White Paper

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Best Practice for Nucleic Acid Thermal Stability Measurements Using the Cary 3500 UV-Vis Spectrophotometer

Thermal melt (T_m) analysis using rapid, precise temperature-dependent UV-Vis absorbance measurements

The Cary 3500 Peltier UV-Vis systems offer the ability to analyze the thermal stability and profiles of biological and other molecules using temperature-controlled UV-Vis spectroscopy.

UV-Vis spectrophotometers have been used widely for nucleic acid quantification and quality control (QC) utilizing the fact that nucleic acids have a maximum absorbance at 260 nm (*1*). The concentration of nucleic acids can be easily estimated using the absorbance at 260 nm and the established absorption coefficient. Often a background correction is also performed, for example collecting a baseline using a solution containing everything but the nucleic acid or by measuring the absorbance at a wavelength that nucleic acids do not absorb.

Double stranded nucleic acids are bound by hydrogen bonds between the base pairs. The temperature at which double stranded nucleic acids denature to become single stranded depends on the:

- sequence and length of the nucleic acid
- the pH and buffer conditions
- and any mismatches in base pairs between the two strands

As such, the melting temperature is very useful analytical tool and can be studied by monitoring the absorbance at 260 nm as temperature is increased or decreased. As the temperature is increased, the hydrogen bonds between the strands are broken and the double stranded nucleic acid separates into two separate strands. When the strands separate, the absorbance at 260 nm increases. The transition temperature is called “melting temperature” (T_m) (*1*).

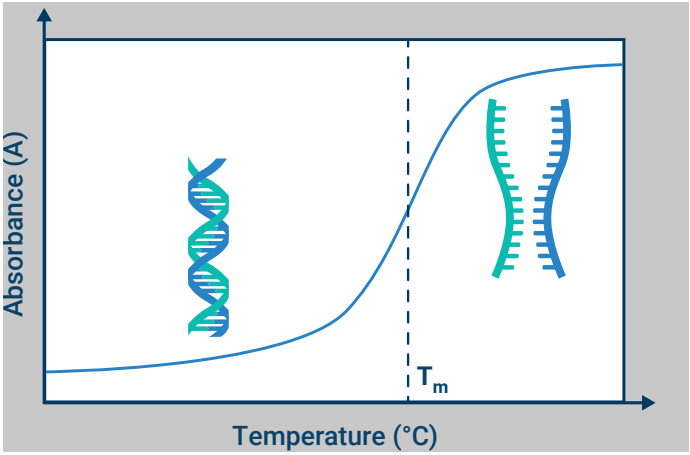


Figure 1. The plot represents a melting curve of a DNA sample.

The melting profile of other molecules/compounds can also be analyzed using UV-Vis spectroscopy. Some examples include polymer cloud-point determination, drug-protein interactions, and protein thermal denaturation.

This document describes how to optimize experimental conditions for thermal melt measurements to achieve high-quality data with confidence. Here are our top tips:

Use the right cuvette

The choice of cuvette will depend on the volume of sample to be analyzed, the z-height of the UV-Vis spectrophotometer[1] and the wavelengths used. The most widely used cuvette in UV-Vis spectroscopy has a pathlength of 10 mm and volume of 3.5 mL.

Thermal melt measurements are typically done at UV wavelengths and therefore the cuvette needs to transmit UV light. Quartz cuvettes are recommended for this reason. Polymethyl methacrylate (PMMA), polystyrene, and normal glass cuvettes should be avoided as these cuvettes are only transparent in the visible wavelength range.

While taking measurements, the cuvette should be placed in the cuvette holder such that the transparent sides are aligned with the beam path. If only a small amount of sample is available, consider a smaller volume cuvette to ensure that the sample is in the beam path (see Table 1).

The Cary 3500 system is ideal for measuring small volume nucleic acid samples as the instrument has a highly focused beam of less than 1.5 mm width (see Figure 2). The instrument's factory-aligned optics require no adjustment before taking measurements, even when measuring multiple cuvettes at the same time.

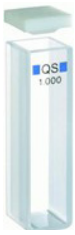
Figure 2. The highly focused beam of the Cary 3500.

If measuring more than one sample at a time, it is recommended to use the same type of cuvette in all the cuvette positions. Heat transfer from the Peltier heating elements to the sample depends on the physical characteristics of the cuvette, so using different cuvette types can introduce an unwanted variable.

Table 1 describes the different cuvette options.

[1]*The z-height is the distance from the base of the cuvette to the middle of the light beam.*

Table 1. Cuvette types that can be used with the Cary 3500 instrument. The Agilent part number for each cuvette is shown in column 1, e.g., 5061-3387. The Cary 3500 can control the temperature of samples inside cuvettes using either an in-cuvette temperature probe (indicated with 'Probe' in the Temperature Control column) or by relying on the temperature of the cuvette holder in which the cuvettes sit (indicated with 'Block'). If using cuvettes that cannot be fitted with a temperature probe, the maximum temperature ramp rate should be set to ≤ 0.5 °C/min.

