Course   : PG Pathshala-Biophysics

Paper 10   : TECHNIQUES USED IN MOLECULAR BIOPHYSICS II (Based on Spectroscopy)

Module 10 : *Absorption spectroscopy of nucleic acids: DNA and Rna, Nucleic acid bases; Estimation of concentration, DNA purity, homogeneity*

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Introduction

Absorption spectroscopy of nucleic acids (DNA and RNA) is a very useful tool in molecular biology. The reason is simplicity of experimental setup needed and possibility of fairly reliable estimation of concentration and purity of nucleic acids. Latter is a pre requisite for study of reactions that use DNA and RNA. The spectrophotometric estimation of nucleic acid concentration is based on the fact that absorption by DNA, RNA and their constituents follow a particular pattern. Quantification can be done on the basis of Beer-Lambert law.
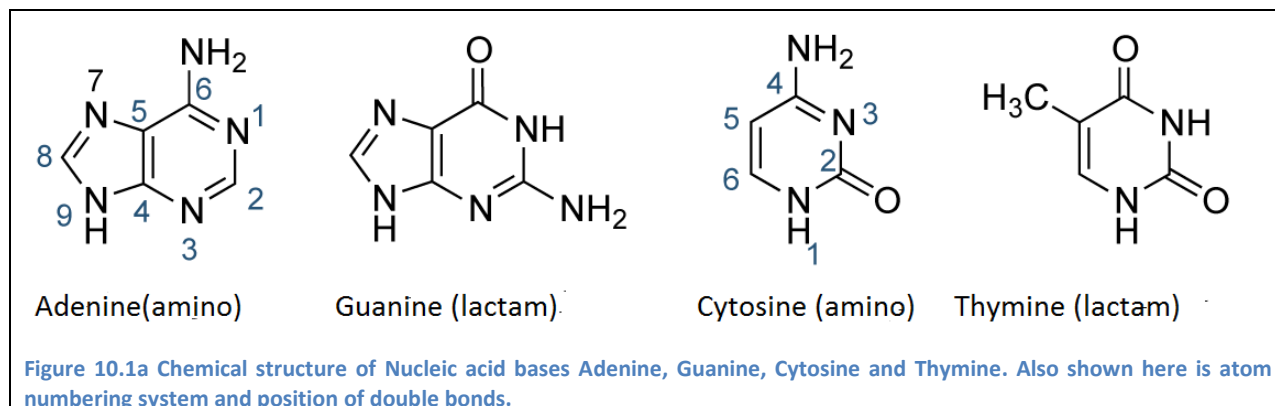
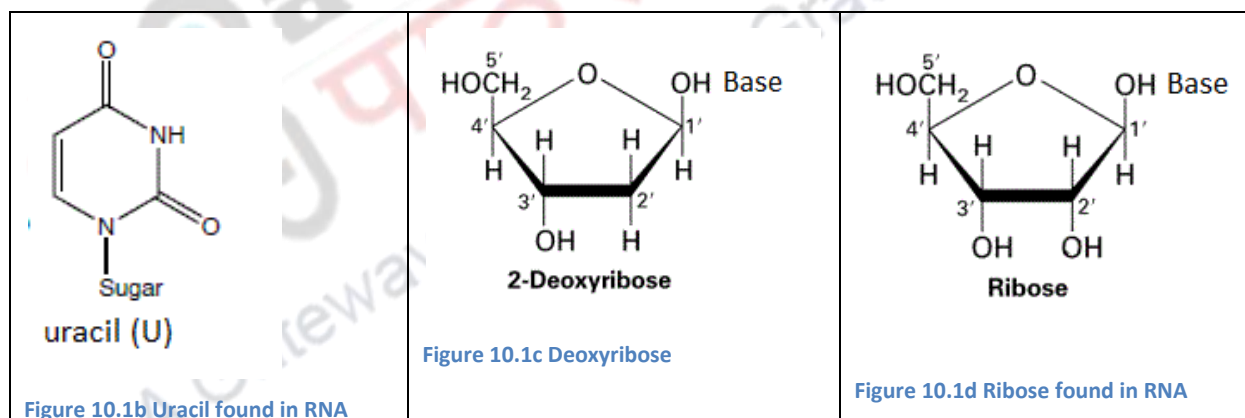*Objectives*

