a strong household -etergent liquid) per 100 mL extraction buffer
- about 50-70 grams of whole white onions, or frozen peas or dried peas per 100 mL of extraction buffer
- an electric blender, or mortar and pestle
- crude screen or cheesecloth
- Proteinase K (10mg/ml) degrades DNAases, RNAases, and proteins (meat tenderizer will works fine).
- 95% and 70% (v/v) ethanol
- small centrifuge for washing DNA pellets

General Extraction Proecdures

1. Prepare (if necessary) and heat 100 mL of extraction buffer containing 10 mL SLS, 3-5 g NaCl solution to 60°C. If SLS is not available use a good home dishwashing liquid and add about 10-15 mL of it.
2. Dice and weigh 50.-60. g of finely diced frozen onion and place into the heated buffer.
3. Gently pour the contents into a blender and blend intermitantly at low speed for 3-5 seconds. If no blender is available use a mortar and pestle to grind the cells.
4. After 20 minutes at 60°C chill the beaker containing the onion solution to 20-25°C by placing it into a freezer or an ice bath.
5. Filter and collect the solution through a screen or cheesecloth
6. Collect the filtrate containing dissolved DNA which should be around 25-60 mL of solution. Add 4-5 mL of papain solution.
7. Carefully add -20°C 95% ethanol down the side of the beaker with no mixing. It is essential that the ethanol and homogenate form separate layers. The DNA will selectively precepitate in the ethanol layer and appear as a white stringy mass.
8. Spool the DNA onto a glass rod and allow the excess ethanol drip off or press it out gently against the side of the beaker. Place the spooled DNA into a small 50 mL beaker.
9. Proceed with a several washing steps by adding 15-20 mL of 70% ethanol solution. This will wash away water soluble salts, proteins and clean up the DNA.
10. Place the contents into test tube and centrifuge for 5 minutes. Repeat process 3 times by resuspending pellet and rising again with 70% ethanol.
11. Dissolve pellet in distilled water for UV spectrophotometry analysis.

C. Concentration Measurements of Nucleic Acids and Proteins in a Sample

A fast, convenient and routine way to evaluate DNA concentration and purity is to use both UV spectrophotometric measurements and agarose gel electrophoresis. DNA, and most of the common contaminants found in preparations have absorbances in the region 190 nm to 320nm so measurement of the absorbances in this region allows measurement of the DNA concentration, protein concentration and provides information about the contaminant levels. The absorbance at of duplex, single strand and RNA are well characterized and as follows:

$$\text{DNA (duplex) } (\mu\text{g/ml}) = A_{260} \times (50 \mu\text{g DNA/ml}) / (1 A_{260} \text{ unit}) \times (\text{dilution factor})$$

$$\text{DNA (single) } (\mu\text{g/ml}) = A_{260} \times (33 \mu\text{g DNA/ml}) / (1 A_{260} \text{ unit}) \times (\text{dilution factor})$$

$$\text{RNA } (\mu\text{g/ml}) = A_{260} \times (40 \mu\text{g RNA/ml}) / (1 A_{260} \text{ unit}) \times (\text{dilution factor})$$

The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The $A_{260}:A_{280}$ is a popular measure of the purity of a DNA sample; it should be between 1.65 and 1.85 after subtraction of background absorbance at 320 nm. The absorption of light at 280 nm by proteins is due to the presence of aromatic amino acids; the absorbance per mass can vary because it is dependent upon the presence of tyrosine, phenylalanine, and tryptophan, which account for a small and variable portion of the amino acids in proteins. Additionally, the extinction coefficient for protein is much lower than the extinction coefficient for nucleic acid. Therefore, the $A_{260}:A_{280}$ is not a very sensitive indicator of protein contamination. The $A_{234}:A_{260}$ ratio is a better indicator. Nucleic acids have an absorbance minimum at 234 nm, and protein contamination causes an increase in this ratio. An $A_{234}:A_{260}$ ratio greater than 0.50 is indicative of protein contamination. 320 nm: DNA and proteins do not absorb at this wavelength. A_{320} provides a general measurement of the turbidity of the sample and is normally subtracted from the A_{260} value as a background reading for the calculation of DNA concentration. The absorbance at 320 nm should be less than 5% of the A_{260} . Excessive values may indicate non-specific contamination, dirty cuvettes, air-bubbles or large scattering particles in solution. The average absorptivity constants for proteins and nucleic acids lead to the following relationships:

- If a sample containing pure double-stranded DNA has a A_{320} corrected absorbance of 1 at 260 nm, then it contains approximately 50 $\mu\text{g/mL}$ of double -stranded DNA.
- If a sample containing pure single-stranded DNA has an absorbance of 1 at 260 nm, then it contains approximately 33 $\mu\text{g/mL}$ of DNA.
- If a sample containing pure RNA has an absorbance of 1 at 260 nm, then it contains approximately 40 $\mu\text{g/mL}$ of RNA.
- Values for proteins vary. A very rough rule is that if a sample containing pure protein has an absorbance of 1 at 280 nm, then it contains approximately 1 mg/mL of protein.

