Application Note

Biopharmaceuticals

Agilent
Trusted Answers

Fast Determination of Thermal Melt Temperature of Double-Stranded Nucleic Acids by UV-Vis Spectroscopy

Reproducible melting temperature (T_m) data at fast ramp rates using the Agilent Cary 3500 UV-Vis

Authors

Wesam Alwan and
Mathieu Rault
Agilent Technologies, Inc.

Introduction

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

During drug discovery, development, and manufacturing of nucleic acids, the T_m is often used as a secondary identification test (QC check) by researchers and manufacturers. The melting point is typically measured by UV-Vis spectrophotometry, since the absorbance of single-stranded nucleic acids is higher than for double-stranded nucleic acids at 260 nm, the maximum absorption peak for nucleic acids.[1] Using herring sperm DNA as an example, Figure 1 shows that the absorbance at 260 nm is significantly higher at 90 °C compared to 25 °C.

Nucleic acid thermal melt experiments are typically performed by measuring the change in absorbance at 260 nm. The temperature of the sample is gradually increased under controlled pH and ionic strength conditions typically using a 0.5 °C per minute ramp rate.[2–4] A slow temperature ramp rate is used to ensure accurate and reproducible data.

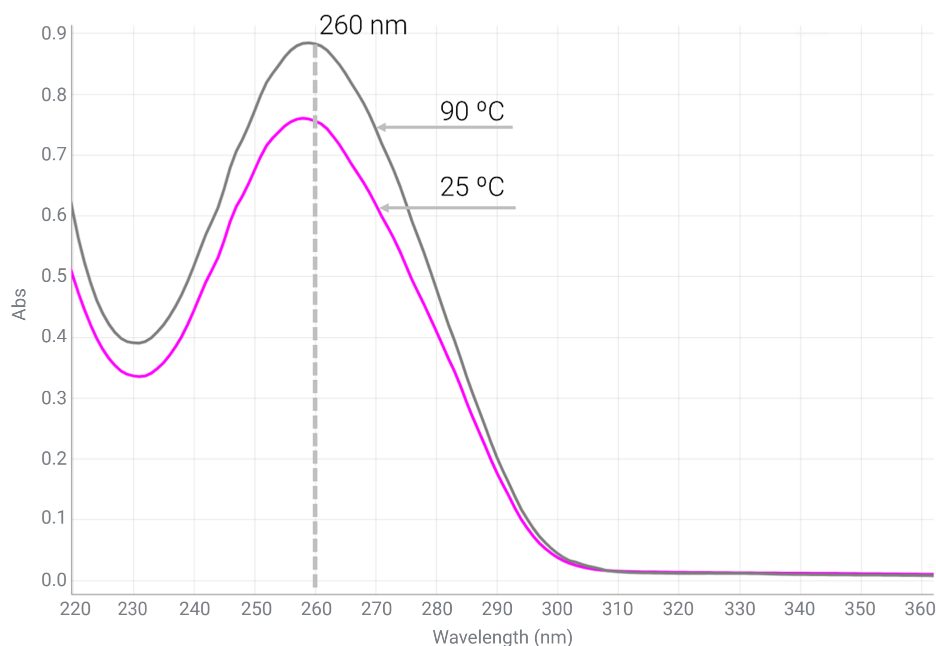

Figure 1. Wavelength scan of a herring sperm DNA sample at 25 °C (double-stranded, purple) and 90 °C (single-stranded, grey).

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/min 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.

However, reannealing experiments should be performed at a slower rate to allow time for complete reannealing of single-stranded nucleic acids.

The Agilent Cary 3500 Multizone UV-Vis spectrophotometer uses integrated in-cuvette temperature probes to accurately control the temperature of the solutions during thermal melt experiments. Water-free, air-cooled Peltier devices are used 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 T_m of herring sperm DNA using a Cary 3500 Multizone UV-Vis spectrophotometer.