The objective of the present module is:

 a) To introduce the reader to UV-Vis absorption by DNA and RNA,

 b) To discuss absorption by nucleic acid bases and backbone,

 c) To elaborate on estimation of concentration of DNA and RNA,

 d) To discuss application of UV-Vis spectroscopy for determination of purity of nucleic acids,

 e) To enumerate use of UV-Vis absorption for studying homogeneity of DNA.
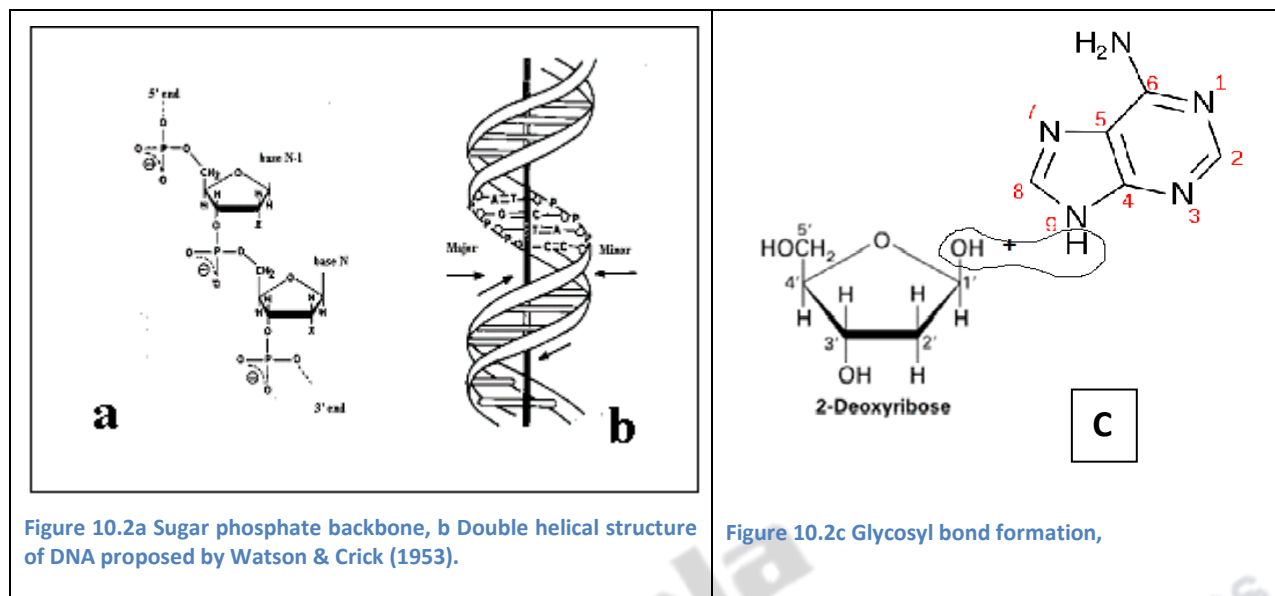
10.1 UV-Vis absorption by DNA and RNA

Nucleic acids DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) are made up of nitrogenous bases: adenine, guanine, thymine, cytosine and uracil (generally we refer these only as 'bases' and designate these using either three letter or one letter symbols: Ade(A), Gua(G), Thy(T), Cyt (C) and Ura(U) (figure 10.1a,b), phosphate and a sugars (deoxyribose/ribose) groups (figure 10.1c, d). The polymer is built by attaching 'bases' to 'sugar phosphate backbone' (figure 10.2a and b) (Watson and Crick 1953, Hoppe et al 1983).

Figure 10.1a Chemical structure of Nucleic acid bases Adenine, Guanine, Cytosine and Thymine. Also shown here is atom numbering system and position of double bonds.

Adenine and guanine belong to the heterocyclic double-ring (five-six fused rings) class of molecules called 'purines' and abbreviated as 'R'. Thymine, cytosine and uracil have six member aromatic rings, are called pyrimidines. Pyrimidines are abbreviated as 'Y'. Uracil (U) has the same ring structure as thymine but lacks a methyl group at position 5 (figure 10.1b). Ribose found in RNA is a normal sugar with one oxygen atom attached to each carbon atom. It is a monosaccharide with formula $H-(C=O)-(CHOH)_4-H$. Deoxyribose found in DNA lacks one oxygen atom. Hence it gets the name 'deoxy'. It is also a monosaccharide with the formula $H-(C=O)-(CH_2)-(CHOH)_3-H$. The sugar phosphate backbone of DNA runs in opposite direction with respect to orientation of 5' and 3' hydroxyl groups which are attached to C5' and C3' atoms of sugars(refer to figure 10.2 for nomenclature). These have overall helical structure and coil around each other (figure 10.2b).

