Application Note

DNA Testing

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Achieving Faster Ramp Rates for Thermal Melt Experiments

Same result achieved independent of ramp rate, using the Cary 3500 UV-Vis spectrophotometer

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Introduction

The separation of double stranded nucleic acids into single strands can be induced by raising the temperature. At a certain temperature, the hydrogen bonding between the base pairs is broken. A thermal melt experiment exploits the difference in the number of hydrogen bonds between adenosine to thymine (A=T) and guanine to cytosine (GΞC) nucleotides for DNA, or between adenosine to uracil (A=U) and G to C for RNA. As the GΞC nucleotides contain three hydrogen bonds, the heat energy required to dissociate is greater than that of the double bonded pairs. This means that DNA and RNA containing more GΞC pairs will melt at a higher temperature. The melting temperature (T_m) gives an accurate indication of the base composition (ratio of the GΞC vs the A=T / U=T) in the sample.

The melting point is measured using UV-Vis spectrophotometry, utilizing the fact that the absorbance of single stranded nucleic acids is higher than for double stranded nucleic acids at 260 nm (*1*). Figure 1 shows an example of this using a short interfering RNA (siRNA) sample; the absorbance at 260 nm is significantly higher at 85 °C, compared to 25 °C.

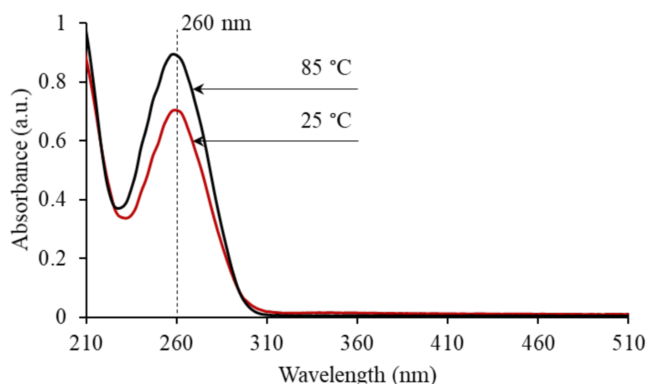

Figure 1. Wavelength scan of an siRNA sample at 25 °C (red) and 85 °C (black).

Thermal melt experiments are typically performed by measuring the absorbance at 260 nm. The temperature of the sample is then gradually increased under controlled pH and ionic strength conditions. The temperature is usually increased at 0.5 °C per minute (*2*, *3*, *4*). This very slow temperature ramp rate is required to ensure accurate and reproducible data. Unfortunately, the slow temperature ramp rate also means that experiments take a long time. For example, changing the temperature from 20 to 95 °C at 0.5 °C per minute takes 2.5 hours. Laboratories often repeat these measurements to ensure reproducible results, so a complete thermal melt experiment can take a significant amount of time.

There are various approaches to reduce the time taken for thermal melt measurements. For example, some instrumentation allows an experiment to be divided into stages, with each stage using a different temperature ramp rate. A fast ramp rate can be used at the start and end stages, with a slower rate over the temperature range where the sample will denature.

Recent advances in spectrophotometric instrumentation offer significant reductions in elapsed times for thermal melt measurements, as well as higher temperature accuracy than previously possible. The Agilent Cary 3500 Multizone UV-Vis spectrophotometer uses integrated in-cuvette temperature probes to accurately control the temperature of the solutions during the experiment. The multicell holder is built into the instrument and uses water-free, air-cooled Peltiers to control the temperature of samples between 0 and 110 °C.

This study assessed the impact of increasing the temperature ramp rate on the calculated melting temperature (T_m) of an siRNA sample, using a Cary 3500 Multizone UV-Vis spectrophotometer.

Experimental

Samples

A sample of siRNA was provided by the Agilent Nucleic Acid Solutions Division. A solution of ≈ 0.3 mg/mL of the siRNA was prepared in a buffer solution containing 100 mM NaCl, 0.1 nM EDTA, and 10 mM sodium phosphate, adjusted to pH 7.0.

Standard, 3.5 mL volume, 10 mm optical pathlength quartz cuvettes were used. Neat phosphate buffer was used as the reference solution.

Instrumentation and method

A Cary 3500 Multizone UV-Vis spectrophotometer was used for all measurements. The method parameters are shown in Table 1.

The only parameter that was changed during the study was the temperature ramp rate. A total of eight ramp rates were used: 0.5, 1, 5, 10, 15, 20, 25, and 30 °C per minute.

All measurements used at least three aliquots of the sample, measured simultaneously, under identical conditions. Each sample cuvette was paired with a reference and placed in the eight-position multicell holder. An in-cuvette temperature probe was inserted into each sample cuvette (Figure 2) and could be used to control the experimental temperature.

Figure 2. The in-cuvette temperature probe, used to control experimental temperature during the measurements.

Data was collected every 0.5 °C and the signal was averaged for 1 second before each data point was recorded. At a temperature ramp rate of 30 °C per minute the measurement took approximately five minutes.

The stirring was set to 500 rpm for these tests to ensure a sufficiently uniform temperature throughout the cuvette.

Table 1. The method parameters.

Parameter	Setting
Wavelength (nm)	260
Spectral bandwidth (nm)	5
Signal averaging time (s)	1
Data interval (°C)	0.5
Start temperature (°C)	25
End temperature (°C)	86
Return temperature (°C)	25
Number of stages	1
Hold time (min)	0.5
Temperature ramp rate (°C/min)	Various
Stirring speed (rpm)	500
No. temperature zones	4
Temperature control	Temperature probe

Results and Discussion

T_m values at different temperature ramp rates

Data analysis was performed in a third party software package. The absorbance data collected during the eight temperature ramp rates is shown in Figure 3. The data was normalized from 0 to 1 absorbance unit and stacked for ease of viewing. The first derivative of each was calculated and is also shown. The peak of each first derivative plot identifies the midpoint of the melting curve and thus, the Tm value.

