Improved Thermal Melt Analysis Workflow with the Cary 3500 UV-VIS Spectrophotometer

Agilent
Trusted Answers

Scott Melis, PhD
Application Scientist, Molecular Spectroscopy
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Outline

- UV-VIS technology
  - A staple of every lab
  - Applications for many forms of characterization
  - Don't want it to be a bottleneck
- Thermal Melt Applications
  - How they have traditionally been done
  - How the workflow can be changed
  - Doesn't have to be a bottleneck!
- Expanding Temperature Control
  - With great temperature performance, other applications benefit as well
  - Enzyme assay studies
  - Quantification
- Summary
  - Agilent UV-VIS

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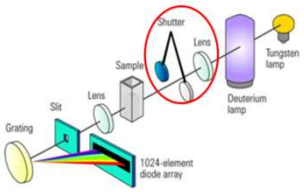
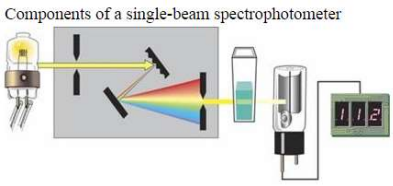
UV-VIS Spectrometers

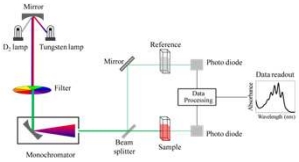
Established Technology

UV-VIS Spectrometers

Many Shapes and Sizes!







Diode Array

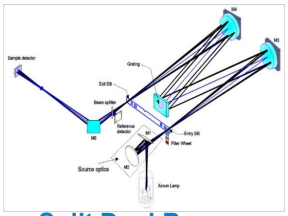
Fast, White light on sample / 4 X less Stable

Single Beam

4 X less Stable

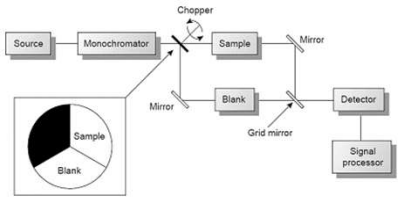
Split Double Beam

Stable 50% Shared Energy



Split Dual Beam

Stable 95% Energy

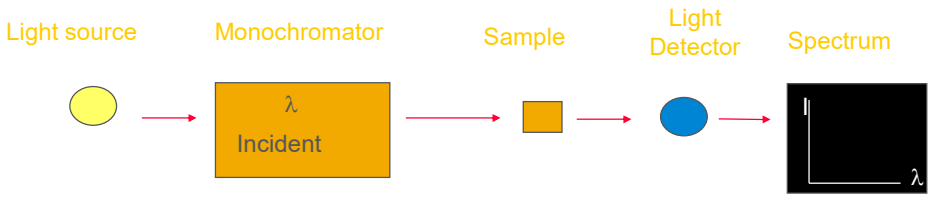


Time Shared Double Beam

Stable 100% Energy Time Shared

UV-VIS Spectrometers

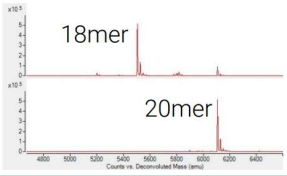
At the core…



How can we improve upon this?

Biomolecules Characterization and Analysis

- **Purification/Separation**
  - Polyacrylamide gel electrophoresis
  - Ion-paired reversed-phase high-performance (IP-RP-HPLC)
- **Mass characterization**
  - Mass spectrometry (MS)
  - Electro-spray ionization mass spectrometry (ESI-MS)
- **Structural characterization**
  - X-ray crystallography
  - NMR



- **Stability Studies**
  - Thermal melts
  - Buffer and pH suitability
  - Kinetics
- **Quantification – QC/QA**
- **Enzyme Assays**

https://www.agilent.com/cs/library/posters/public/TP434_ASMS2020_High-throughput_Mass_Spectrometry_Analysis_of_Synthetic_Oligonucleotides.pdf

Characterization and Analysis with UV-VIS

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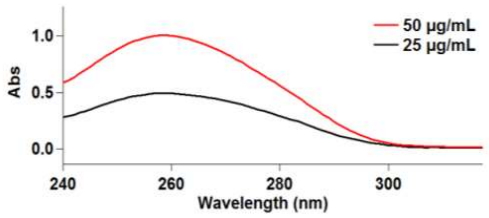
Quantification and QC

- UV-Vis absorbance at 260 nm can be used to provide estimate of nucleotide concentration.
- Other wavelengths provide indication of purity *e.g.* 260/280 ratio to identify nucleic acid contamination and 260/230 ratio to identify phenols contamination.
- QC is critical due to downstream use of nucleotides – sample preparation can be source of experimental variability.