Figure 10.1b Uracil found in RNA

Figure 10.1c Deoxyribose

Figure 10.1d Ribose found in RNA

Figure 10.2a Sugar phosphate backbone, b Double helical structure of DNA proposed by Watson & Crick (1953).
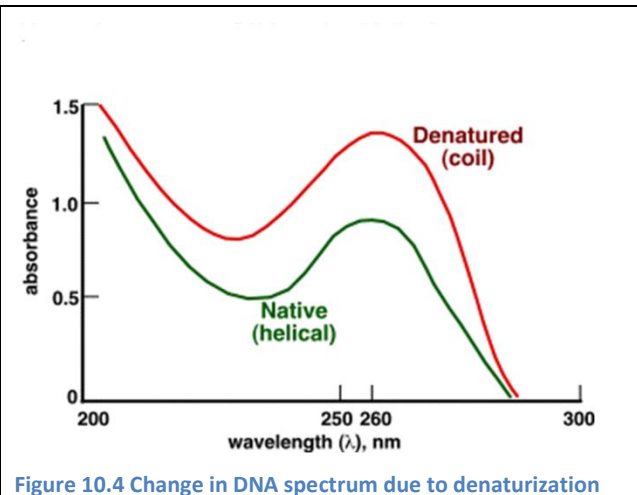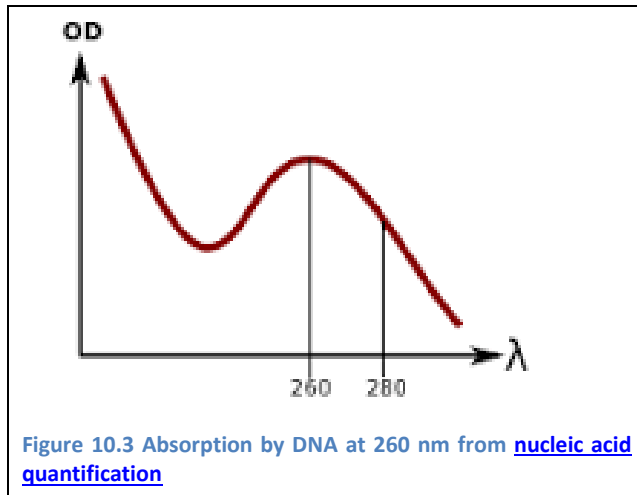
Figure 10.2c Glycosyl bond formation,

Bases are connected to backbone by a glycosyl bond between C1' atoms of sugars and N9 of purines or N1 of pyrimidines. During the 'glycosylation' there is a loss of one water molecule (figure 10.2c). In DNA the bases flank outside the backbone in such a way that complementary bases (A-T) and (G-C) face each other and hydrogen bond with two and three hydrogen bonds respectively.

The bases are aromatic in nature and have alternate double bonds (figure 10.1a and b), which leads to planarity of their structures. These have conjugated π electron system (also named as 'ring current' in some literature). Structure of RNA can be helical or more complex with some helical regions in between, where bases hydrogen bond and stack over each other.

Nucleic acids show strong absorption in the region 240-270 nm (figure 10.3). It arises as a combination from number of transitions due to aromatic nature of bases A,T,G,C and U. Nucleic acid absorption peak is around 260 nm. However, depending on the base composition and environment, the peak can shift from 255 to 265 nm.

The bases can be protonated. As a result, spectra are sensitive to pH of the medium. At neutral pH the absorption maxima for guanosine shifts to 253 nm and for cytidine to 271 nm. This is the reason why polymeric DNA and RNA show a strong absorption at 260 nm. In native DNA the bases are stacked in the hydrophobic core of the double helix and therefore their absorbance is considerably decreased compared to absorbance by a single stranded DNA (figure 10.4).

Figure 10.3 Absorption by DNA at 260 nm from **nucleic acid quantification**

Figure 10.4 Change in DNA spectrum due to denaturization

The denatured DNA has higher absorbance. This phenomenon is known as 'hyperchromicity'. This takes place when DNA is heated and two strands separate out (figure 10.5). One can plot absorbance at 260 nm with respect to temperature. The temperature at which there is 50 % increase in absorbance is called 'melting temperature' or ' T_m'(figure 10.6).