Cuvette type		Nominal volume	Minimum volume	Temperature control	Stirrer can be inserted
10 mm quartz cuvette (5061-3387)		3.5 mL	With stirrer: 1.5 mL Without stirrer: 2.0 mL	Block	Yes
10 mm quartz cuvette (5061-3387), with temperature probe (G9889-60005)		3.5 mL	With stirrer: 1.5 mL Without stirrer: 2.0 mL	Probe	Yes
10 mm quartz cuvette (5062-2477)		3.0 mL	2.0 mL	Block	No
Semi micro cell (5063-6559) with temperature probe (G9889-60005)		1.0 mL	400 µL	Probe	No
Semi micro cell with PTFE stopper (5063-6561)		1.0 mL	800 µL	Block	No
Rectangular semi-micro cell, pair, with PTFE stopper (6610012700)		900 µL	400 µL	Probe	No
Ultra micro cell with PTFE stopper (5062-2496)		50 µL	50 µL	Block	No
Low Head Space cell (Starna cuvette catalog[2])		10 to 160 µL	*refer to* Starna cuvette catalog	Block	No

[2]*https://www.starna.com/images/cell_cat_2014_v22042014.pdf*

Keep cuvettes clean

It is important that cuvettes are clean and free from scratches. The cuvette cleaning process should prevent contamination and air bubble formation. An alkaline cleaning solution[3] should be used. Both cuvettes and stoppers should be washed, rinsed (preferably with the solvent or buffer used for the analysis), and air dried.

Optimize buffer and pH

To ensure reliable and reproducible analysis, as well as minimize variabilities of measurements, biological samples need to be dissolved in a suitable buffer with the right pH and salt content. Buffer and pH optimization studies are recommended during any biological sample analysis (*2, 3*).

Optimum buffer conditions should be checked at different temperatures (*2*). The same buffer type should be used for blanks and samples. Freshly prepared buffer is recommended.

Avoid condensation on cuvettes

Condensation on the outside of the cuvette can occur:

- When decreasing the temperature of a sample below ambient (i.e., room temperature), there is a risk of condensation forming on the outside of the cuvette and interfering with the measurement.
- When biological samples/buffers, which are often stored at low temperatures, are placed directly into a cuvette at room temperature.

To prevent condensation, ensure that the sample and cuvette are at room temperature when preparing the sample for measurement. During the measurement, if temperatures below ambient are required, purge the sample compartment with nitrogen gas to remove any condensation that forms. See the user guide of the Cary 3500 UV-Vis spectrophotometer for purging instructions[4].

Figure 3 presents examples of a melting experiment in presence of condensation. The plot shows noisy traces and absorbance variations not related to the sample.

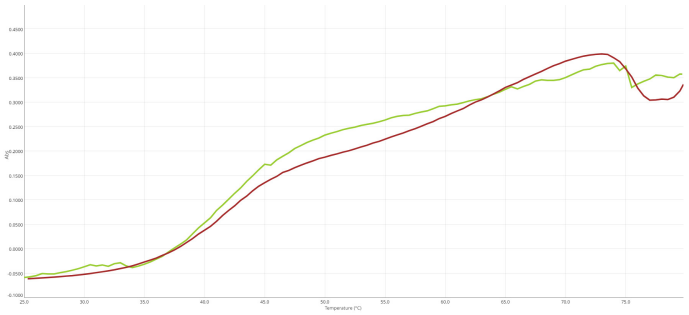


Figure 3. Two examples of melting curve absorbance artefacts caused by condensation on the cuvette wall.

Optimize the sample concentration

If a sample is very concentrated, dilution may be needed to keep the measurement within the linear range of the spectrophotometer. Before starting a thermal denaturation experiment, perform a wavelength scan of the sample to ensure an appropriate concentration and check the quality of the sample. A good starting absorbance for nucleic acids is 0.5 Abs at 260 nm. Th absorbance will increase by approximately 15% to 20% as the sample denatures (*1*).

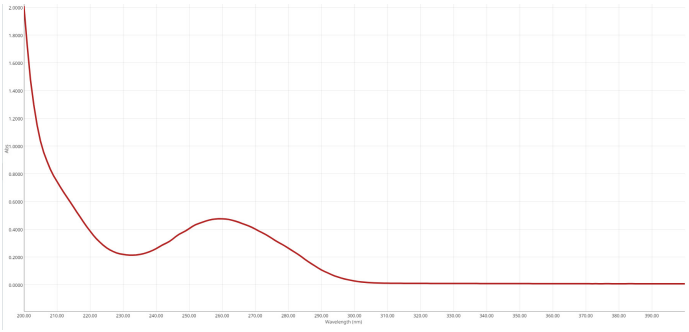


Figure 4. A wavelength scan of a DNA sample showing an absorbance level around 0.5 Abs at 260 nm.

