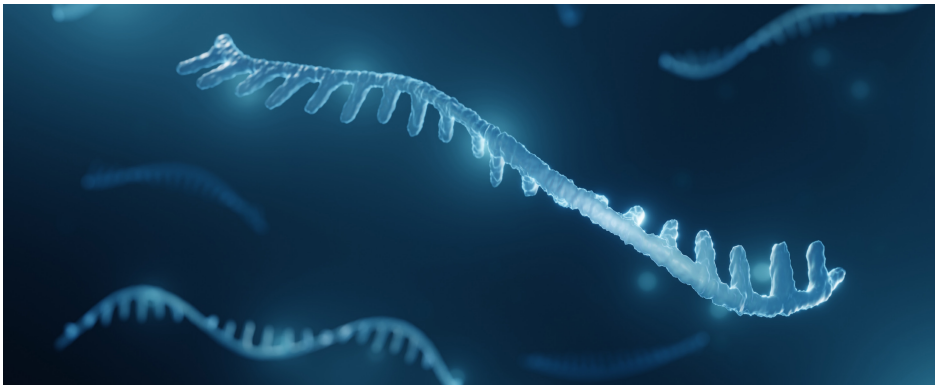
White Paper

Biopharmaceutical

Optimization of Thermal Stability Measurements for the Identification of Oligonucleotide APIs

Fast and precise thermal melt (T_m) measurements using the Agilent Cary 3500 UV-Vis spectrophotometer



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Introduction

To ensure drug safety, all starting materials of drug products must be subjected to analytical identity (ID) determination as part of the release, before their introduction into the pharmaceutical manufacturing process. This regulatory requirement also includes the active pharmaceutical ingredient (API) of drug products. Simple and quick to perform, yet highly specific analytical procedures are desired to confirm the ID. Oligonucleotides, which are a relatively new class of APIs, have created major challenges regarding ID determination. Commonly used and well-established methods such as chromatographic or electrophoretic methods are ideal for size confirmation; for sequence confirmation a technique that can distinguish base pair changes is needed.[1] Furthermore, these methods are less selective regarding short oligonucleotides, as similar oligonucleotides of the same length may result in similar HPLC chromatograms and retention times, irrespective of the oligonucleotide sequence.

Figure 1. The Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer.

However, melting temperatures (T_m) are highly oligonucleotide-sequence specific and determination of the T_m can be used for ID confirmation when combined with intact mass measurements.[1,2] Therefore, an ultraviolet (UV)-based spectrometric procedure that enables reliable and precise determination of oligonucleotide melting temperatures was implemented at Vetter using the Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer with multizone software add-on (Figure 1).

Melting temperatures of oligonucleotides

Double-stranded nucleic acids, including oligonucleotide duplexes, are thermodynamically stabilized by intermolecular, non-covalent hydrogen bonds (H-bonds) between complementary bases and by the stacking of the aromatic rings in the bases of nucleotides. By thermally denaturing (i.e., heating) an oligonucleotide duplex sample, its H-bonds and base-stacking interactions are dissolved, and its strands are separated (Figure 2). The exact temperature at which 50% of all duplexes are separated into single-stranded oligonucleotides corresponds to the melting temperature of the oligonucleotide and represents a measure of its stability.[3]

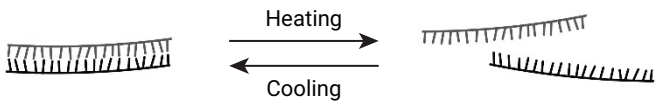

Figure 2. Schematic illustration of thermal oligonucleotide melting. The reversible and temperature-controlled process of association and dissociation of an oligonucleotide duplex into its complementary single strands is called "melting".