Figure 10.5 Increase in UV absorption of DNA due to heating

Figure 10.6 Measurement of DNA melting temperature Tm
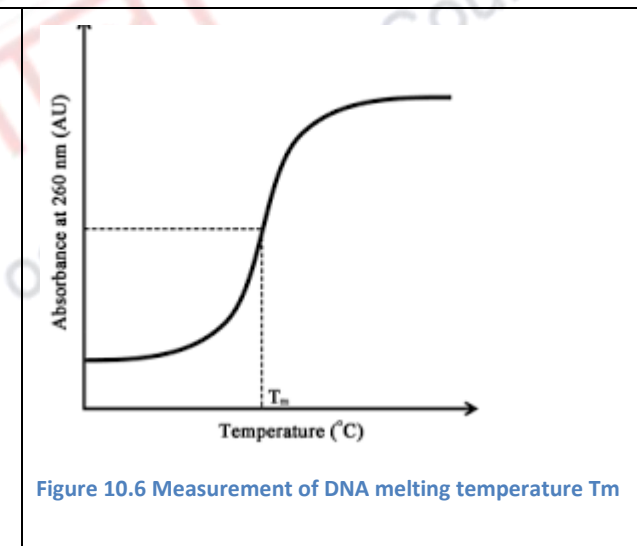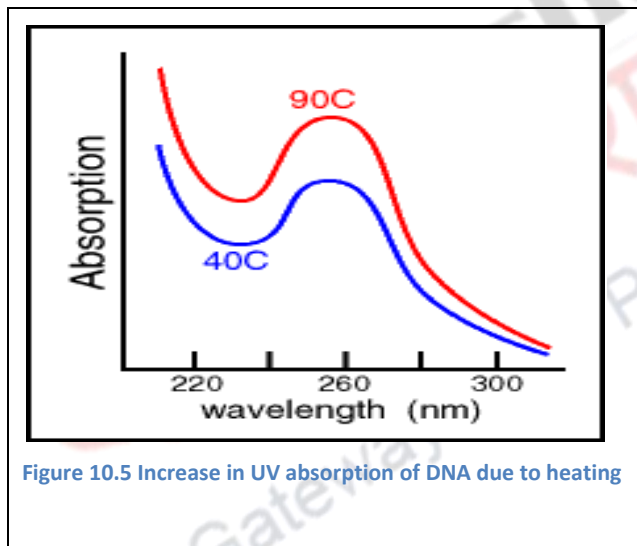
10.2 *Absorption by nucleic acid bases and backbone*

UV spectra of bases show λ_{max} from 259 to 274 nm. Typical absorption spectra of bases A, G, T and C are shown in figure 10.7. Table 10.1 lists λ_{max} and molar extinction coefficients for bases at λ_{max} and 260 nm.
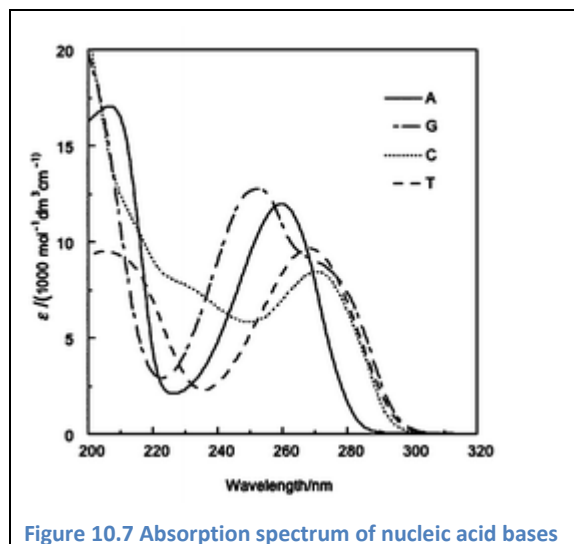
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Figure 10.7 Absorption spectrum of nucleic acid bases

Table 10.1

Value for λ_{max} and molar extinction coefficient ε of nucleic acid bases at pH7.0 (from Methods in Enzymology Gray et al 1995, Cavaluzzi & Borer2004)

Bases	λ_{max}	ε x10^{-3} (M^{-1} cm^{-1})	ε x10^{-3} (M^{-1} cm^{-1}) at 260 nm
Adenine	260.5	15.3	15.2
Cytosine	267	7.6	7.05
Guanine	275	12.2	12.01
Thymine	264.4	8.7	8.4
Uracil	259.5	10.2	8.9

The sugar-phosphate backbone is considered as the most important for conformational changes in double stranded DNA (A and B forms). Backbone PO2 stretching bands can be followed only using FT-IR spectroscopy. These are visible from at 836 and 860 cm^{-1}, respectively. A comparison of the integrated absorptions of these bands $A_{836}/(A_{836} + A_{860})$ can be used for quantitative estimations of B-A forms (Serec et al 2016).