[3]*Cell Cleaning Supplies for UV-Vis & UV-Vis-NIR – Agilent part numbers: 5190-0530 and 9300-0761*
[4]*Cary 3500 UV-Vis purge kit – Agilent part number: G9864-68000*

Prevent sample evaporation

As the temperature increases during a denaturation experiment, sample evaporation could occur. As the buffer evaporates, the concentration of nucleic acid in the remaining solution will increase, resulting in the absorbance increasing over time, as shown in Figure 5.

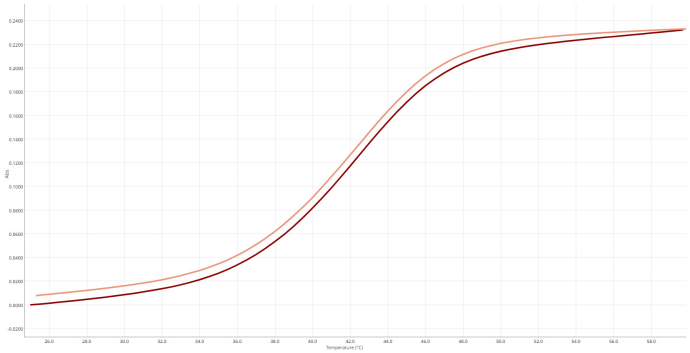


Figure 5. Absorbance at 260 nm as the temperature ramps up (dark red) and ramps down (amber). The absorbance level for the ramp down is slightly higher than the ramp up at the same temperature due to evaporation increasing the concentration.

In addition, highly concentrated samples that evaporate during the measurement may cause salt deposition on the wall of the cuvette. After measuring a sample, check that no droplets are sticking to the wall of the cuvette. Droplets are a sign that evaporation has occurred. Use the following approaches to prevent evaporation:

- Always use stoppered cuvettes.
- Add a few drops of mineral oil (Agilent part number FS-SMO15) to float on top of the sample in the cuvette. Using a different oil type, for example silicone oil, is not recommended.
- Use a low head space cuvette to reduce the dead space between the sample surface and the bottom of the stopper (as listed in Table 1)
- Place Parafilm between the top of the cuvette and its stopper.

Prevent contamination

To avoid sample contamination from nucleases or other particles found on human skin, wearing gloves and a face mask is recommended.

Measure replicates

To ensure measurement accuracy, replicate samples, or replicate measurements of the same sample, are recommended.

Degas samples

Samples will naturally degas when heated. This process will generate bubbles that perturb the light beam going through the sample, as shown in Figure 6. The problem is more likely to occur when the temperature used during thermal melt experiments is close to the solvent boiling point.

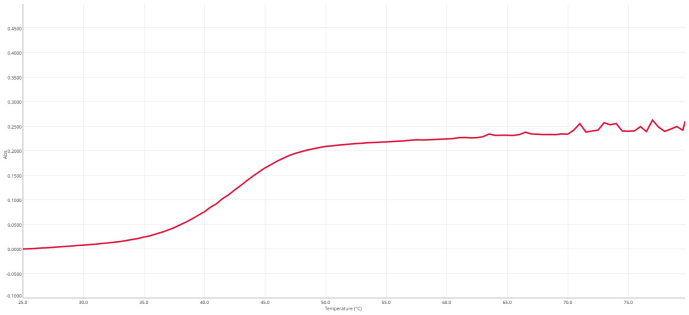
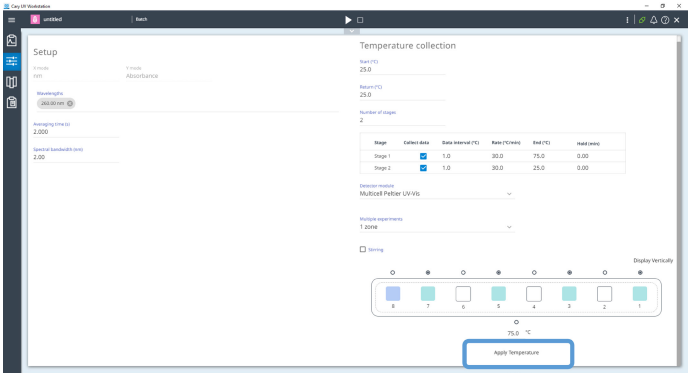


Figure 6. Melting curve showing spikes at high temperatures due to the sample degassing

To reduce this phenomenon, it is recommended to preheat the sample or degas the sample under vacuum at room temperature if the sample is prone to degassing with temperature (*3, 4*). There are different ways to apply the preheating step:

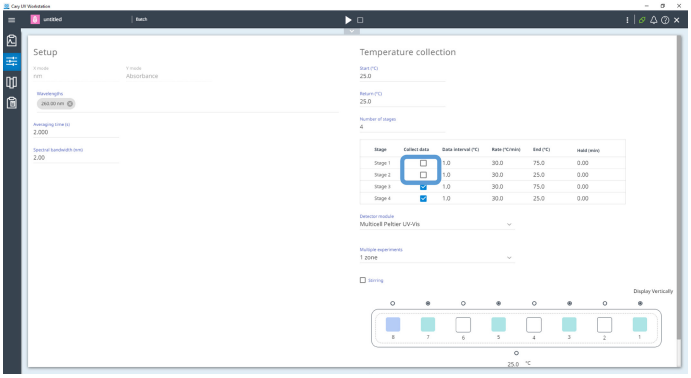
Degassing option 1

Hold samples at the upper temperature of the experiment for five minutes before starting data collection. Use the Apply Temperature control (highlighted in the screen, following) in the software to do this.