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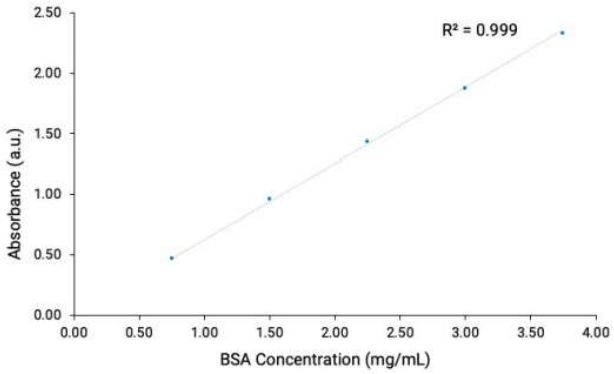
Quantification



Pure protein samples

- Absorbance at 280 nm for pure, well characterized proteins using Beer-Lambert law:

$$A_\lambda = \varepsilon c L$$

A_λ	Absorbance
ε	Molar absorption coefficient
c	Molar concentration
L	Optical pathlength

- Deviations in linearity can be caused by scatter
- Effect of light scattering (*e.g.* by aggregates) can be corrected.

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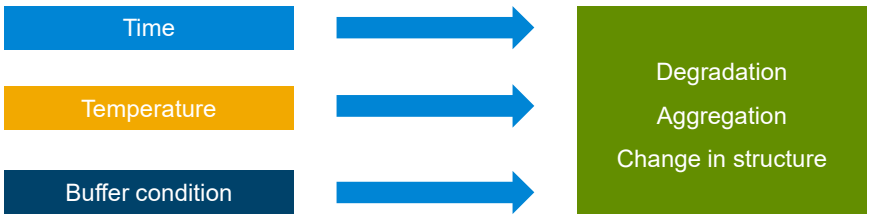
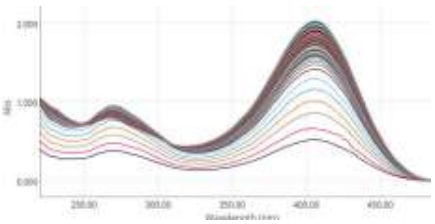
Stability studies

UV-Vis used to study stability of nucleotides or proteins by monitoring change in absorbance.





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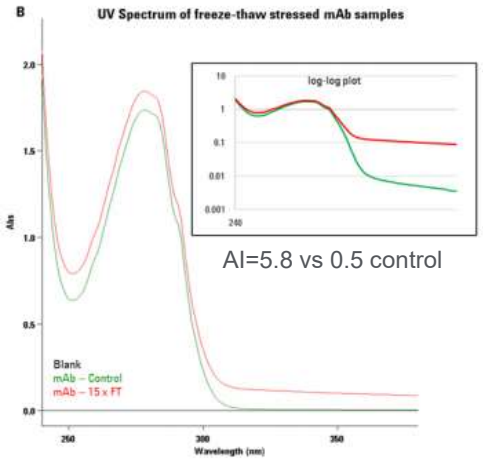
Aggregation Studies

Analysis by UV-Vis

- Simple test to identify proteins that aggregate.
- Example: change buffer concentration then detect if aggregation has occurred.
- UV-Vis: proteins absorb at 280 nm and not at 350 nm.
- Absorbance at 350 nm indicates the sample is scattering light: aggregation has occurred.



AI=5.8 vs 0.5 control

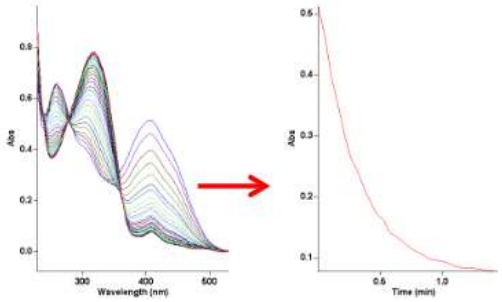
aggregation index (AI): OD350/(OD280–OD350)×100