10.3 Estimation of concentration of DNA and RNA

The most common technique for determination of concentration of DNA is UV absorbance at 260 nm. It may be argued that fluorescence is more accurate than absorbance measurements; however, absorbance is simpler and needs common laboratory equipment such as: spectrophotometer equipped with UV lamp, UV-transparent cuvettes (depending on the spectrophotometer type) and a solution of purified DNA. Moreover the method is non destructive and rapid. The only problem is the sensitivity of the method, which is not very high and one needs at least 1 μg/ml sample for proper estimation of DNA and RNA. Another problem is that the method cannot distinguish between DNA and RNA. Third problem is it cannot be used with crude preparations.

The absorbance readings are performed at 260 nm (A_{260}), where DNA absorbs light very strongly (figure 10.3); the number generated allows one to determine concentration. One has to ensure that the measurements are in linear range of the equipment (generally between .1 -1.0). DNA concentration is estimated by measuring A_{260} and adjusting the measurement for turbidity (discussed later). An OD ~1 corresponds to 50 μg/ml of double stranded DNA (dsDNA), ~40 μg/ml of single stranded RNA and ~ 33 μg/ml of single stranded DNA or oligo nucleotides.

10.3.1 Correction for turbidity

One can take care of turbidity in the DNA solution by measuring absorbance ε at 320 nm and calculating OD using relation

Optical density (OD) = log (intensity of incoming light / intensity of out coming light)= log (I_0/I)

One has to multiply this observation by dilution factor, and use A_{260} at OD 1.0 = 50 μg/ml (dsDNA).

Concentration μg/ml = (A_{260} reading $-A_{320}$ reading) $\times$ dilution factor $\times$ 50 μg/ml

Total yield is obtained by multiplying DNA concentration by final total purified sample volume using relation:

DNA yield (μg) = DNA concentration $\times$ total sample volume (ml.)

The calculation is quite simple.

10.3.2 Correction for different path length

In a 10 mm cuvett if OD is =2.0 for double stranded DNA

DNA Concentration = 100.0 μg/ml

When using a path length (cuvett size) that is shorter than 10 mm, the resultant OD would be reduced by a factor 10/path length.

Thus if the cuvett size is 3 mm,

OD for 100 μg/ml sample would be = 2/(10/3)= .6

For any OD with smaller cuvett you have to multiply the observed concentration by 10/ path length. If OD = .6 for 3 mm cuvett the concentration would be= (.6 $\times$10/3) $\times$50 = 100.0 μg/ml

In other words if you have smaller path length, the same OD would yield higher concentration.

10.3.3 Correction for DNA base composition

Since the absorbance of each base is different, base composition and sequence context influence the absorbance. For example, 1.0 OD_{260} of d(CCCCCCCCCCCC) (homopolymeric deoxycytidine) has a mass of 39 μg while 1.0 OD_{260} of d(AAAAAAAAAAAA) (homopolymeric deoxyadenosine) has a mass of 25 μg.

This can be easily taken care of by calculating molar extinction coefficient ε based on composition of DNA.

The extinction coefficient (ε_{260}) describes the relationship between concentration and UV absorbance. It can be calculated for any sequence using nearest neighbor method, although greatest accuracy is achieved when the exact value of ε_{260} is calculated for each oligo. It is also possible to take into account the presence of modified groups, such as fluorescent dyes, which have significant

absorbance at 260 nm. For details refer to [Molar extinction coeffient](). The values can be obtained from (the formula given below (Cantor et al (1977), Cavaluzzi,M.J. and Borer,P.N 2004)

$$\varepsilon_{260} = \left(\sum_{1}^{n-1} \varepsilon_{nearestnei\,ghbour} \right) - \left(\sum_{2}^{n-1} \varepsilon_{individual} \right) + \left(\sum_{1}^{n} \varepsilon_{mod\,ification} \right)$$

A typical example of oligo-nucleotide sequence 5'd(ApTpCpG)3' is given below (Gray et al 1995).