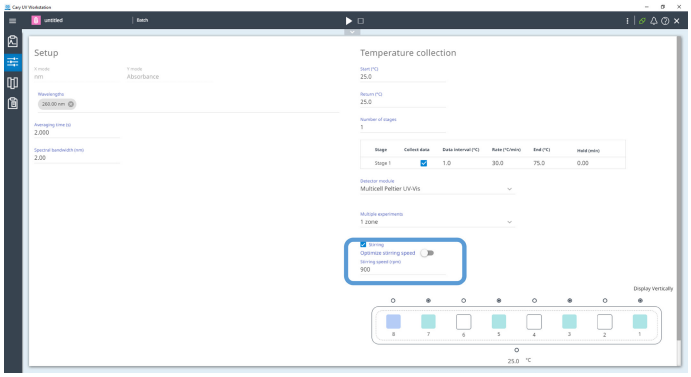
Degassing option 2

Ramp the temperature of the sample up and down, using the maximum temperature settings that will be used for the measurement with “Collect data” boxes unchecked. (highlighted in the screen following). The measurement will be done during the following stages with the “Collect data” boxes checked.



Remove bubbles

Stir speeds of approximately 900 rpm yield the best temperature uniformity within standard 3.5 mL cuvettes that are filled with liquid of similar viscosity to water. Star stirrers (Agilent part number 7418000400) are recommended for standard cuvettes (Agilent part number 5061-3387). Rod type stirrers are not recommended as they can create and/or trap air bubbles. Air bubbles can interfere with the light passing through the cuvette and sample and create spikes or other abnormalities in the UV-Vis measurement. As the sample is stirred, the bubbles may move around in the cuvette, passing in and out of the light path. To remove air bubbles on the stirrer, it is recommended to stir the sample before starting the measurement and visually inspect the cuvette to ensure no bubbles remain. With the Cary 3500, this can be done using the “optimize stirring speed” option in the Cary UV Workstation software that turns on the stirring before data collection (see the screen capture, following).



Set stirring speed to suit sample viscosity

Stirring speed should be increased when measuring more viscous samples. Often, bubbles accumulate on the inner surface of the cuvettes, as dissolved gases have less solubility in high temperatures. To ensure accurate measurements, tap the base of the cuvettes gently on the benchtop with the caps on. This action should remove bubbles.

Stirring is also helpful during the annealing process. Stirring will increase the interaction of single stranded nucleotides for reannealing. If samples are not stirred, particularly when using standard cuvettes (which have a relatively large volume), the melting temperature during the ramp up could appear at a higher temperature than for the ramp down. This is due to the slow diffusion of single stranded nucleotides through the cooler solution.

Understand temperature-based measurement parameters

The Cary 3500 UV-Vis spectrophotometer is available in three different thermally controlled options:

- Cary 3500 Compact Peltier
- Cary 3500 Multicell Peltier
- Cary 3500 Multizone

The Cary 3500 Compact Peltier is designed around a single Peltier block for 2 cuvette positions (one reference channel and one sample channel). The temperature can be controlled either from the sample inside the cuvette using the temperature probe or by relying on the temperature sensor of the cuvette holder in which the cuvette sits. The radio button under the different channels represents the Peltier block sensor. The radio buttons above the channels represent the positions where a temperature probe can be connected.

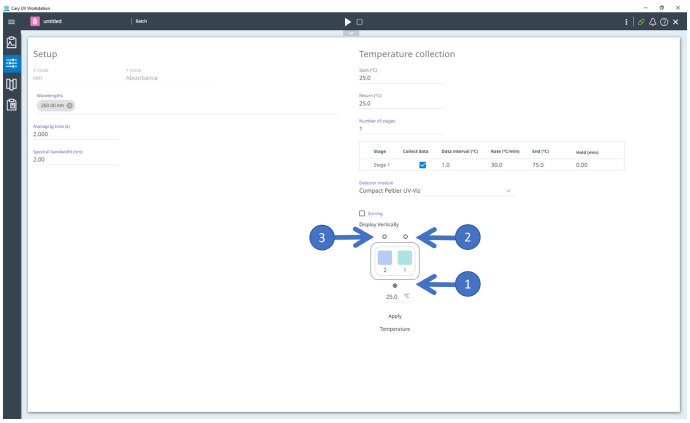


Figure 7. The Cary UV Workstation Thermal application interface for the Cary 3500 Compact Peltier. The temperature control mode is represented by the radio button selected. If the button labelled (1) is selected then the temperature is controlled by the Peltier block thermal sensor control. If the button labelled (2) is selected then the temperature is controlled through the sample channel. If the button labelled (3) is selected then the temperature is controlled through the reference channel.