https://www.agilent.com/cs/library/applications/cary_60_5991-7974EN-mAb-app.pdf

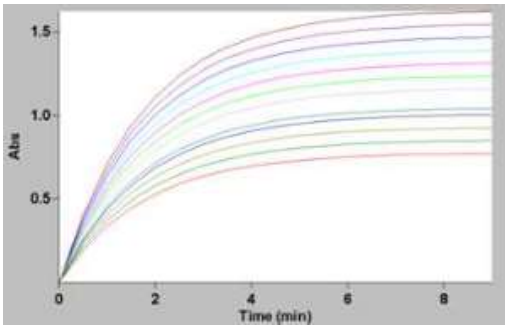
Enzyme assays

With UV-Vis spectroscopy

- Monitor enzyme reactions by measuring the changes in the intensity of light absorbed or scattered by the reaction solution.
- Determine the reaction rate constant or catalysis efficiency.



Wavelength scans over time



Absorbance at a single wavelength over time

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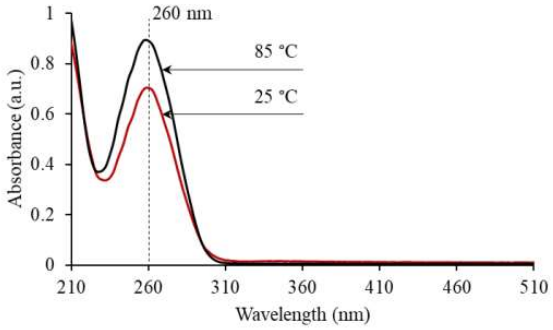
Thermal melts (T_m)

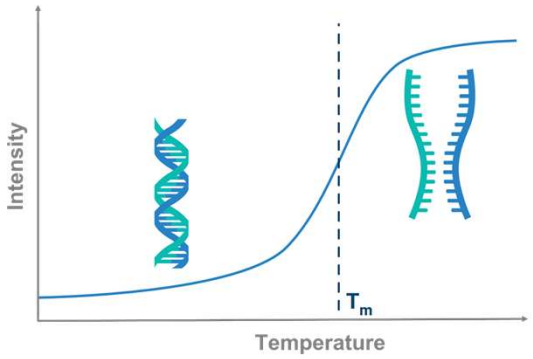
- Double stranded nucleotides become denatured into single strands as they are heated.
- Temperature at which this occurs is dependent on nucleotide composition and sequence.
- Thermal melts monitor the absorbance with changes in temperature gradually under controlled conditions.
- Thermal stability applications: DNA/RNA structure and protein-nucleic acid interactions, identification confirmation, screening for thermostability.



UV-Vis Thermal Melts

Thermal Melts - Goals

- Thermal Melts consist of measuring absorbance as a function of temperature
- We are looking for indications that a sample undergoes a change at high temperatures.
- These changes can be reversible or irreversible



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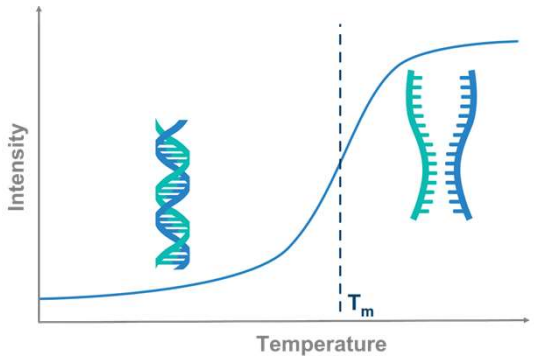
Melting Temperature Determination



- The melting temperature is the inflection point of the melting curve
- It can be found by collecting a melting curve and taking the first derivative
- It is the temperature that corresponds to the maximum of the first derivative curve.