D. Estimation of the Purity of a Nucleic Acid Preparation

It is possible to use UV-spectrophotometry to estimate the purity of a solution of nucleic acids. This method involves measuring the absorbance of the solution at two wavelengths, usually 260 nm and 280 nm, and calculating the ratio of the two absorbances:

- An $\frac{A_{260}}{A_{280}}$ ratio of 2.0 is characteristic of pure RNA.

- An $\frac{A_{260}}{A_{280}}$ ratio of about 0.6 is characteristic of pure protein
- An $\frac{A_{260}}{A_{280}}$ of 1.8 is characteristic of pure DNA.
- An $\frac{A_{234}}{A_{260}}$ of > 0.5 indicates protein contamination
- A good quality DNA sample should have a A_{260}/A_{280} ratio of 1.7-2.0 and an A_{260}/A_{230} ratio of greater than 1.5,
- A ratio of 1.7 - 2.0 is desired when purifying nucleic acids. (Note that this method does not actually distinguish DNA and RNA from one another.) A ratio less than 1.7 means there is probably a contaminant in the solution, typically either protein or phenol.
- Beer's Law is accurate when the absorbance varies between 0.1 and 1, so dilution of concentrated samples may be required.

E. Inaccuracies

It is common for analysts to estimate and report the purity of a sample and/or its concentration based on absorbances at 260 and 280 nm. In fact, these calculations are performed so routinely that many spectrophotometers can perform them automatically and display the resulting values. It is important to be aware, however, that the values obtained by these methods are approximations only. Reasons for inaccuracy include:

- The A_{260}/A_{280} method is based on the spectral characteristics of average proteins and nucleic acids. In reality, proteins and nucleic acids vary from one another and so their spectra may vary from one another.
- A_{320} provides a general measurement of the turbidity of the sample and is normally subtracted from the A_{260} value as a background reading for the calculation of DNA concentration. The absorbance at 320 nm should be less than 5% of the A_{260} . Excessive values may indicate non-specific contamination, dirty cuvettes, air-bubbles or large scattering particles in solution.

F. Measuring the Absorbance Values

1. Turn the UV-Vis Spectrophotometer at least 30 minutes before the start of the laboratory (instructor or TA will do this). The instructor or TA will show you how to use the instrument. You will use quartz cuvettes in the UV range. Please handle them with care and caution!
2. Use lens paper or Kimwipes to clean the surfaces of the quartz cuvette. Rinse the cuvette chamber with a small amount of 70% ETOH. Be sure to remove all of the ETOH after the wash. Place 100 μ l sample of your blank (H_2O , TE, whatever your DNA or RNA sample is dissolved in) in the cuvette chamber. Place the cuvette in the holder and place the lid on the holder.
3. Record a blank measurement using buffer if a single beam instrument is used. For a double beam instrument prepare a second cuvette and place in the reference path.
4. Dilute and record the dilution factor of your DNA sample.
5. Measure the A_{220} , A_{260} , A_{280} and A_{320} for each sample. Calculate your DNA concentration, total mass of DNA from your extraction, yield, ratio of A_{260}/A_{280} and A_{234}/A_{260} ratios. If your spectrum goes off-scale, it means the sample is too concentrated and it should be diluted further. Explain what each tells you about your sample.

G. Thermodynamics of DNA Melting

The purpose of this lab is to evaluate thermodynamics of double helical DNA and determine: the dominant forces of interaction in DNA from its melting curve and the thermodynamic parameters of the single strand to helix transition and compare thermodynamic properties of DNA duplex of different sequences. You will measure thermodynamic properties (ΔH° , ΔS° , and ΔG°) of short DNA duplexes by melting the ordered native structure (duplex or double helix) into the disordered, denatured state (single strands) while monitoring the transition using ultraviolet (UV) spectrophotometry.

Melting of DNA

Since the A:T base pair is held together by only two hydrogen bonds, while the G:C pair has three. It is reasonable to think that the T_m would be a function of quantity of the types of base pairs. Generally this true. DNA rich in A:T base pairs exhibit a low melting temperature. The curve of absorbance versus temperature will then increase slowly, the broad nature of the transition indicating compositional heterogeneity.