Experimental

Samples

A solution of ~15 µg/mL of the herring sperm DNA (D6898) was prepared in phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C). Neat phosphate buffered saline was used as the reference (blank) solution. Agilent quartz semimicro cells (part number 5063-6559) with temperature probe (part number G9889-60005), 600 µL sample volume, and 10 mm optical pathlength were used (Figure 2). To minimize sample evaporation, a few drops of mineral oil (FS-SMO15) were floated on top of the sample in the cuvette.

Instrumentation and method

A Cary 3500 Multizone UV-Vis spectrophotometer was controlled using the Agilent Cary UV Workstation software. Measurements were performed using the method parameters shown in Table 1. The only parameter changed during the study was the temperature ramp rate. A total of six ramp rates were used: 1, 5, 10, 20, 30, and 40 °C/min. 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 the feedback from the probe was used to control the experimental temperature.

Data were collected every 1 °C and the signal was averaged for 0.1 seconds before each data point was recorded. At a temperature ramp rate of 30 °C/min, the measurement took approximately 10 minutes.

Table 1. Agilent Cary 3500 UV-Vis method parameters.

Parameter	Setting
Wavelength	260 nm
Signal Averaging Time	0.1 s
Data Interval	1 °C
Start Temperature	25 °C
End Temperature	100 °C
Return Temperature	25 °C
Temperature Ramp Rate	1, 5, 10, 20, 30, and 40 °C/min
Number of Stages	1
Temperature Control	Temperature probe
Smoothing Filter	11
Smoothing Data Interval	0.5 °C
Derivative Filter	11
Derivative Data Interval	0.5 °C

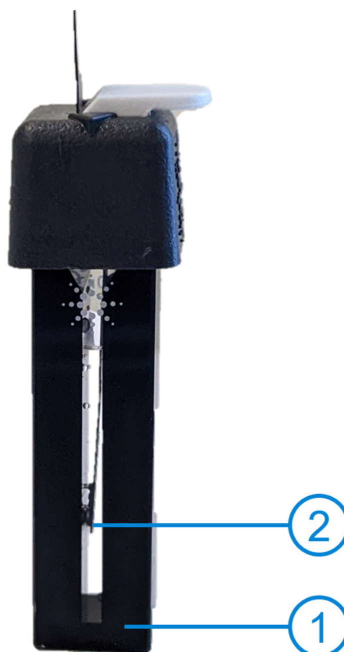

Figure 2. Agilent semimicro cell (1) and the in-cuvette temperature probe (2) used to control experimental temperature during the measurements.

Results and discussion

T_m values at different temperature ramp rates

The absorbance data collected at the six temperature ramp rates (1, 5, 10, 20, 30, and 40 °C/min) is shown in Figure 3. The first derivative of each scan was calculated and is also shown in Figure 3. The maximum peak of each first derivative plot identifies the midpoint of the melting curve, and, therefore, the T_m value. As shown in Figure 3 and Table 2, the measured T_m values of the herring sperm DNA samples was within ±0.2 °C for all six temperature ramp rates used in the experiments.

Table 2. The measured T_m values for the herring sperm DNA sample at each temperature ramp rate.

Entry	Ramp Rate (°C/min)	Replicate 1 T_m (°C)	Replicate 2 T_m (°C)	Replicate 3 T_m (°C)	Average T_m (°C) (n = 3), Each Ramp Rate	Standard Deviation T_m (°C) (n = 3), Each Ramp Rate
1	1	87.1	87.1	87.1	87.1	0.0
2	5	87.0	86.6	86.5	86.7	0.2
3	10	86.7	87.1	87.0	86.9	0.2
4	20	87.1	87.1	87.1	87.1	0.0
5	30	87.1	87.0	86.6	86.9	0.2
6	40	86.6	87.0	87.0	86.9	0.2
Average T_m (°C) (n = 6), All Ramp Rates		86.9	87.0	86.9		
Standard Deviation T_m (°C) (n = 6), All Ramp Rates		0.2	0.2	0.2		