Table 2. The measured T_m values for the siRNA sample at each temperature ramp rate.

	Ramp rate (°C/min)	Replicate 1 T_m °C	Replicate 2 T_m °C	Replicate 3 T_m °C	Average Tm (°C) (n=3)
Experiment 1	0.5	78.5	78.5	78.5	78.50
Experiment 2	1	78.5	78.5	78.0	78.33
Experiment 3	5	77.5	78.0	77.5	77.67
Experiment 4	10	78.0	77.5	77.9	77.80
Experiment 5	15	78.4	78.5	77.9	78.27
Experiment 6	20	78.4	78.3	78.5	78.40
Experiment 7	25	78.7	77.8	78.3	78.27
Experiment 8	30	79.0	78.9	78.2	78.70

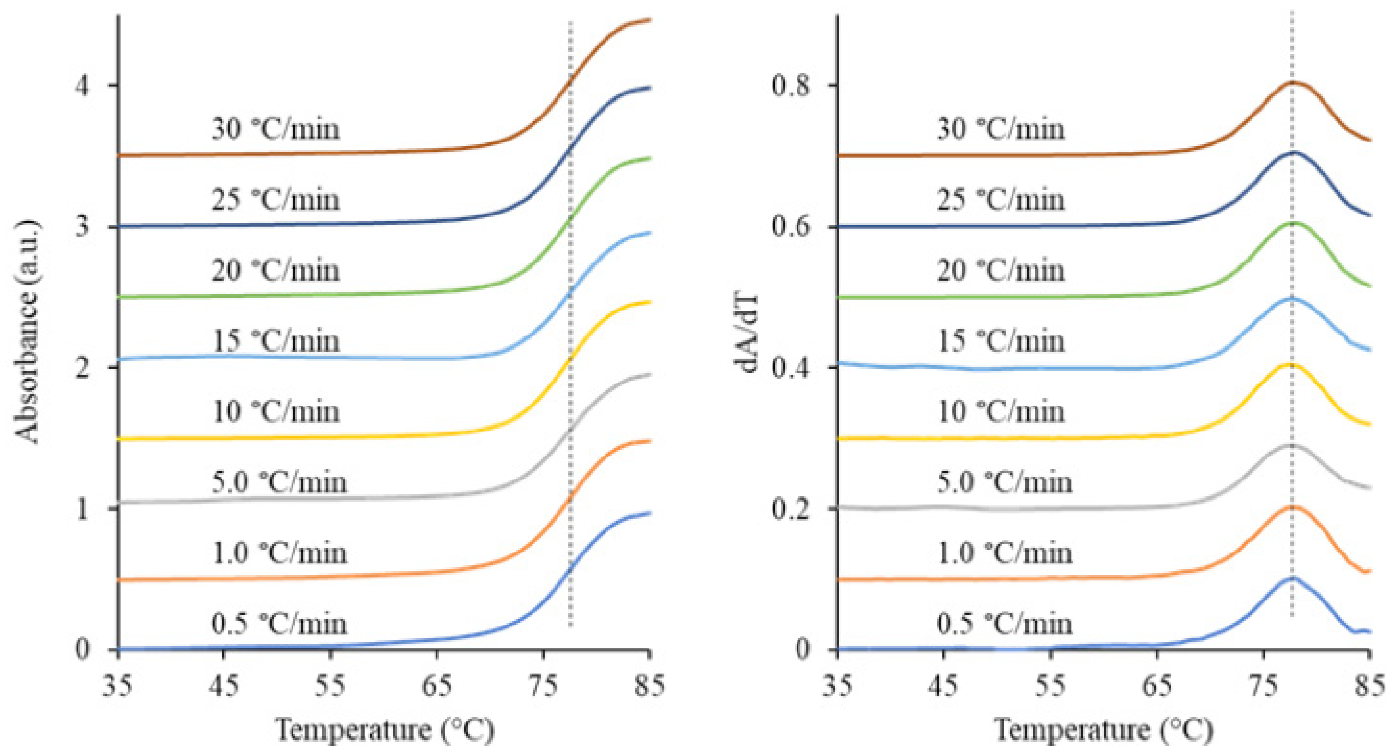

Figure 3. siRNA (left) absorbance versus temperature and (right) the corresponding first derivative as a function of temperature ramp rate.

As shown in Figure 3, and Table 2, the measured T_m value of the siRNA samples was within ±1 °C for the eight temperature ramp rates used in the experiments.

Conclusions

The Cary 3500 Multizone UV-Vis spectrophotometer was used to measure the melting point of the same siRNA sample at eight different temperature ramp rates. The measured melting points at each of the ramp rates were all within ±1°C. This means that laboratories could use temperature ramp rates faster than the common protocol of 0.5 °C/min. Experiment time could be significantly reduced by using a higher ramp rate without compromising the result. This study demonstrated that an experiment that previously took 2.5 hours could be measured in approximately five minutes using the Cary 3500. The simultaneous measurement of at least three replicates of the sample also represents additional, considerable time saving.

The use of fast temperature ramp rates extends to other measurements of UV-Vis absorbance as a function of temperature, offering significant productivity benefits for laboratories conducting temperature-controlled experiments. The ability to measure all eight cuvette positions simultaneously offers further productivity improvements for laboratories interested in studying the response of liquid samples to temperature changes.

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