ε d(ApTpCpG)2 = (1/4) [2ε(ApT) + 2ε (TpC) +2ε (CpG) −ε(Tp) −ε(Cp)]

           = (1/4)[2(11,420) +2(11,780) +2(11,420) −(8700)- (15340)]

           =11,300 M^{-1} cm^{-1}

In this relation we multiply each molar extinction coefficient by 2 because of we have a dimer and not a monomer. There are thus two moles of each monomer. We subtract extinction coefficients of monomers because each monomer (except the end ones), are used twice (once with each neighboring dimer). Finally the sum is divided by 4 (the number of monomers in the oligomer 4 in the case of tetra-nucleotides) to obtain extinction coefficient per mol.

It is also possible to use oligo extinction calculators. We show in figure 10.8 oligo extinction calculator by [Williamson lab]().
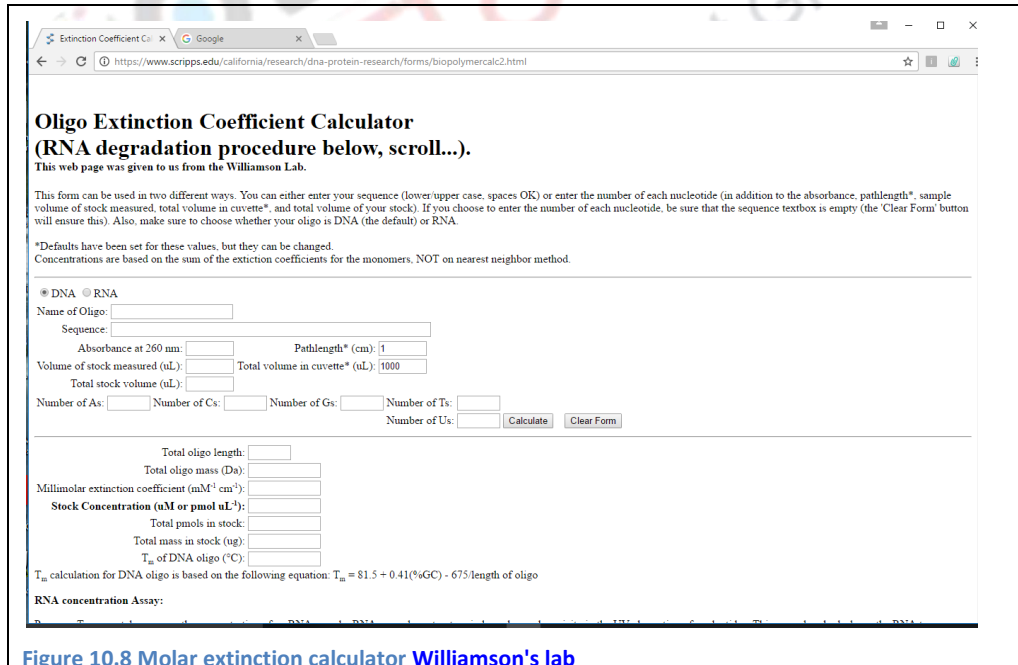

Figure 10.8 Molar extinction calculator [Williamson's lab]()

You can either enter your sequence or enter the number of each nucleotide (in addition to the absorbance, path length, sample volume of stock measured, total volume in cuvett and total volume of your stock). If you choose to enter the number of each nucleotide, be sure to keep the sequence text

box empty (the 'Clear Form' button will ensure this). Also, make sure to choose whether your oligo is DNA (the default) or RNA. Defaults values for DNA have been set in the calculator, but these can be changed.

Concentrations in this method are based on the sum of the extinction coefficients for the monomers, not the nearest neighbor method discussed earlier.

RNA secondary structure induces hyperchromicity in the UV absorption by nucleotides. This procedure described by the authors hydrolyzes the RNA to nucleotides to remove the hyperchromicity and allows for repeated measurements to reduce the inaccuracy associated with the procedure (mostly pipetting error). For details refer to Williamson's lab.