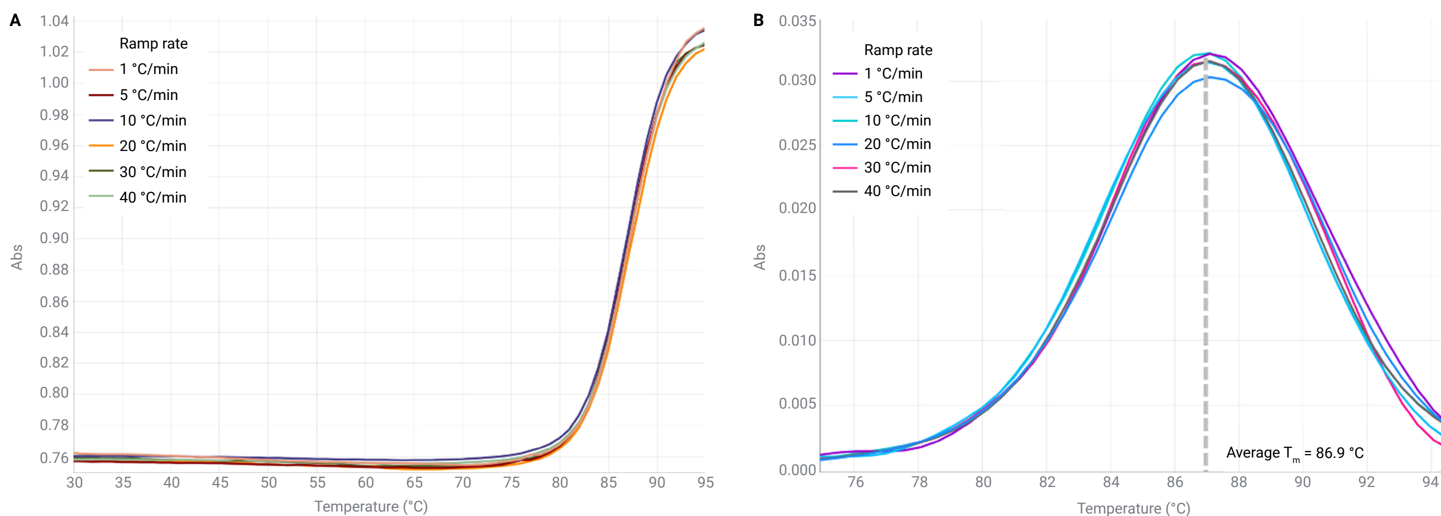

Figure 3. Herring sperm DNA absorbance versus temperature (A) and the corresponding first derivative (B) as a function of temperature ramp rate.

Cary UV Workstation built-in smoothing and derivative calculator

The Cary UV Workstation software includes smoothing and derivative functions to help calculate the melting temperature. Smoothing can be applied to reduce interference and noise in the spectra and the derivative function calculates the first derivative.

The smoothing and derivative functions use the Savitzky–Golay technique.[5] Both functions require a filter and interval sizes. The filter size defines the number of points that are used to generate each output point. If a different interval value is used for the data analysis than the collection interval, the calculated data will be set to the specified interval. It is recommended to match the smoothing and derivative intervals to the collection interval.

The smoothing and derivative calculations can be saved within a method. The calculations will then be applied automatically at the end of the data acquisition (Figure 4).