Despite the oligonucleotide length and overall base composition (proportions), the exact nucleotide order within the sequence characterizes its T_m. Additionally, chemical properties and modifications of oligonucleotides, ambient salt, and strand concentration can influence the melting temperature.[3]

Melting processes can be monitored and the T_m can be determined using UV spectroscopy, as the absorbance at 260 nm (A_{260}) of single-stranded oligonucleotides in solution is higher than the absorbance of duplex-oligonucleotides. By constantly measuring the absorbance of an oligonucleotide sample during its thermal denaturation, a melting curve can be plotted (Figure 3). Within this curve, the inflection point corresponds to the specific melting temperature of an oligonucleotide. The T_m can easily be determined by calculating the first derivative of the melting curve and identifying the maximum.[3]

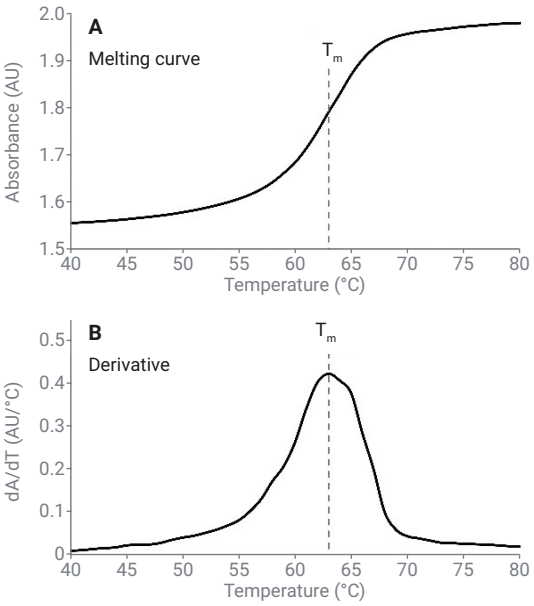

Figure 3. Melting curve and derivative. A typical oligonucleotide melting curve is obtained when the absorbance at 260 nm is plotted against the rising sample temperature (A). The maximum of the first derivative of the melting curve corresponds to the T_m (B).

Determination of T_m using the Cary 3500 UV-Vis spectrophotometer

Sample preparation

For T_m determination using a Cary 3500 Multicell Peltier UV-Vis spectrophotometer with multizone software add-on, oligonucleotide samples were prepared according to Table 1 and measured using a thermal method that was set up in the Agilent Cary UV Workstation software. The sample solutions were vortexed thoroughly and incubated for approximately 20 minutes at 2 to 8 °C before T_m measurements. A randomly chosen 20-mer oligonucleotide without modifications and its complementary strand were used for the method development experiments (Table 2). These high purity salt free (HPSF) oligonucleotide sequences were synthesized by Eurofins Genomics (Ebersberg, Germany) and reconstituted in 1x Tris-EDTA (TE) buffer (pH 8.0, Fisher Bioreagents, order no. BP2473) to obtain 100 µM oligonucleotide stock solutions.

Table 1. Composition of sample used for T_m measurements.

Component	Final Concentration
Oligonucleotide (100 µM)	4 µM
Complementary Oligonucleotide (100 µM)	4 µM
10x Tris-EDTA (TE) Buffer (pH 7.4) (Fisher Bioreagents, Order No. BP2477)	1x
2 M NaCl (Prepared from NaCl (Sigma-Aldrich, Order No. S3014)	0.1 M
Nuclease-Free Water (Invitrogen, Order No. AM9937)	Not applicable

Table 2. Sequence of the oligonucleotide used in this study.

Description	Sequence (5' → 3')
Oligonucleotide	CCA CGA CAG TAA CAT TCT CA
Complementary Oligonucleotide	TGA GAA TGT TAC TGT CGT GG

Method development and optimization

In all T_m determination experiments, baseline correction was performed using TE buffer in the reference beam path and used sample beam paths of the Cary 3500 Multicell Peltier UV-Vis spectrophotometer with multizone software add-on. The oligonucleotide samples were then measured against the TE buffer in the reference beam path. To identify the optimum instrument settings and experimental setup for the determination of T_m, several of the adjustable instrumental parameters were varied during method development (Table 3). In total, nine methods were used and compared to assess the ideal parameter settings (Table 3). After the measurements, the first derivatives and T_m were calculated by the Cary UV Workstation Software using a smoothing and derivative filter (smooth/derivative filter: 31, smooth/derivative interval: 0.1).