The Cary 3500 Multicell Peltier is built with 4 independent Peltier blocks for a total of seven sample channels and one reference channel. Each pair of cuvettes (cuvettes 1 and 2, cuvettes 3 and 4, cuvettes 5 and 6, and cuvettes 7 and 8) is positioned in its own independent Peltier block. The temperature is controlled exclusively with the thermal sensors for each Peltier block. A thermal probe can be used in position 8 only to read the temperature for that position. It doesn't control the temperature of the system.

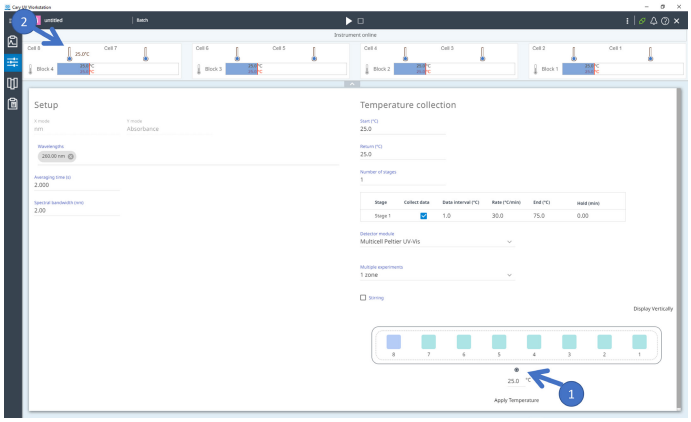


Figure 8. The Cary UV Workstation Thermal application interface for the Cary 3500 Multicell Peltier. The temperature control mode is represented by the selected radio button, here (1) is selected, and represents the Peltier block thermal sensor control for each Peltier block. (2) represents the reading from a temperature probe connected to the reference channel.

The Cary 3500 Multizone software Add-on enables direct monitoring of the sample temperature. This system is also built with 4 independent Peltier blocks like the Cary 3500 Multicell Peltier configuration with the additional ability to control each individual Peltier block by a temperature probe.

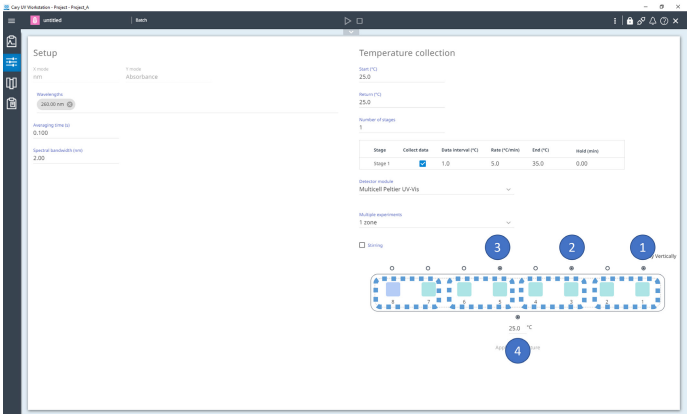


Figure 9. The Cary UV Workstation Thermal application interface for the Cary 3500 Multizone. Each independent Peltier block is represented with a blue dashed box and each of them is driven either by a temperature probe or the Peltier block thermal sensor. From right to left, the first three Peltier blocks (1,2,3) are monitored by a temperature probe represented with selected radio buttons. The Peltier block on the far left has no temperature probe selected (unselected radio buttons), it means it is driven by the Peltier block thermal sensor (4) represented by the selected radio button.

Note: Each of the four Peltier blocks can be controlled by a single source i.e., the Peltier block inbuilt temperature sensor, or a temperature probe connected to one of the two positions within a Peltier block. Two temperature probes cannot be connected on the same Peltier block.

The Cary 3500 can be used for static temperature experiments i.e., kinetic applications, or for ramping temperature experiments. For static temperature experiments, the Multizone software Add-on will enable the cuvette pairs to be set at different temperatures simultaneously. Setting the instrument to use two or four zones in this way could be relevant if different buffers are used for different experiments, each at a different static temperature. These experiments can be run simultaneously.

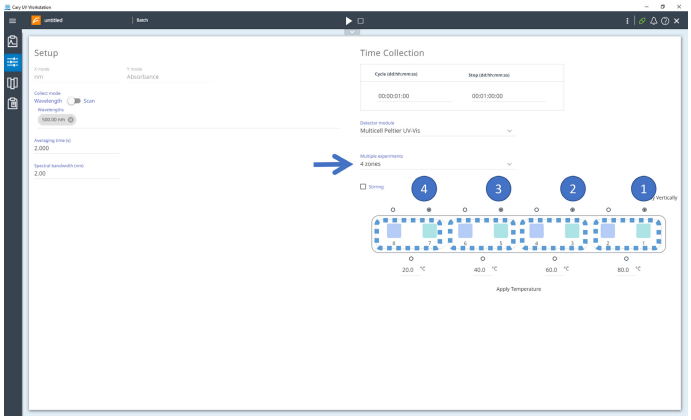


Figure 10. The Cary UV Workstation Kinetic application interface for the Cary 3500 Multizone. The system has been set up to use 4 independent zones (blue arrow). A kinetic experiment will be run simultaneously with four zones at different temperatures (1,2,3 and 4).

