

Thermal Denaturation of DNA in Semimicro Cells

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Background and Introduction

Deoxyribonucleic acid (DNA) is the origin of the central dogma of biochemistry (DNA → RNA → Proteins) and the template from which all of life is structured. There is no doubt that the study of DNA is key to the understanding of many biological processes, therefore DNA is widely researched in both the academic and industrial settings. DNA technology has resulted in several therapeutics, such as recombinant insulin, used every day by millions of diabetics all over the world. The completion of the rough draft sequence of the human genome is only the beginning of the quest to find more diagnostic and pharmaceutical therapeutic applications of this important biopolymer. Therefore, research into DNA structure and function continues in laboratories all over the world.

The change in structure that accompanies the temperature increase of any polymer is called melting. Careful observation of the melting process provides insight into the physical attributes of the polymer. Specifically, DNA is a biopolymer that is double helical in structure. It consists of two complementary strands composed of four unique bases (adenine, cytosine, guanine, and thymine) held together by hydrogen bonds according to a scheme known as Watson-Crick base pairing. In this bonding scheme, adenine and thymine (AT) form two hydrogen bonds and guanine and cytosine (GC) form three hydrogen bonds. When heated, the hydrogen bonds that hold the two complementary DNA strands together are broken and the two strands separate. When the temperature is sufficiently high, the two strands completely separate from each other and exist in solution as single-stranded DNA. When the melted DNA is returned to a lower temperature, the separated strands will renature, thus returning to the original double-stranded, double-helical structure.

The melting of DNA can be monitored very efficiently using UV-Vis absorption spectroscopy. Because of their aromatic structure, each of the four DNA bases has a characteristic absorption spectrum. The sum of the absorption spectra of these four aromatic bases has an absorption maximum near 260 nm. When DNA melts and the two strands separate, the electronic interactions between the bases are modified and the entire absorption spectrum increases in intensity. Illustrated in Figure 1, this phenomenon is called the hyperchromic effect.¹ The increase in absorption due to melting is usually between 30–40%.

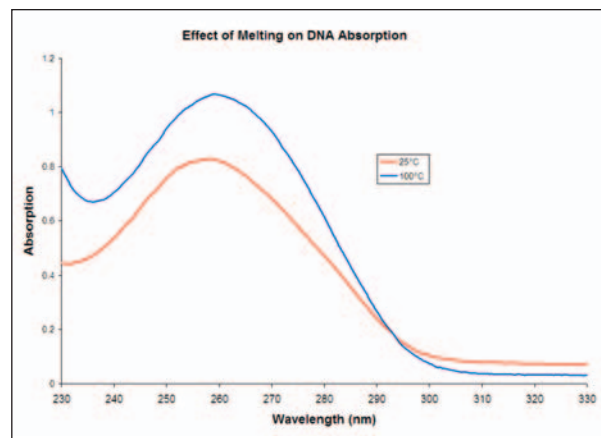
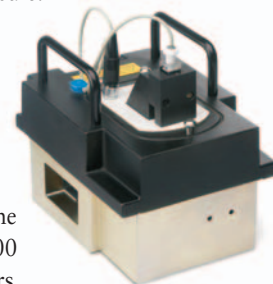


Figure 1: The UV absorption spectra of calf thymus DNA recorded at 25 °C and 100 °C. The increase in absorption due to DNA denaturation is known as the hyperchromic effect.

The melting of DNA occurs over a narrow temperature range and the midpoint of this transition is called the melting temperature or T_m .² The T_m depends strongly on the percentage of the stronger hydrogen bonding GC base pairs present, therefore, DNA with a higher GC content will have a higher T_m .⁴ The T_m is also used to determine the GC content of DNA fragments whose exact sequence is not known. The T_m value for a particular DNA will be reproducible as long as the pH, ionic strength, and buffer conditions do not change.⁴ Subtle changes to any of the previously mentioned solution conditions can alter the T_m value of a particular DNA molecule.³

DNA thermal melting experiments require the temperature of the sample to be precisely controlled during the absorption measurements. The Smart Peltier Thermostatted Single Cell Holder accessory for the Evolution™ 300 and Evolution 600 UV-Vis spectrophotometers offers the ability to measure both the temperature of the cell block and the temperature of the solution inside the cell. To reduce sample volume, denaturation experiments can be performed in 4.0 mm semimicro cells. The extended temperature range of the Peltier accessory allows thermal equilibrium at 100 °C to be achieved in 4.0 mm semimicro cells. The temperature probe is compact enough to accommodate these reduced volume cells.