A good practice guide has been developed to provide more information about optimizing the experimental conditions for T_m measurements.

The mean melting temperatures obtained using methods 1 to 9 were comparable (Figure 4), with results between 60.7 °C (method 8) and 62.9 °C (method 6).

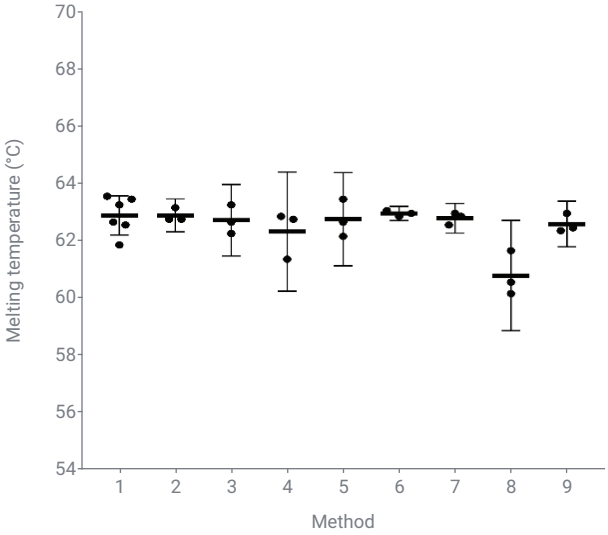

Figure 4. Measured T_m data obtained using methods 1 to 9. T_m determination was conducted in TE buffer containing 4 µM oligonucleotide, 4 µM complementary oligonucleotide, and 0.1 M NaCl. The graph shows mean T_m (center; n = 6 for method 1, n = 3 for methods 2 to 9), single T_m of replicas (dots) and 95% confidence intervals (bars).

Table 3. Methods used during method development.

Parameter [Setting Range]	Method								
	1	2	3	4	5	6	7	8	9
Ramp Rate (°C/min) [0.1 to 40.0]	10	10	10	10	10	20	1	10	10
Data Interval (°C) [0.1 to 10.0]	0.5	0.5	0.1	1.0	0.5	0.5	0.5	0.5	0.5
Averaging Time (s) [0.004 to 1,000.000]	1.0	0.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Spectral Bandwidth (nm) [0.10 to 5.00]	2	2	2	2	5	2	2	2	2
Cuvette Type [Semi-Micro/Micro/Macro]*	Semi-micro	Semi-micro	Semi-micro	Semi-micro	Semi-micro	Semi-micro	Semi-micro	Micro	Macro
In-Cuvette Stirring (rpm) [Off/400 to 1,400 rpm]	Off	Off	Off	Off	Off	Off	Off	Off	500
Temperature Probes [Yes/No]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Start Temperature (°C) [–5.0 to 110.0]	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
End Temperature (°C) [–5.0 to 110.0]	80.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0
Number of Stages [1 to 10]	1	1	1	1	1	1	1	1	1

* Volumes used: 0.8 mL (Semi-micro), 0.4 mL (Micro), and 2.0 mL (Macro).

Evaluation of the methods

A reliable method for the determination of T_m that can be used as part of the ID testing in a pharmaceutical environment requires high measurement precision. Measurement precision is quantified by calculating the confidence intervals (CI). The smaller a CI, the more precise a measurement is. Also, the time needed for a single measurement and general handling should be considered when choosing an appropriate method for ID testing.

Ramp rate

In methods 6 and 7, varying ramp rates were compared to the ramp rate of 10 °C/min (methods 1 to 5 and 8 to 9), which requires approximately 10 minutes measurement time. Applying a faster ramp rate of 20 °C/min (method 6) resulted in a run time of approximately 8 minutes. Considering that the ramp rate was twice as fast as the normal setting, the time difference seemed marginal. However, if the time required for a measurement is critical, method 6 could be used. T_m determinations with method 7 (ramp rate = 1 °C/min) took approximately one hour, without any analytical benefit. This shows the excellent accuracy level of the Cary 3500 UV-Vis. Using a faster ramp rate will shorten T_m experiments without sacrificing data quality.