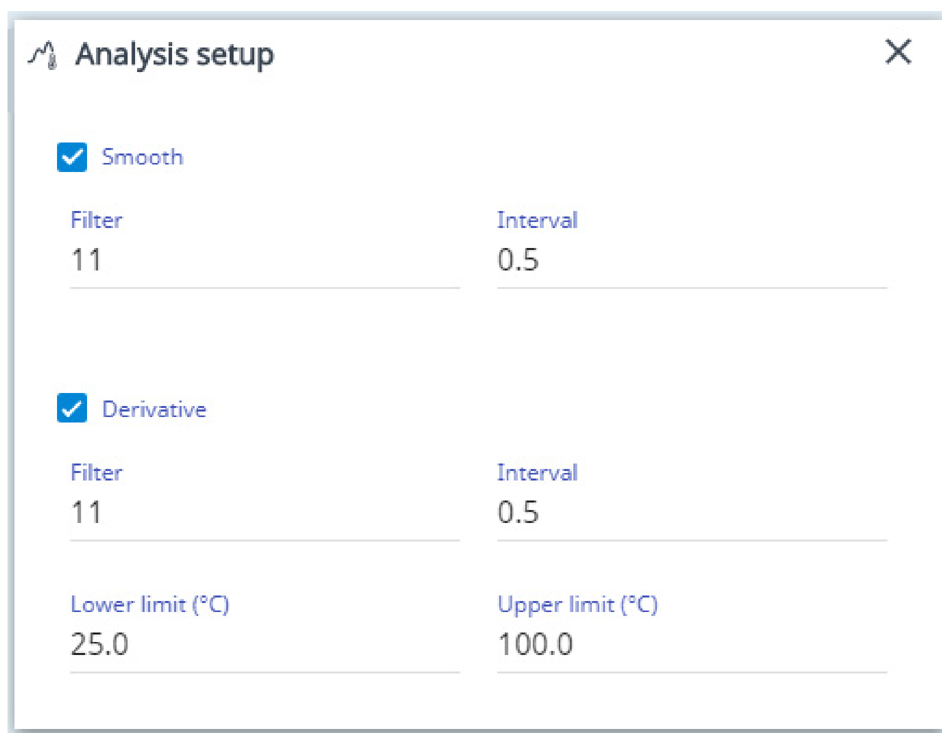

Figure 4. Agilent Cary UV Workstation built-in smoothing and derivative calculator.

Conclusion

The Agilent Cary 3500 Multizone UV-Vis spectrophotometer was used to measure the melting point (T_m) of herring sperm DNA samples at six different temperature ramp rates. The measured melting points at each of the ramp rates were all within ±0.2 °C. This level of reproducibility means that laboratories can use faster temperature ramp rates than the standard protocol of 0.5 °C/min, significantly reducing experimental times without compromising the quality of the results. The study demonstrated that an experiment that previously took 2.5 hours could be measured in approximately 10 minutes using the Cary 3500 UV-Vis.

The eight-position multicell of the Cary 3500 UV-Vis enabled the simultaneous measurement of at least three sample replicates and reference, which also reduced overall experimental measurement times.

The Agilent Cary UV Workstation software includes built-in capabilities for DNA melting temperature calculations (smoothing and derivative) that quickly provide actionable results. The calculations, which can be saved within a method, are automatically applied and displayed after data collection.

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 without other experimental variables.

The Cary UV Workstation software can also be integrated with the Agilent OpenLab software. OpenLab provides technical controls to securely acquire, process, report, and store data. These controls are needed in laboratories that must follow the compliance guidelines of FDA 21 CFR Part 11, EU Annex 11, GAMP5, as well as ISO/IEC 17025 and EPA 40 CFR Part 160.

References

1. Shen, C-H. *Diagnostic Molecular Biology*, Chapter 7 - Detection and Analysis of Nucleic Acids, Academic Press: 2019; pp 167–185.
2. Chetana, P. R. *et al.* New Ternary Copper(II) Complexes of L-Alanine and Heterocyclic Bases: DNA Binding and Oxidative DNA Cleavage Activity. *Inorganica Chimica Acta* **2009**, *362*, 4692–4698.
3. Rao, R.; Patra, A. K.; Chetana, P. R. Synthesis, Structure, DNA Binding and Oxidative Cleavage Activity of Ternary (L-leucine/isoleucine) Copper(II) Complexes of Heterocyclic Bases. *Polyhedron* **2008**, *27*, 1343–1352.
4. Davis, T. M. *et al.* Melting of a DNA Hairpin Without Hyperchromism. *Biochem*. **1998**, *37(19)*, 6975–6978.
5. Savitzky, A.; Golay, M. J. E. Smoothing and Differentiation of Data by Simplified Least Squares Procedure. *Anal. Chem*. **1964**, *36*, 1627–39.

www.agilent.com

DE80572371

This information is subject to change without notice.

© Agilent Technologies, Inc. 2022

Printed in the USA, August 15, 2022
5994-0384EN

Agilent
Trusted Answers