10.4 Use of UV-Vis spectroscopy for estimation of purity of DNA and RNA

DNA is not the only molecule that absorbs at 260 nm. Since RNA has a greater absorbance at 260 nm and aromatic amino acids absorb at 280 nm, both contaminants if present would contribute to the absorbance at 260 nm. Presence of guanidine would lead to higher absorbance at 260 nm. This means that if A_{260} is used for DNA estimation it would lead to overestimation of DNA concentration.

In order to evaluate DNA purity one should measure absorbance from 230- to 320 nm to determine contaminants. The first step is to account for turbidity by measuring absorbance at λ_{320} nm.

10.4.1 Purity calculation using (A_{260}/A_{280})

The most common purity calculations use ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}) because it is a very good indication of presence of proteins. The ratio changes from .57 to 2.0 depending on the percentage of nucleic acids (from 0 to 100) (table 10.2). Good quality DNA would have ratio equal to 1.7-2.0. A measurement of 1.6 does not render DNA unsuitable for any experiments. However lower ratios indicate more contaminant. If there is significant contamination by protein or phenol the ratio ($OD_{260}:OD_{280}$) will decrease and accurate quanitation of DNA concentration will not be possible. Before calculating the ratio ($OD_{260}:OD_{280}$) one has to correct absorbance measurements for turbidity discussed earlier. One can estimate DNA purity using relation

DNA purity (A_{260}/A_{280}) = (A_{260} reading −A_{320} reading) ÷ ((A_{280} reading −A_{320} reading)

Table 10.2

Absorbance of nucleic acids and protein (from http://www.csus.edu/indiv/r/rogersa/quant3.pdf)

% nucleic acid	OD $_{260}$:OD$_{280}$	% nucleic acid	OD $_{260}$:OD$_{280}$	% nucleic acid	OD $_{260}$:OD$_{280}$
0	.57	35	1.78	70	1.94
5	1.06	40	1.81	75	1.95
10	1.32	45	1.84	80	1.97
15	1.48	50	1.87	85	1.98
20	1.59	55	1.89	90	1.98
25	1.67	60	1.91	95	1.99
30	1.73	65	1.93	100	2.0

Glasel (1995) gave a relationship for estimation of DNA concentration using data in table 10.2

%N= P (11.6.R-6.32).(2.16-R) where R is equal to OD $_{260}$:OD$_{280}$

Note: The relationship is valid only if the preparations are free from phenol**.**

Low value of the ratio A$_{260}$:A$_{280}$ means residual phenol or other reagents used for extraction protocol or a very low concentration of nucleic acid (less than 10 μg/ml). The high value means residual RNA from extraction. This is not normally an issue. If the ratio OD $_{260}$:OD$_{280}$ is lower than 1.8 or higher than 2.0 it is good idea to reprecipitate the sample.

10.4.2 Purity calculation using (A_{260}/A_{230})

A strong absorbance at 230 nm, indicate presence of organic compounds or chaotropic salts in the purified DNA. Table 10.3 gives relationship between purity of nucleic acid and the ratio A$_{260}$:A$_{230}$.

Table 10.3 The ratio A$_{260}$:A$_{230}$ for RNA from (Nucleic Acid quantitation)

% nucleic acid	A$_{260}$:A$_{230}$
100	2
95	1.99
90	1.98
70	1.7

A$_{260}$:A$_{230}$ ratio lacks sensitivity for protein contamination in nucleic acids (table 10.3 is for RNA, 100% DNA is approximately 1.8).