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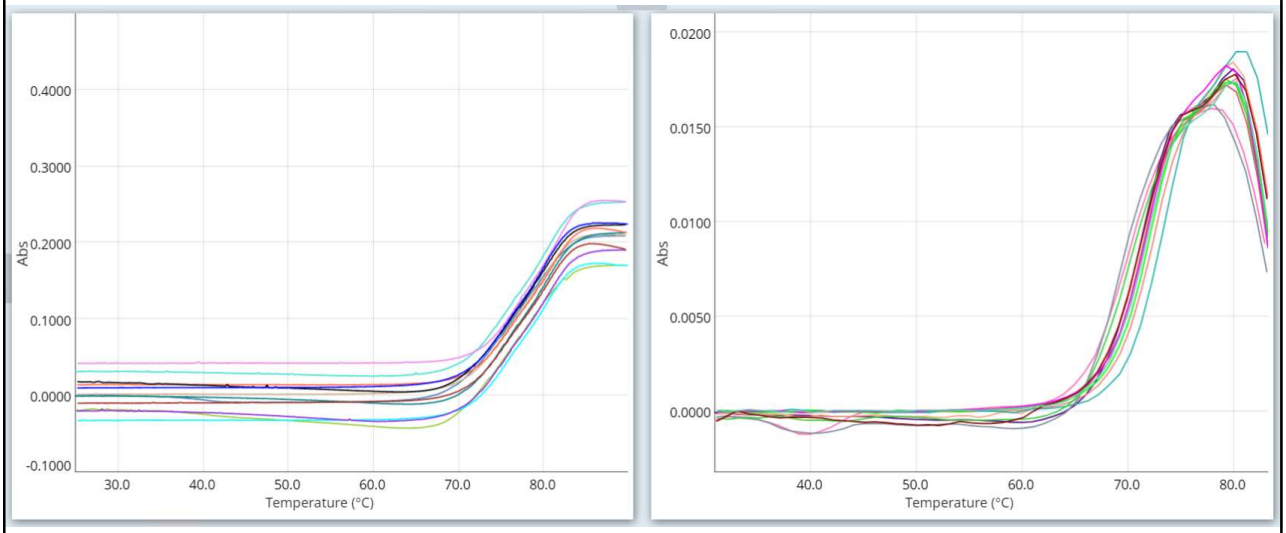
Melting Temperature Determination



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The Original Process

- Heating samples was achieved using a Peltier block.
- It was very easy to measure the Peltier block's temperature
- Sample absorbance would be plotted versus the block's temperature
- Temperature ramp rates were slow
  - We needed the time for the cuvette to equilibrate
  - 0.1 – 1°C per minute

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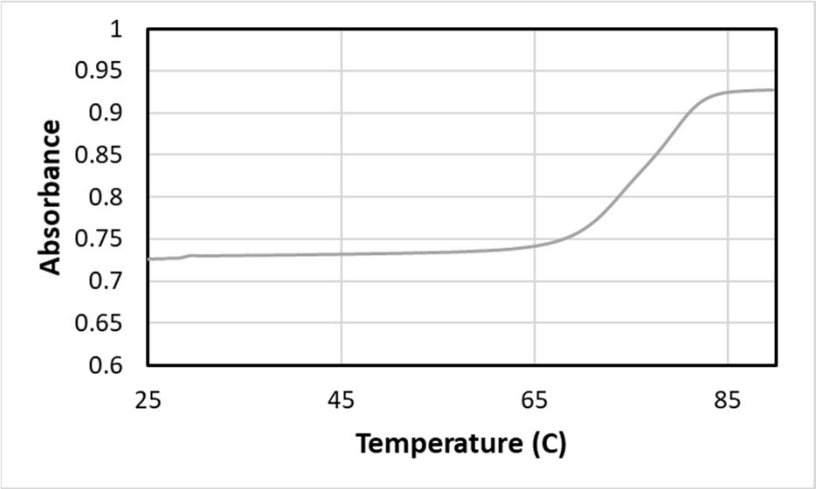
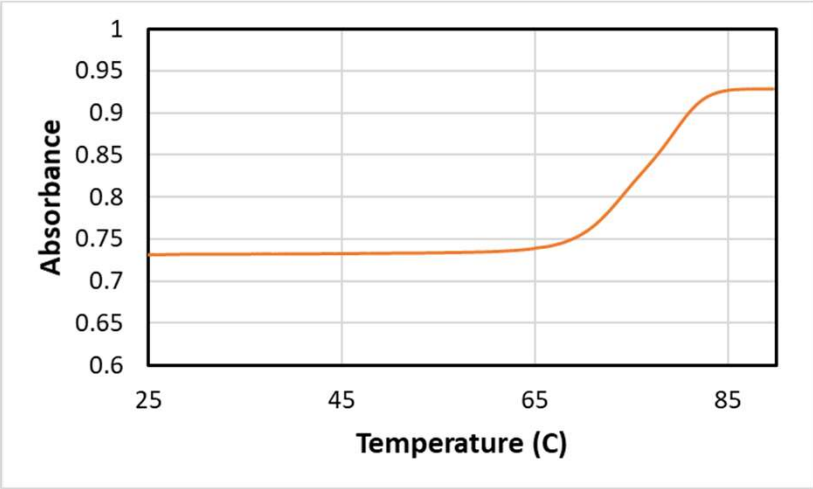
0.1 Degrees Celsius per Minute (~10-11 hours of data collection)