Key Words

- Denaturation/ Renaturation
- DNA
- DNA Melting
- GC Content
- Semimicro Cells
- T_m
- UV-Visible

The use of smaller volume semimicro cells has several distinct advantages over larger vessels. These advantages include:

- Less sample required for measurement
- Little or no dilution required for measurement
- Higher temperatures can be achieved by reducing reflux action present in larger cells
- Cells are accommodated by standard rectangular sample holders, no adapters required

Perhaps the most distinct advantage of small volume cells is the reduction of the reflux action that occurs at higher temperatures. While condensation of the solution can occur at any temperature, this effect is greater at temperatures near the boiling point, where a small amount of buffer solution from the melting experiment will evaporate from the solution. The quartz surface above the solution level is not in thermal equilibrium with the quartz inside the solution level, therefore vapor striking the surface above the solution will immediately condense, cool, and return to the top surface of the liquid, decreasing the temperature of the solution slightly.

A Smart Peltier Thermostatted Single Cell Holder accessory is available for use with the Evolution 300 UV-Visible spectrophotometer. This accessory has many features that make it convenient for use in the life science laboratory:

- A temperature range of 5 ° to 110 °C
- Ramp rates adjustable from 0.1 to 6.0 °C per minute
- A temperature probe to record the temperature of the solution during the experiment
- No external circulating water is required to maintain the Peltier effect
- Automatic recognition by VISION*pro*[™], VISION*life*[™], and VISION*security*[™] software upon installation
- The unique serial number of the accessory is automatically recorded by VISION*pro* and VISION*security* software

VISION*life* software adds to VISION*pro* or VISION*security* the ability to monitor reaction kinetics and DNA denaturation and renaturation. For DNA melting experiments, multiple temperature ramps can be programmed into a single method, allowing the temperature to be cycled up or down or held at a specified temperature for up to an hour. This flexibility in temperature ramping allows several denaturation/renaturation experiments to be performed within a single experiment method. The data from each temperature cycle can be analyzed individually using three pre-programmed fitting methods available in the VISION*life* software. Both horizontal and sloping intercept methods are available, as well as the inflection point method. Data fitting can be automated and T_m values determined immediately after the data acquisition. Using UVcalc, a spreadsheet like functionality of VISION*pro* and VISION*security* software, additional calculations can be performed with the melting data, such as %GC content determination or statistical comparisons of T_m values.

This application note describes the denaturation of calf thymus DNA in 4.0 mm semimicro cells. We demonstrate differences in T_m determination by comparing methods using a maximum block temperature of 100 °C to a method using a maximum block temperature of 110 °C. Given the dependence of the T_m value on the molecular weight of the DNA, we demonstrate the clear advantage of the Smart Peltier Thermostatted Single Cell Holder accessory for determining T_m values for high molecular weight DNA.

Experimental

High molecular weight calf thymus DNA, purified by phenol/chloroform-extraction and lyophilized from DNase-free and RNase-free distilled deionized water, was used for the studies presented here. The DNA was mechanically sheared into fragments of approximately 50k base pairs. Previous studies have shown that mechanically shearing the DNA into smaller fragments does not alter the T_m .⁴ The lyophilized DNA was dissolved into a saline sodium citrate (SSC) buffer (0.15 M NaCl, 0.015 sodium citrate, pH 7.0) at a concentration of approximately 2 mg/mL. The solution was stirred overnight to ensure complete dissolution. For analysis, 50 μ L of the concentrated DNA solution was diluted in SSC buffer to a total volume of 1200 μ L.