For ramping temperature experiments using the Cary 3500 Multizone, the temperature rate can be set between 0.1 to 40 °C/min. The temperature of all independent Peltier blocks will be driven at the same temperature rate. For the best accuracy and precision, use a temperature probe inserted directly into the sample and use this probe to control the temperature change (this functionality is available on the Cary 3500 Compact Peltier and Cary 3500 Multizone. The Cary 3500 Multicell Peltier doesn't offer temperature controlled with probes, it controls the temperature of the cuvette holder in which the cuvettes sit). Temperature probes provide the most accurate temperature measurement as they are monitoring the temperature of the sample close to where the measurement is being taken.

Figure 11. Cary in-cuvette temperature probe has a low mass, large surface area, and a super fast feedback loop.

Note: When controlling the sample temperature in a Cary 3500 Multizone using temperature probes with a fast ramp rate, it is recommended to place the sample where a temperature probe will be used. Leave the other position on the same Peltier block empty. The figure, following, shows how to set up samples in positions 1, 3, 5 and 7 and how to turn off the positions 2, 4 and 6. Hover the mouse over each cuvette position and select either Sample or Unused.

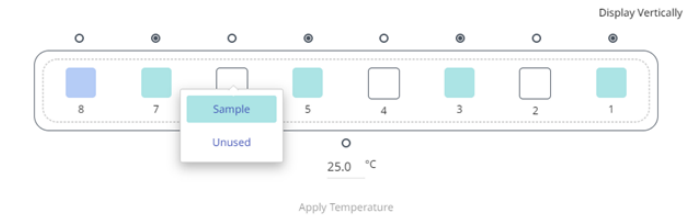


Figure 12. The Cary 3500 multizone interface. Temperature probes are in positions 1, 3, 5 and 7 and represented by the selected radio buttons. Positions 2, 4, and 6 are set to “unused”.

Note: In the Cary 3500 multicell/multizone configuration all sample positions are measured simultaneously. This is different to a typical UV-Vis spectrophotometer motorized multicell accessory that will sequentially measure each sample and risk missing vital data points between readings.

Alternatively, the temperature of the Peltier block can be used to control the temperature change. In this case, test experiments are recommended to find the optimum ramp rate for a given sample. Generally, using a slower temperature change rate (e.g., 0.1 to 0.5 °C/min) will yield higher accuracy for thermal melting experiments. A slower rate of temperature change will reduce the lag in temperature between the sample in the cuvette and the Peltier block in which the cuvette sits in.

Absorbance readings will be taken at the data interval defined in the software. If a large shift in absorbance is observed between different temperatures, a small data interval is recommended so that more data points are captured across the temperature change.

Up to 10 stages can be programmed in a thermal melt experiment with a Cary 3500 instrument. Each stage includes:

1. An end temperature
2. The data interval
3. The temperature change rate
4. The hold time.

The hold time determines how long the sample will be held at the end temperature before starting the next stage. If a long hold time is used, sample evaporation must be considered. See "Prevent sample evaporation".

Ramping the temperature down too quickly may result in incomplete annealing due to the time-based nature of the re-annealing process (*1*).

Understand instrument parameter settings

The software interface of the Cary 3500 thermally controlled configurations allows control of multiple parameters, including:

- Analysis wavelengths
- Signal averaging time
- Spectral bandwidth
- Start and end temperatures
- Number of temperature stages
- Number of temperature zones (depending on the instrument type)
- Stirring
- Temperature control method

These parameters are shown in the image (right) and more detail is provided in Table 2.

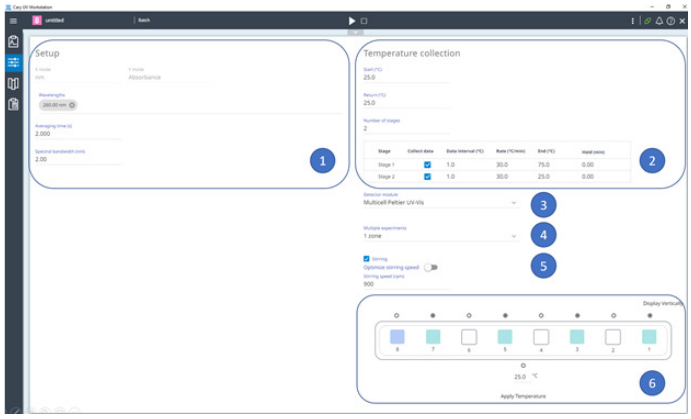
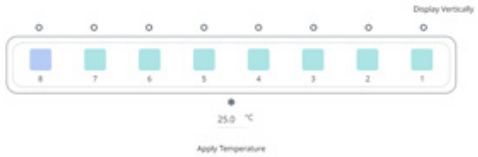
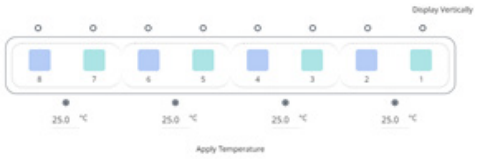


Table 2. Cary UV Workstation Thermal application parameters and settings.