The Evolution of the Workflow

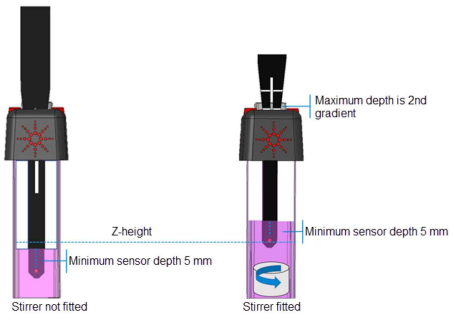
- We know where to expect the interesting effects to be.
- We need slow temperature ramp rates to determine the melting temperature, but we just need to survey for impurities/effects elsewhere
- Variable ramp stages as an option
  - We can define multiple ramp stages throughout the measurement
  - We can ramp quickly to survey away from the desired location
  - We can slow it down where we need to

Variable Ramp Rate (~1-2 hours of data collection or longer)

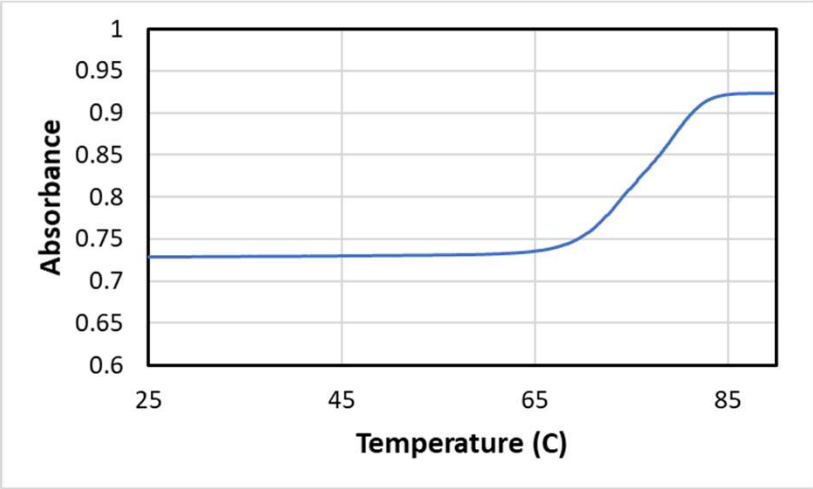


The Next Steps

- Accurate temperature probes allow for measurements of the sample directly
  - No longer need use the block temperature
  - Can also stir samples to help with equilibration
- Improvements in technology allow for faster ramp rates of Peltier heaters as well.
  - Equipment ramp rates can push 40C/min
- With temperature probes and technology improvements, these workflows can be drastically improved
  - Not a bottleneck!



10 Degrees Celsius per Minute (~10 minutes of data collection)



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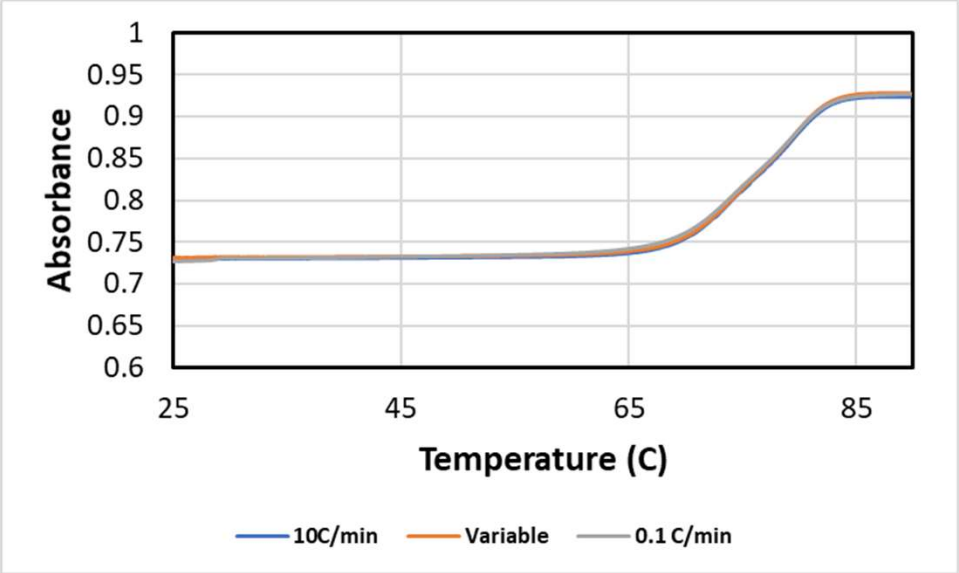
Overlay of 3 data sets