An Evolution 300 UV-Visible spectrophotometer equipped with a Smart Peltier Thermostatted Single Cell Holder accessory was used for the thermal melting experiment. 600 μ L of the diluted DNA solution was transferred to a quartz 4.0 mm self-masking semimicro cell (10 mm pathlength). The small Temperature Probe accessory was inserted into the cell, displacing the DNA solution and leaving about 1.5 mm of bare quartz surface above the solution level for thermal expansion. The temperature of the solution was monitored via the temperature probe accessory and recorded by VISION*life* software.

The sample was inserted into the Peltier accessory and the block temperature was set to 65 °C. The sample and the block were allowed to equilibrate for approximately 30 minutes before the start of the experiment. The final temperature of the solution was 60 °C, typical of the ~10% differential observed in other thermal experiments with 4.0 mm semimicro cells. After equilibration, the Peltier block temperature was ramped at a rate of 0.2 °C/min to a final temperature of 110 °C. The solution was held at 110 °C for 15 minutes. The final solution temperature reached 99.1 °C, again indicative of the ~10% differential typically observed. For comparison purposes, the same experiment was repeated with the same conditions, except the maximum Peltier block temperature was set to 100 °C.

The absorption of the DNA solution at 260 nm was recorded at 40 second intervals using a spectral bandwidth of 2.0 nm and a 2.0 second integration time. The T_m was calculated using the three pre-programmed fitting methods available in VISION*life* software. Additionally, the %GC content of the DNA was determined from the T_m by entering the following equation⁴ into UVcalc:

$$\%GC = \frac{T_m - 69.3}{0.41}$$

The UVcalc spreadsheet like functionality of VISION*pro* software containing the %GC calculation is saved alongside the experimental method, automating the calculation of the %GC content each time the method is performed.

Results and Discussion

Figure 2 shows the melting curve of calf thymus DNA when the temperature of the Peltier block is ramped from 65 °C to 110 °C. The initial decrease in absorption observed from 60 °C to 80 °C is a common artifact of DNA melting and can be accounted for by using the sloping intercept fitting model. The transition from the double stranded to denatured structure is very steep, occurring over the range of approximately 83 °C to 93 °C. After melting is complete, a slight decrease in absorption is observed. Again, this is a common in DNA melting experiments and accounted for in the sloping intercept fitting model. Also shown in Figure 2 is the T_m determined by the sloping intercept model and the %GC determined from the T_m using the UVcalc functionality. The T_m and %GC content data for this experiment is presented in Table 1. The maximum solution temperature achieved in the microcell is 99.1 °C, with a Peltier block temperature of 110 °C.

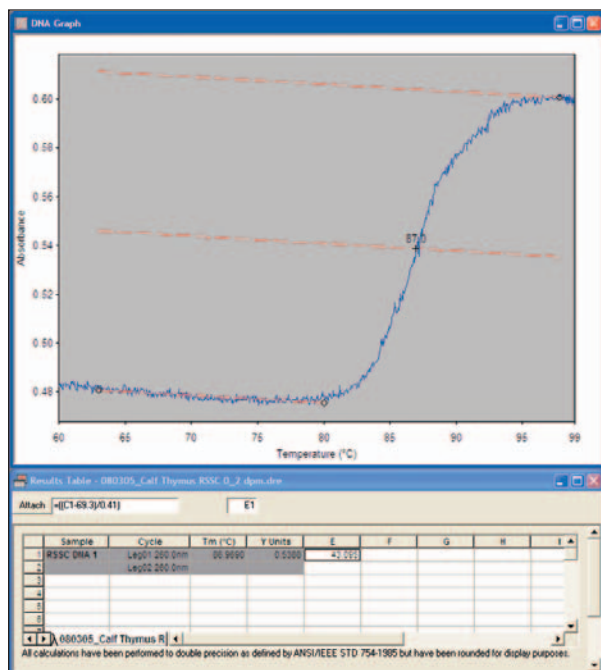


Figure 2: The thermal denaturation curve of calf thymus DNA obtained with a maximum Peltier block temperature of 110 °C. The curve is fit with the sloping intercept model and the T_m value shown in Table 1. The %GC content is also calculated using the UVcalc functionality of the VISION*life* software.