A ratio of (A$_{260}$ / A$_{230}$) with lower value than 1.5 indicates presence of more thiocyanate. Low value of the ratio A$_{260}$:A$_{230}$ means carbohydrate carryover (often problem with plants, residual phenol from nucleic acid extraction, residual guanidine often used in column based kits), glycogen used for precipitation etc. High reading means Blank measurements on dirty pedestal, using inappropriate solution for blank measurements. Latter should have same pH and ioninc strength as the sample solution. For example water for the blank measurement for sample dissolved in TE may give a low value.
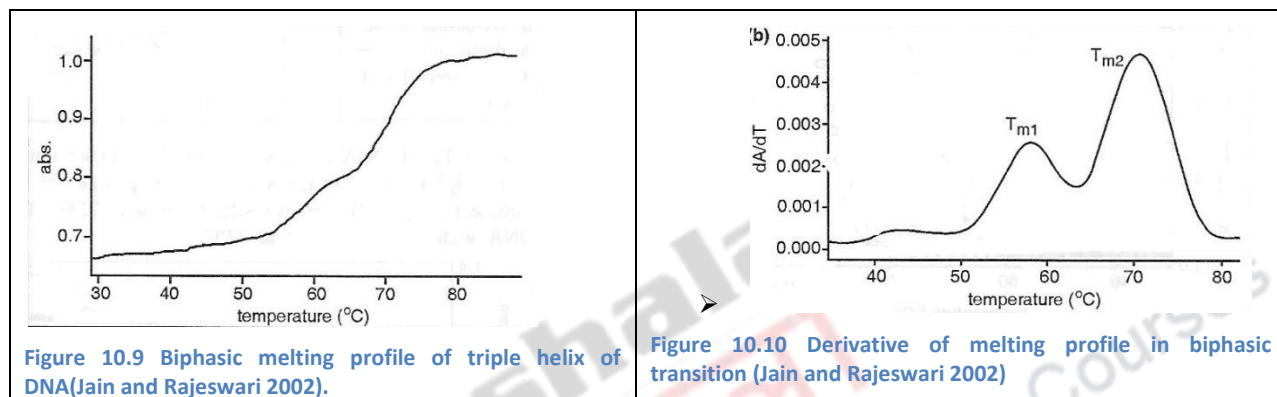
10.4.3 Other contaminants

Phenol absorbance peak is at 270nm with ratio A$_{260}$:A$_{280}$ equal to 1.2. Phenol used in extraction is the greatest contaminant. Other contaminants are phenolate, thiocynate and organic compounds. For pure RNA the ratio A$_{230}$: A$_{260}$:A$_{280}$ should be around 1:2:1 While for pure DNA it should be 1:1.8:1.

Absorption at 330 nm and higher indicates particulate contaminating the solution causing scattering. The value in pure nucleotides should be zero.

Negative values could result if an incorrect solution was used as a blank. These values could arise due to fluorescence of a dye in the solution.

10.5 Use of UV-Vis absorption for studying DNA homogeneity

The DNA molecule when heated the two strands would separate. Melting temperature T_m is sensitive to composition of DNA, presence of metal ions, change in salts concentration and presence of other molecules in the vicinity etc. For example presence of salts has a stabilizing effect on DNA. It is possible to monitor these changes following DNA melting curve and study inhomogeneity in DNA sample. For homogeneous DNA the melting curve should be normally 'monophasic' as depicted in figure 10.6. If not, it indicates inhomogeneity. A typical example of biphasic transition in triple helix is shown in figure 10.9 (Jain and Rajeswari, 2002). One can notice the difference between the curves (figure 10.6 and 10.9).

Figure 10.9 Biphasic melting profile of triple helix of DNA(Jain and Rajeswari 2002).	**Figure 10.10 Derivative of melting profile in biphasic transition (Jain and Rajeswari 2002)**

The melting curve can be analyzed by plotting 'derivative of the absorption dA/dT with respect to temperature (figure 10.10). We see here a bi-phasic transition (Jain and Rajeswari (2002). The figure shows two species of DNA: one melts at 57° and other at 70°. One can study transitions in triple helix, quadruplexes, segmental melting and conformational changes in DNA following UV-Vis absorption at 260 nm with temperature. It is one of the most useful tools for studying drug-DNA and protein-DNA interactions. These topics would be discussed elsewhere.

Quadrant II

Summary

The module introduces reader to basic building blocks of nucleic acids and UV-Vis absorption by DNA and RNA and their constituents. We have depicted differences in the absorption spectrum of nucleic acid bases adenine, guanine, thymine and cytosine and introduced the reader to vibrational spectra due to backbone P-O stretching. We have elaborated on measurement of concentration of DNA and RNA using absorbance at 260 nm. We have described method for correcting errors due to turbidity based on measurement of absorption at 320nm. Also we have described method of correcting OD for different cuvett size. We have given a formula for calculation of molar extinction coefficient of any single stranded or double stranded DNA based on nearest neighbor approximation. We have discussed possible contaminants which affect absorption at 260 nm and lead to erroneous measurement. A method for correcting DNA concentration measurements based on the ratio of absorbance at 260, 280 nm (A_{260}/A_{280}) and 260, 230 nm (A_{260}/A_{230}) is given. We have enumerated use of UV-Vis spectroscopy of DNA for studying DNA homogeneity as a result of presence of ions, salts and other molecules in DNA solution.