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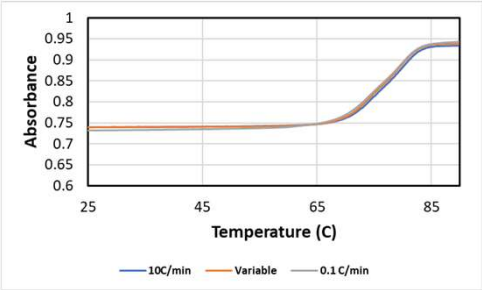
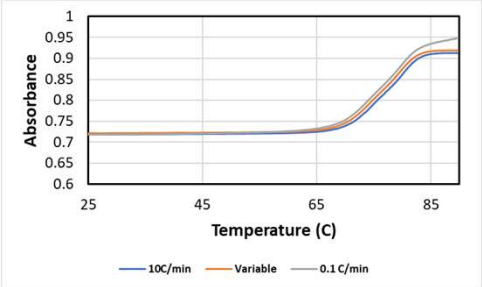
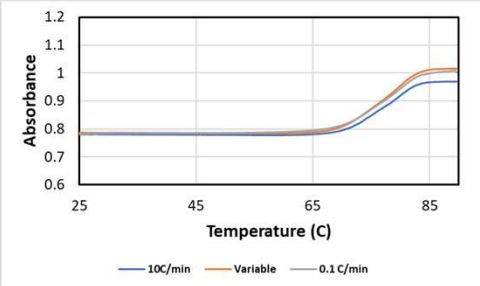
Data is reproducible







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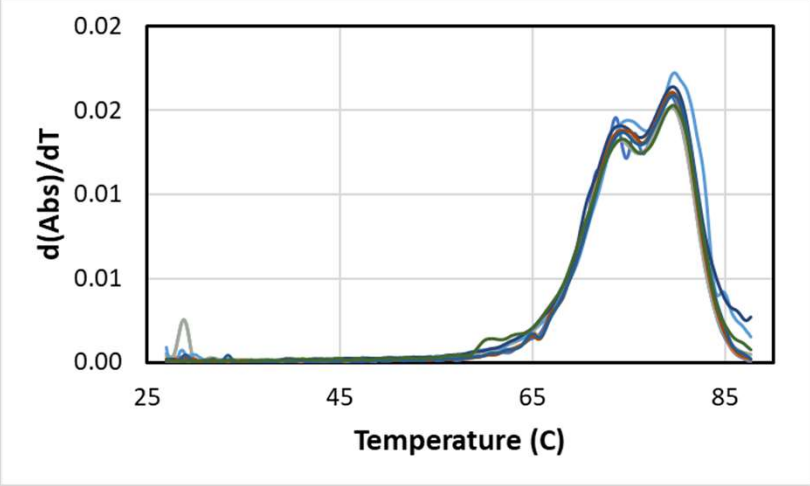
Determination of Melting Temperature



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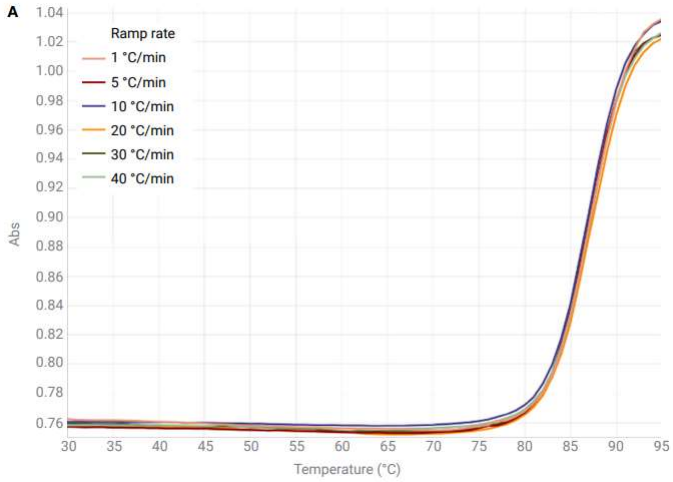
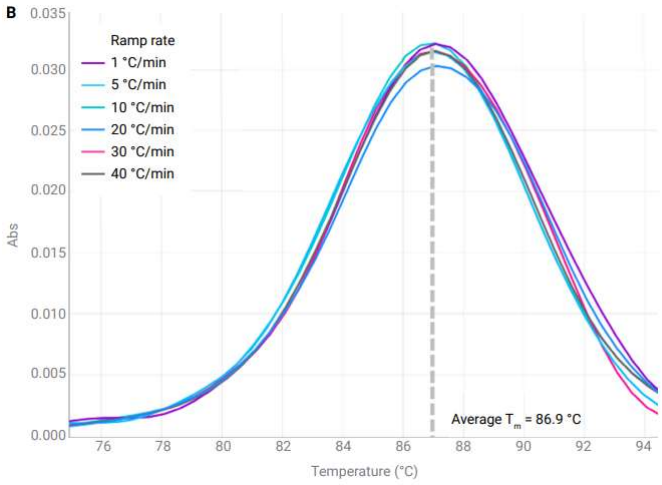
Different Ramp Rates Comparison