Table 1: T_m and %GC content data from the melting of calf thymus DNA (65 °C – 110 °C)

	Fitting Method		
	Sloping Intercept	Horizontal Intercept	Inflection
T_m Observed (°C)	87.0	87.0	87.6
T_m Literature (°C)	87.0	87.0	87.0
Error (PPT)	0.46	0.46	6.78
%GC Observed	43.1	43.1	44.6
%GC Literature	42.0	42.0	42.0
Error (%)	2.6	2.6	6.2

Figure 3 shows the melting curve for calf thymus DNA when the Peltier block temperature is ramped from 65 °C to 100 °C with a 100 °C hold time of 15 minutes. Like the melting curve shown in Figure 2, initially the absorption of the solution at 260 nm decreases. The melting transition begins to occur around 83 °C, as expected; however, the maximum temperature achieved in solution is 92.2 °C. Therefore, is it not clear whether or not the melting of the DNA is complete. The advantage of the extended temperature range of the Smart Peltier Thermostatted Single Cell Holder accessory is evident when the data in Figure 2 is compared to Figure 3.

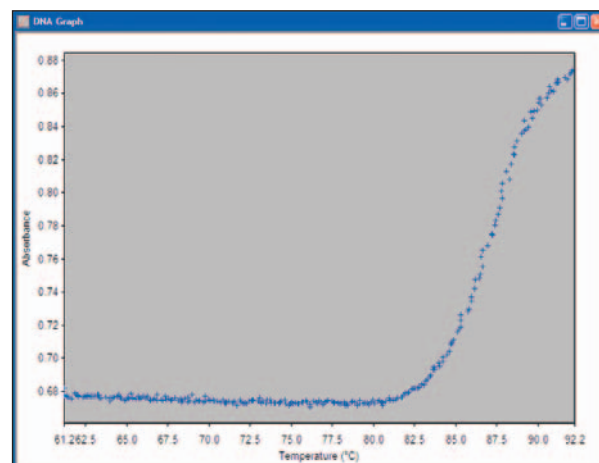


Figure 3: The thermal denaturation curve of calf thymus DNA obtained with a maximum Peltier block temperature of 100 °C

The horizontal and sloping intercept methods of determining the T_m of calf thymus DNA give the most accurate results when compared to the literature values (Table 1). Here a variation from the literature value less than 1 part per thousand is observed. The %GC content of the DNA is also determined accurately with an error of 2.6%. The T_m determined by the inflection point method has a higher error than the other two models. Increasing the sampling rate of the melting experiment can increase the accuracy of the inflection point method by determining the rate of change of the melting curve from a greater number of points. For extremely sensitive melting point analysis, thermal denaturation experiments can be performed at rates as slow as 0.1 °C per minute, with high sampling rates, some melting transitions not typically seen at higher ramping rates can be resolved.

The 54k base pair fragment of calf thymus DNA is very complex and therefore requires a significant amount of time to return to a renatured state. However, for shorter sequences or less complex DNA fragments, renaturation experiments are easily accommodated by the multiple temperature ramps offered in the DNA application of the VISIONlife software. When high resolution denaturation/renaturation curves are required, VISIONlife offers a simple solution to automate this process, allowing multiple lengthy experiments to be performed with no user intervention. The data is separated by each cycle of the temperature ramp allowing the researcher great flexibility in data fitting. Both denaturation and renaturation are accommodated by the T_m algorithms.

Conclusion

In this application note we have demonstrated use of our Evolution 300 UV-Visible spectrophotometer equipped with a Smart Peltier Thermostatted Single Cell Holder accessory for determining the T_m and %GC content of calf thymus DNA in 4.0 mm semimicro cells. This note also explains the advantages of using semimicro cells for DNA analysis. In these experiments, the temperature of the solution is measured directly with a temperature probe, allowing the exact temperature of the solution to be known at each absorption reading. This allows sophisticated denaturation/renaturation experiments to be performed with Peltier block ramp rates as low as 0.1 °C per minute. VISIONlife software also allows multiple temperature ramps to be performed in a single experiment with the ability to analyze each segment of the ramp independently.

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