Data interval

Methods 3 and 4 were used to evaluate the influence of the data interval. Comparing data intervals of 1.0 °C (method 4) and 0.1 °C (method 3) resulted in a smaller CI for the T_m measurement with method 3, i.e., a higher precision. However, a ramp rate of 10 °C/min seems to be too fast for a data interval of 0.1 °C. Therefore, these two methods were eliminated, and a mean value of the data interval (0.5 °C) was applied in all other methods.

Averaging time

In method 2, the alternative shortened signal averaging time (SAT) of 0.1 seconds did not lead to any beneficial effects compared to using a SAT of 1.0 seconds. The SAT determines how long the signal will be averaged before moving onto the next data point. In general, a longer SAT improves the signal-to-noise ratio (S/N) by decreasing background noise and smoothing the signal. The Cary 3500 UV-Vis has low noise, and high quality data can be obtained with short SAT.

Spectral bandwidth

The spectral bandwidth (SBW) can be adjusted to control the light throughput and thereby affect the SNR and spectral resolution. At a larger SBW, more light will pass through the sample, improving the SNR. In method 5, there were no obvious effects of increasing the SBW from 2 to 5 nm during the measurements or on the calculation of the T_m. Since 5 nm represents the upper limit of this parameter, the midrange setting of 2 nm was preferred.

Cuvette type and in-cuvette stirring (rpm)

From a practical point of view, method 8 seemed convenient, as very low sample volumes are needed when using microcuvettes (minimum sample volume of 0.4 mL). However, in the best practice guide, microcuvette usage is combined with block temperature control but not the thermal probes, as they have not been designed to fit in microcuvettes. If samples are available in low quantity, microcuvettes can be used with a slow ramp rate, e.g., < 0.5 °C, and block temperature control rather than probe temperature control.

Despite an acceptable CI, method 9 was excluded, as macro-cuvettes need significantly larger sample volumes (minimum sample volumes of 2.0 mL) than semi-micro cuvettes (minimum of 0.8 mL). If sample volume is not critical, the use of larger sample volumes is advised because in-cuvette stirring within macro-cuvettes ensures homogeneous temperature distribution of the sample solution.

Temperature probes

In all measurements, an offset of the sample temperature and the heating block temperature was observed (Figure 5). Inherently, in any system that heats a sample, there is a time delay. This delay is due to the heat transfer process from the block to the sample solutions through the quartz glass of the cuvette, which explains the observed temperature differences. Therefore, it is crucial to use in-cuvette temperature probes for accurate T_m measurements, at least when moderate to high temperature ramp rates are set (typically for ramp rates ≥ 0.5 °C).

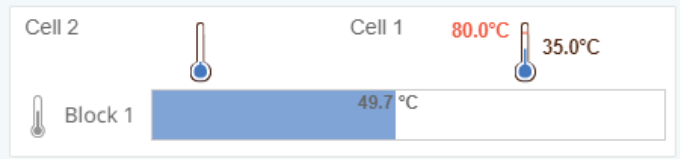

Figure 5. Example temperature information from the Agilent Cary UV Workstation software during T_m measurement. The software provides detailed information about the system temperature control, including the monitoring of the Peltier block and sample temperatures. The screenshot shows the actual temperatures of the heating block (49.7 °C), sample solution (35.0 °C), and the set temperature (80.0 °C) using the Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer with multizone software add-on and method 1.

Based on a process of elimination, methods 1 and 9 were judged to be the most appropriate and favored methods for reliable T_m determination of oligonucleotide samples. In summary, these methods exhibited good overall precision and fast measurements due to the setting of the ramp rate. One more advantage of method 1 is the use of small sample volumes due to the semi-micro cells. An exemplary melting curve recorded using method 1 and the corresponding smoothed derivative are shown in Figure 6.