Fast Determination of Thermal Melt Temperature of Double-Stranded Nucleic Acids by UV-Vis Spectroscopy. Agilent application note. Aug 2022. 5994-0384EN

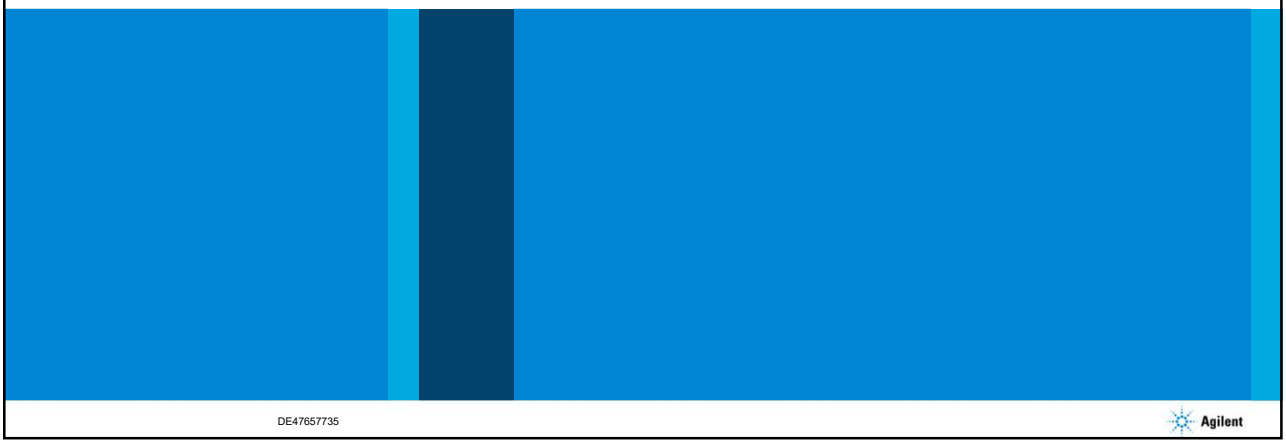
Different Ramp Rates Comparison – Determining Melting Temperature



Ramp Rate (°C/min)	Average T_m (°C) (n = 3), Each Ramp Rate	Standard Deviation T_m (°C) (n = 3), Each Ramp Rate
1	87.1	0.0
5	86.7	0.2
10	86.9	0.2
20	87.1	0.0
30	86.9	0.2
40	86.9	0.2
Average T_m (°C) (n = 6), All Ramp Rates	86.9	0.13

Fast Determination of Thermal Melt Temperature of Double-Stranded Nucleic Acids by UV-Vis Spectroscopy. Agilent application note. Aug 2022. 5994-0384EN

Other Temperature Applications



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Other Applications Benefit From Good Temperature Control

- Thermal Stability
  - Determine melting temperature
  - Study sample aggregation
- Reactions
  - Reaction rate depends on temperature of the sample
  - Have stable control of temperature from probe feedback during measurement
- Identification and Quantification
  - Measure sample's spectrum at fixed temperatures
  - Determine sample concentrations