Number	Parameter description
1.	Choose the analysis wavelength(s), signal averaging time, and spectral bandwidth. See Table 3.
2.	Define the thermal parameters: start and end temperatures and the number of stages. Each stage is defined by the data interval, ramp rate, the end temperature, and the hold time. See "Understand temperature-based measurement parameters"
3.	Select the instrument module, either a Compact Peltier or Multicell Peltier.
4.	For Cary 3500 Multizone only, define the number of temperature zones (the temperature of all the zones will be driven at the same rate). The system can use a single zone, i.e., one reference channel with seven samples. Or the system can be split into two zones (two pairs of one reference channel with three samples) or four zones (four pairs of one reference channel with one sample channel). The reference position is shown in blue in the diagrams, following  One zone configuration  Two zone configuration  Four zone configuration
5.	Select stirring capabilities, if necessary. See "Set stirring speed to suit sample viscosity"
6.	Define the temperature control method, either temperature probe or block. The selected radio buttons in the images, preceding, represent the Peltier blocks being selected. See "Select which temperature reading to use"

Optimize instrument parameters

When performing thermal melts using UV-Vis spectroscopy, the following parameters should be optimized as summarized in Table 3.

Table 3. Thermal melt experiments recommended parameters.

Parameter	Recommendation	How to Optimize
Spectral bandwidth (SBW)[5]	1 to 2 nm	If a higher signal throughput is needed, increase the spectral bandwidth, as a wider setting increases the amount of light through the sample. As SBW increases beyond a certain value (typically around 2 nm), separation of wavelengths is less well defined and the absorbance at the maxima may decrease. It is a good idea to experiment with different spectral bandwidth settings to determine the optimum setting for a given sample. 1-2 nm is ideal for most biological samples.
Signal averaging time (SAT)	>2 seconds	This setting determines how long the signal will be averaged before moving onto the next data point. In general, a longer SAT improves signal-to-noise ratio (SNR) by decreasing background noise and smoothing the signal. For thermal melt experiments, an averaging time of 2 to 3 seconds is recommended as a good starting point. This parameter should be optimized in parallel with the ramp rate.
Ramp rate	Block control: ≤ 0.5 °C/min Probe control: ≤ 40 °C/min	This setting represents how fast the system will ramp for a given stage. Higher the ramp rate is faster the system will reach to the stage end temperature.
Data interval	≤ 1 °C	This parameter defines how often data will be collected by the system. For example a data interval of 1 °C means a data point is collected every degree. Smaller data intervals tend to provide better result quality. A simple melting experiment can use a single stage to either ramp up or down. More sophisticated melting experiments can be performed in multiple stages. For example, a ramp up will be split into 3 stages: 1 stage for the plateau before the transition, 1 stage for the transition, and 1 last stage for the second plateau. The plateaus before and after the transition could use a high data interval as the absorbance changes slowly. During the transition, the data interval should be smaller as the absorbance changes quickly.
Probe control: temperature feedback from the sample	Available with the Multizone or Compact Peltier	Using temperature probe control will ensure an instantaneous reading of the temperature from inside the cuvette. See "Select which temperature reading to use".
Block control: temperature feedback from the Peltier	Available with all Peltier modules	If using the temperature reported by the Peltier block the cuvette sits in, use a slow temperature ramp rate. This will ensure that the heat transfer through the cuvette is sufficient to ensure sample temperature equilibrium at the desired temperature. It is recommended ≤ 0.5 °C/min. For larger sample volumes, the temperature ramp rate will need to be optimized to ensure thermal equilibrium between the Peltier block and the sample. Stirring will help achieve temperature equilibrium more quickly.
Stirring	Available when using 3.5 mL cuvettes	The stirring feature of the Cary 3500 is designed for star-type stirrers (diameter = 9.5 mm and height = 9.5 mm). Stirring helps achieve good temperature equilibrium in a sample.

[5] https://www.agilent.com/cs/library/posters/public/Optimum_Parameters_Poster_38x25_5994-0399EN.pdf

Select which temperature reading to use

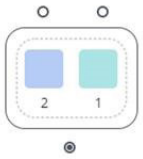
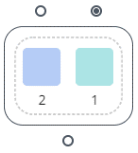
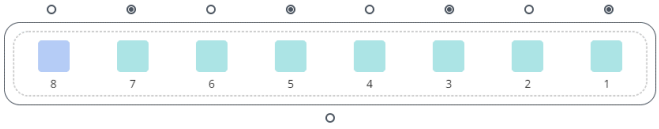
The temperature of the Cary 3500 Multizone can be controlled using readings from either:

- The Peltier block thermal sensor where cuvettes sit in
- Temperature probes.