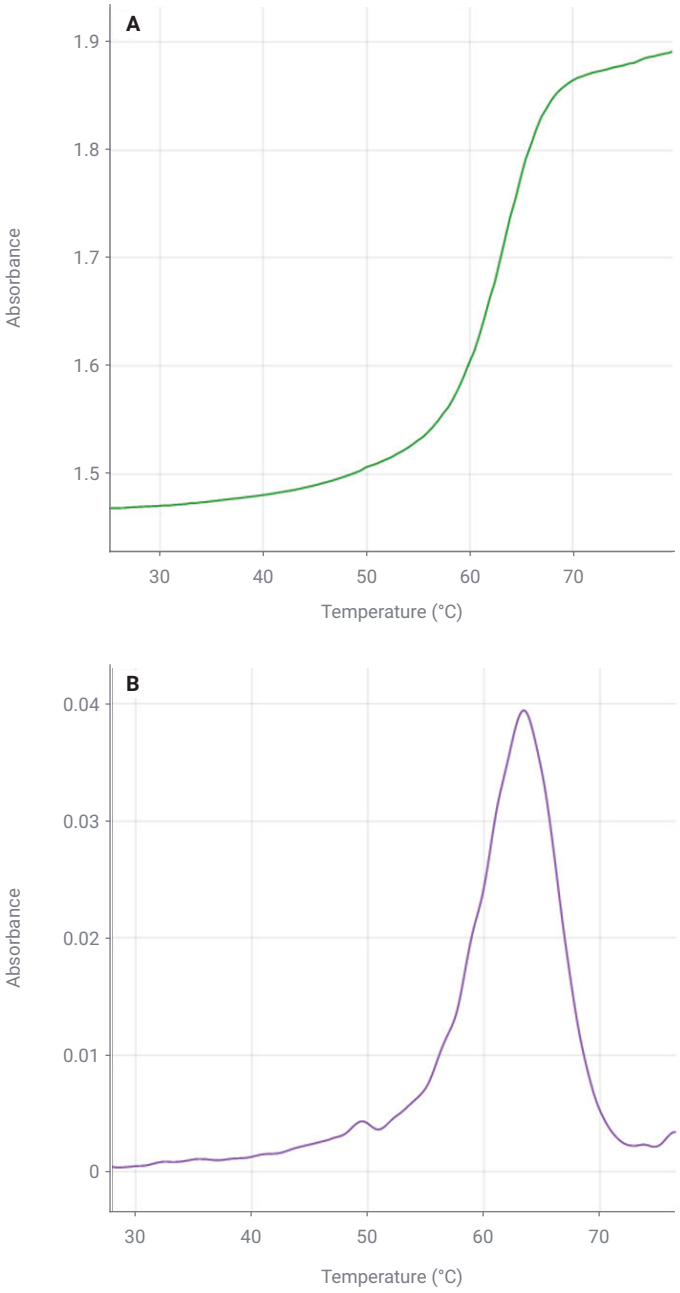

Figure 6. (A) Exemplary melting curve of method 1. (B) Smoothed first derivative plot.

Smoothing used in T_m determination

The Cary UV Workstation software offers the option to apply smoothing and derivative features on melting curves to reduce interference and noise. Smoothing reduces the noise that could be present in the raw data. The Cary UV-Vis Workstation smoothing algorithm uses the Savitzky-Golay technique.[5] The derivative calculation also uses the Savitzky-Golay technique.

Filter and interval size values for smoothing and the derivative can be set manually before T_m calculation. The filter size value defines the number of data points that are used to generate each output point. Larger filter sizes lead to reduced output. The Savitzky-Golay technique requires a uniform interval. The data will be set to the specified interval before performing the smoothing and derivative calculation. Typically, the entered interval should match the data collection interval. The two calculations can be used independently or sequentially, i.e., smoothing followed by a derivative.[4]

Figure 7 shows exemplary first derivatives from method 5. Even if the raw data of the melting curves present low levels of noise (melting curves not shown), the first derivative plots show higher levels of noise if the smoothing and derivative steps are not optimized. For example, Figure 7A represents a plot of method 5 without smoothing and an unoptimized first derivative (derivative interval 0.5 °C, filter 5). The T_m is 62.5 °C. Figure 7B shows the same data with applied smoothing and derivative functions using optimized parameters (smoothing and derivative interval 0.1 °C, filter 31). The T_m is comparable (62.6°C), but the plot is less noisy.