Two strands of a DNA molecule can be dissociate into single polydeoxyribonucleotide strands (the process is also called denaturing or melting) by heat or chemically by the addition of a denaturation agent such as urea. proceeds because of breaking the hydrogen bonds between complementary bases and disrupting the base stacking. Knowing how denaturation proceeds is important for understanding DNA replication and manipulation with DNA in laboratory. Denaturation of DNA can be caused by a number of physical factors such as change in salt concentration, pH or other factors, but importantly for this application, denaturation of DNA can be started by increasing temperature. Melting of DNA by heat is a standard method for preparing "single-stranded DNA" (ssDNA). The denaturation of DNA occurs over a narrow temperature range and causes a number of physical changes. For instance, the buoyant density of the system increases, the viscosity decreases, the optical rotation becomes more negative and, most importantly for this application, the ultraviolet absorption at 260 nm increases. The simplest characterization of DNA denaturing is via melting temperature, T_m , the temperature at which half the melting has taken place. The T_m depends on DNA length, sequence, ionic environment, pH, etc. Because GC pairs consist of three hydrogen bonds, while AT pairs only have two, the temperature at which a particular DNA molecule "melts" usually will increase with higher percentage of GC pairs. One of the better formulas for melting temperature [Richly and Rhoads, *Nucleic Acids Res* 17: 8543, 1989] uses the nearest neighbor thermodynamic data, i.e. ΔH and ΔS , from the same study quoted at the start of this section (c is the oligonucleotide concentration). The relationship between melting temperature (T_m) and GC content for long DNA can be simplified to:

$$T_m = 69^\circ + 0.41(\%G + C) \quad (1)$$

This equation emphasizes that GC pairs are more stable than AT pairs but it oversimplifies the phenomenon. As the ordered regions of stacked base pairs in the DNA duplex are disrupted, the UV absorbance increases. This difference in absorbance between the duplex and single strand states is due to an effect called hypochromicity. Hypochromicity (means "less color") is the result of nearest neighbor base pair interactions. When the DNA is in the duplex state (dsDNA), interactions between base pairs decrease the UV absorbance relative to that of single strands. When the DNA is in the single strand state the interactions are much weaker, due to the decreased proximity, and the UV absorbance is higher than that in the duplex state. On denaturation, the absorbance of the strands more closely approaches that of the free bases and an increase of up to 40% in the UV absorbance is observed. The profile of UV absorbance versus temperature is called a melting curve; the midpoint of the transition is defined as the melting temperature, T_m . The dependence of the melting transition, T_m , on the strand concentration can be analyzed to yield quantitative thermodynamic data including ΔH° , ΔS° , ΔG° for the transition from duplex to single strand. Alternatively, one can get this information by analyzing the whole melting curve. Thermodynamic analyses of this type are done extensively in biochemistry research labs, particularly those involved in nucleic acid structure determination. In addition

to providing important information about the conformational properties of either DNA or RNA sequences (mismatched base pairs and loops have distinct effects on melting properties), thermodynamic data for DNA are also important for several basic biochemical applications. For example, information about the T_m can be used to determine the minimum length of an oligonucleotide probe needed to form a stable double helix with a target gene at a particular temperature.

Thermodynamic Analysis of Small Oligomers Assuming a two-state model, in which denaturing has no intermediate states ("one or the other"), the steep part of the melting curve reflects the double strand (dsDNA) to single strand equilibrium. At any point in time we know that the sum concentrations of the two stands must be equal to the total concentration of the duplex:



The two-state model approximates short (less than 12 base pairs) DNA duplexes well. The association constant at the midpoint of the melting curve (when half of the DNA is single stranded and the other half is helical) is $K_{50} = 4/[C]$, where $[C]$ is the sum of the concentrations of the two single strands which are non-self-complementary. (For self-complementary strands, $K_{50} = 1/[C]$) Since, for any process at equilibrium we can write:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K \quad (3)$$

where $R = 1.987 \text{ cal K}^{-1}\text{mole}^{-1}$. At the midpoint, $T = T_m$ and $K = K_{50} = 4/[C]$. Therefore, for non-self-complementary strands,

$$\Delta H^\circ - T_m\Delta S^\circ = -RT_m \ln \frac{4}{[C]} \quad (4)$$

After rearranging terms, we get

$$1/T_m = R \ln[C]/\Delta H^\circ + (\Delta S^\circ - R \ln 4)/\Delta H^\circ \quad (5)$$

For self-complementary strands, the equation transforms into:

$$1/T_m = R \ln[C]/\Delta H^\circ + \Delta S^\circ/\Delta H^\circ \quad (6)$$

From the concentration dependence of the melting temperature, the standard enthalpy and entropy can be determined using so-called vant Hoff plot of $1/T_m$ versus $\ln[C]$. The standard free energy of duplex formation, ΔG° , at any temperature can then be determined. Information on ΔH° , ΔS° , ΔG° can be also determined from a single melting curve. Indeed, equation for equilibrium in reaction (2) can be expressed via the degree of hybridization: $a = [dsDNA] / [ssDNA]$

as follows: Similarly for self-complementary strands, it transforms into: Upon denaturing (melting) value of a changes from 1 to 0. Its temperature dependence is related to the temperature dependence of K and to ΔH° , ΔS° , in accordance with Eq.(3). You will use the software supplied with Cary 100 spectrophotometer4 to analyze $a(T)$ and calculate ΔH° , ΔS° for your DNA.