

Experiment (3): Characterization of DNA by Spectrophotometric Assay and Melting Temperature (T_m)

Aim:


- Determination of the concentration and purity of extracted DNA using UV spectrophotometer.
- Determination of DNA melting temperature and GC content percentage.

Introduction:

DNA extracts must meet downstream applications requirements. For that after each extraction approach, DNA undergo characterization process, where quantity and quality (purity and intactness) must be measured. The characterization of DNA could be performed with a number of different techniques. In this experiment, spectrophotometric and melting temperature will be used to determine DNA concentration, purity, and GC content.

Characterization of extracted DNA by spectrophotometric assay: DNA concentration and purity can be determined by measuring the absorption of ultraviolet light. The DNA has a maximum and minimum absorbance at 260 nm and 234 nm, respectively and the purines and pyrimidine in nucleic acid are responsible for these absorptions. At 260 nm double-stranded DNA has specific absorption coefficient of $0.02 (\mu\text{g/ml})^{-1}\text{cm}^{-1}$. Moreover, the A_{260}/A_{280} ratio allow to detect nucleic acid purity from proteins contamination since proteins have maximum absorption at 280 nm. Highly purified DNA samples have a 260/280 nm ratio of (1.8-1.9), thus below (1.8) a significant amount of protein impurity may present within the sample. The A_{260}/A_{230} ratio determined to confirm that the sample is pure from carbohydrates, peptides, ethanol or any organic compounds, and it is usually between 2 and 2.2.

DNA melting temperature and GC content: The two strands of the DNA double helix separate when hydrogen bonds between the paired bases are disrupted and this can occur in vitro if the pH of the DNA solution is altered, or if the solution is heated. When DNA is heated, the double-stranded DNA (dsDNA) unwinds and separates into single-stranded (ssDNA) by breaking the hydrogen bonds between the bases ($A=T$ and $G\equiv C$). This process called DNA denaturation and it can be monitored by measuring its absorbance at 260 nm. The absorbance of DNA at 260 nm increases as the DNA becomes denatured, a phenomenon known as the hyperchromic effect. The opposite, a decrease of absorbance is called hypochromic effect. The T_m is the temperature at which 50% of the DNA is unpaired (denatured), and it is depending on both length and GC content of the DNA. The GC content of the DNA that is critical for its stability, and it can be provided by melting temperature (T_m) profile. This profile can be achieved by gradual denaturation of dsDNA into ssDNA.

 PAUSE AND THINK → What is the principle behind hyper/hypochromic effect?

Principle:

When a dilute aqueous DNA solution is heated slowly, the two strands of the double helix gradually separate, leading to the formation of a single stranded DNA (denaturation). It results in an increase in absorbance at 260 nm. Temperature for midpoint of denaturation gives T_m by increasing the temperature slowly and measuring absorbance at 260 nm as melting profile can be generated. The DNA of each species has a specific denaturation curve which is dependent on the % GC content and length. In double stranded DNA, G and C base pairing is more stable and requires more heat energy to break the three hydrogen bonds to separate the strands.

Materials:

The extracted blood and plant DNA from previous experiments.

Preparation of 20 X SSC Buffer

Dissolve 175.3 g NaCl, 88.2 g sodium citrate dihydrate in 800 ml distilled water, adjust PH 7.0 with diluted HCl. Make up the final volume to 1 L by distilled water.

Protocol:

A. Characterization of DNA by Spectrophotometric Assay:

1. Prepare the sample by dissolving the extracted DNA (stock) in 0.1X SSC buffer on a 1:10 ratio with final volume equal to 1 ml.
2. Place the sample in a quartz cuvette, along with a second cuvette contains water as a blank, then set the spectrophotometer as follows:

Nucleic acid → DNA → 10 mm → $\mu\text{g/ml}$ → yes → then enter dilution Factor.

OR

Traditionally, measure the absorbance at 230, 260, then 280 nm

B. Melting Temperature of DNA:

1. In test tube, prepare 1 ml of the sample by diluting your extracted DNA (your stock) to 10 $\mu\text{g/ml}$ with 0.1 X SSC buffer.
2. In another test tube, pipette 2 ml of distilled water (blank)
3. Cover the test tubes of the sample and the blank with aluminium foil.
4. Place the tubes into a water bath at 25°C and allow temperature to equilibrate (5 min).
5. Immediately, transfer the sample and blank into quartz cuvette then read the absorbance at 260 nm.
6. Raise the temperature of the water bath to 50 °C, 60 °C, 70 °C, and boiling, then repeat step 4 and 5.

Results:

A. Characterization of DNA by Spectrophotometric Assay:

Wavelength (nm)	Absorbance of DNA	
	Blood	Plant
230		
260		
280		

- Find out the concentration of the DNA samples using the following equation:

$$\text{Concentration of DNA } (\mu\text{g/ml}) = (A_{260} / \epsilon L) \times \text{Dilution factor (DF)}.$$

- Determine the purity of the DNA samples.

B. Melting Temperature:

Temperature (°C)	DNA Absorbance at 260 nm	
	Blood	Plant
25		
50		
60		
70		
Boiling		

- Plot the value of absorbance vs. temperature and calculate the T_m for sample DNA.
 ➤ Find out the GC content of your sample using the following formula:

$$(G + C)\% = (T_m - 69.3) \times 2.44$$

References:

1. Surzycki S. basic techniques in molecular biology. Springer. (2000).