Unoptimized smoothing and derivative data processing will potentially create noisy curves and artifacts, as shown in Figure 7. Using a filter size that is too large will eventually over smooth the plots and reduce the output. It is quick and easy to optimize the smoothing and derivative parameters using the Cary UV Workstation software. Once the parameters have been optimized, they can be saved as part of a method and applied automatically at the end of a measurement.

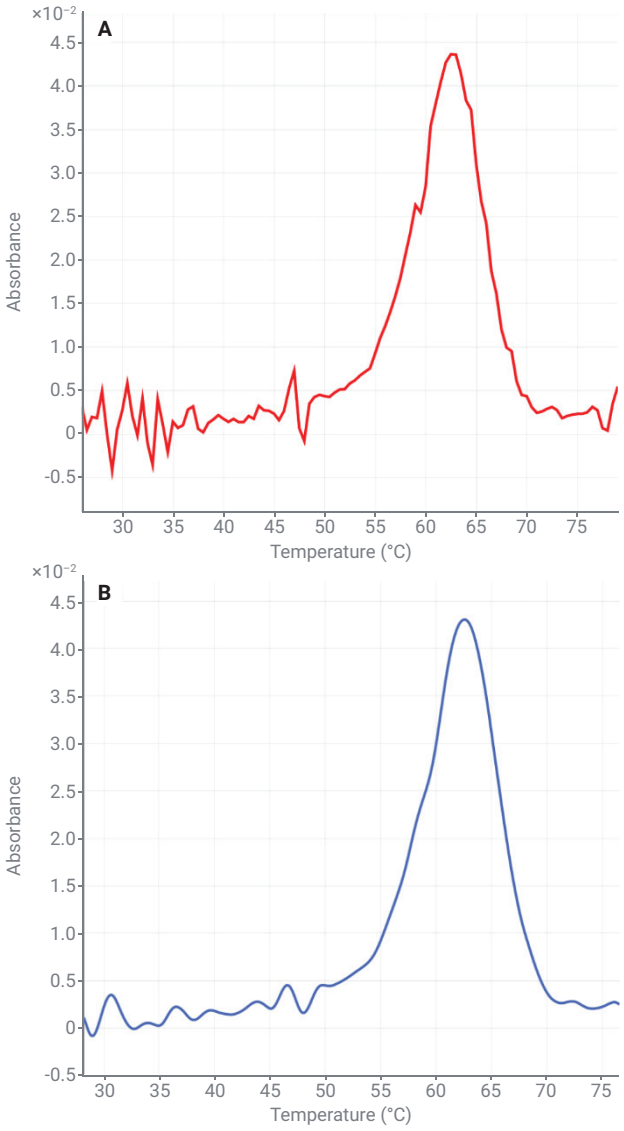

Figure 7. (A) Method 5: first derivative plot without smoothing and unoptimized derivative parameters (derivative interval 0.5 °C, filter 5). (B) First derivative plot with optimized smoothing and derivative parameters (smoothing and derivative interval 0.1 °C, filter 31).

Conclusion

After a thorough evaluation of instrument parameters, a reliable and precise UV-based spectrometric method for the simple and fast determination of oligonucleotide melting temperatures was implemented at Vetter using the Agilent Cary 3500 Multicell Peltier UV-Vis spectrophotometer with multizone software add-on. The Agilent Cary UV Workstation software offers built in smoothing and derivative features that enable optimization of melting curve analysis, improving the evaluation of oligonucleotide melting temperatures.

References

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Further information

- Cary 3500 Multicell UV-Vis Spectrophotometer
- Cary UV Workstation software
- Data Integrity Options for GMP Facilities for the Agilent Cary 3500 UV-Vis Spectrophotometer Series
- UV-Vis Spectroscopy & Spectrophotometer FAQs

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