When using the Peltier block to control the experiment, the temperature of the sample may lag the 'block' temperature, particularly when using fast temperature ramp rates.

Table 4 describes how to set up the temperature control mode within the software for these three different options.

Table 4. Software temperature control options for the three different Cary 3500 options

System Option	Temperature Control Option
Cary 3500 Compact Peltier	 Peltier block control
	 Temperature probe control in position 1
Cary 3500 Multicell Peltier	 Peltier block control
Cary 3500 Multizone	 Peltier block control for each Peltier block
	 Temperature probe control for each Peltier block respectively in position 1, 3, 5, and 7

Insert the temperature probe to the right depth

Figure 13 shows the main parts of the Cary temperature probe and how it sits against the cuvette wall to avoid clipping the light beam.

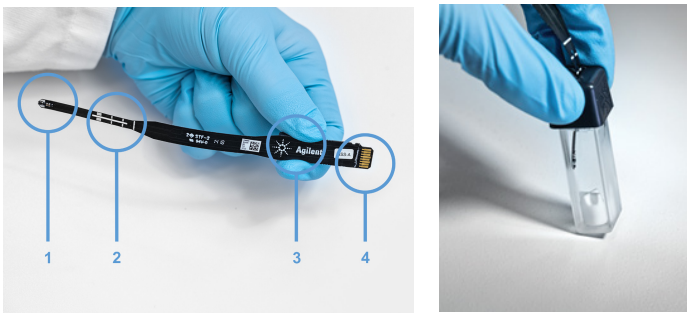


Figure 13. (Left) Cary temperature probe - (1) sensor, (2) height guide, (3) grip, and (4) connector. (Right) The temperature probe is fitted in a cuvette, with a star stirrer in the bottom of the cuvette.

The temperature probe height can be adjusted by gently sliding the probe through the slot in the cap. The recommended temperature probe depth with a 3.5 mL cuvette is when the position 2 marker on the temperature probe just appears at top of the cuvette cap (see Figure 14). This depth positions the tip of the probe at a position next to the center of the light beam. The ribbon-like probe has been designed to sit against the inner side wall of the cuvette. This position means that the probe does not block the light beam passing through the cuvette. Positioning the temperature probe this way ensures that the solution measured by the temperature probe is as close as possible to the solution where the light beam is passing through. This design delivers highly accurate results.

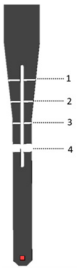


Figure 14. Temperature probe diagram representing the different positions.

Figure 15 shows how the temperature probe connects to the instrument.

Figure 15. The temperature probe connector plugs into the instrument.

The recommended minimum temperature probe submersion depth is 5 mm below the surface of the liquid, regardless of the sample volume. If a stirrer is used, maintain a minimum distance of 3 mm between the bottom of the probe and top of the stirrer. With a stirrer inside a 3.5 mL cuvette, do not place the temperature probe lower than position 2 on the probe depth guide. This position ensures that the probe does not interfere with the stirrer. Stirring at 900 rpm produces the best temperature uniformity throughout the cuvette.

Calculate the melting temperature

Smoothing is an optional feature that can be applied to a melting curve to reduce interference and noise before calculating the derivative of the curve. The smoothing and derivative calculations use the Savitzky-Golay technique. Both functions require two parameters: filter value and an interval value.

The filter value defines the number of points that are used to generate each point in the smoothed curve. The Savitzky-Golay algorithm requires an odd number as the filter value. Larger filter sizes lead to less data points in the curve. The Savitzky-Golay algorithm also requires a data interval to be specified. It is recommended to use the same interval as the data collection interval used to collect the curve.

The lower and upper limit fields (labelled 3 in Figure 16) in the Cary UV Workstation Thermal application are used to specify the temperature range over which the melting temperature calculation is performed. Clicking the recalculate button in the software will generate a thermal calculation table, the T_m value, and the derivative trace of the melting curve.

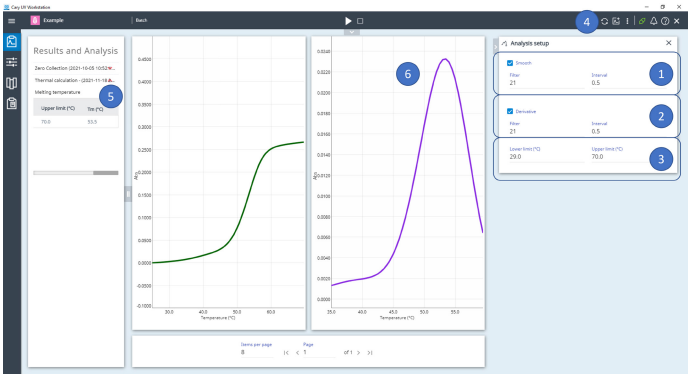


Figure 16. The Cary UV Workstation Thermal application results page with the smoothing parameters (1), the derivative function parameters (2) and the melting temperature calculation limits (3). (4) is the recalculate button. The calculation results in the thermal calculation table and the calculated melting temperature (5) and derivative graph (6).

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