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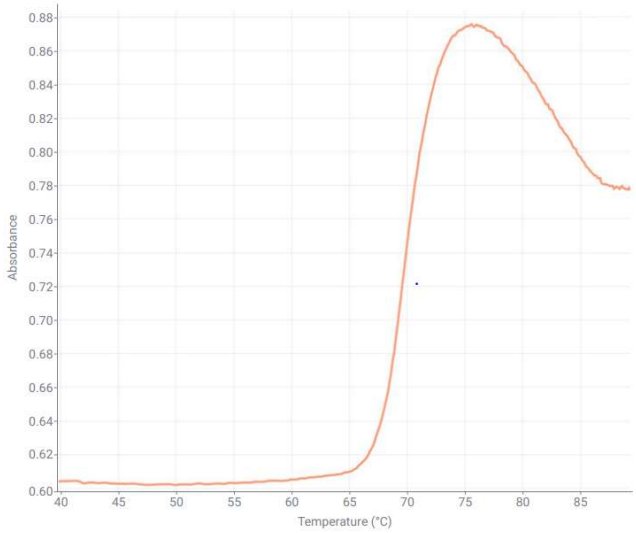
Thermal Stability



Characterization of Protein Thermal Stability Using UV-Vis Spectroscopy. Agilent application note. Aug 2022. 5994-5086EN

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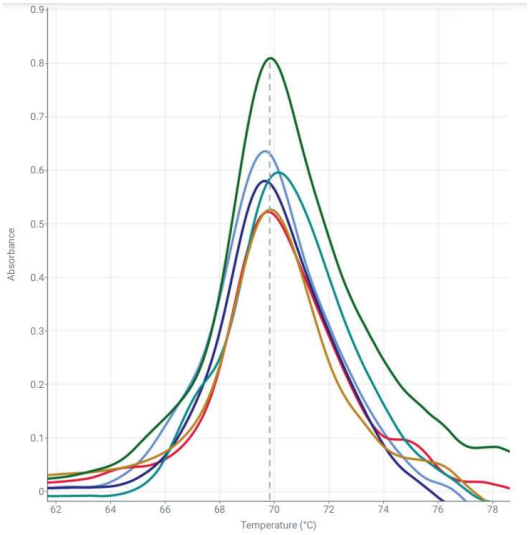
Thermal Stability



Technique	T_m of *Ec*DsbA (°C)
Cary 3500 UV-Vis Spectrophotometer Replicate 1	69.70
Cary 3500 UV-Vis Spectrophotometer Replicate 2	69.80
Cary 3500 UV-Vis Spectrophotometer Replicate 3	69.70
Cary 3500 UV-Vis Spectrophotometer Replicate 4	70.20
Cary 3500 UV-Vis Spectrophotometer Replicate 5	69.90
Cary 3500 UV-Vis Spectrophotometer Replicate 6	69.90
Average	69.86
Standard Deviation	0.16
Literature Value 1[14]	68.55
Literature Value 2[15]	69.25

Characterization of Protein Thermal Stability Using UV-Vis Spectroscopy. Agilent application note. Aug 2022. 5994-5086EN

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Hydrolysis Reactions

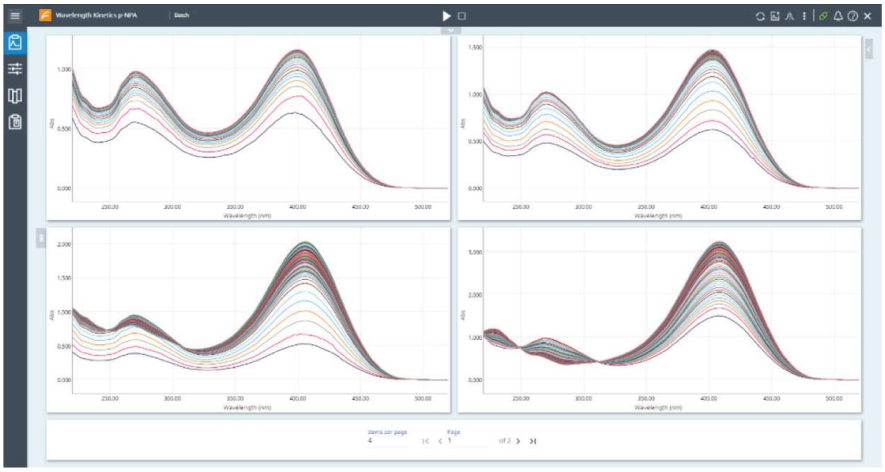


Figure 2. Wavelength scans over time over the wavelength range 220 - 520 nm, collected for 30 minutes after the reaction was initiated by mixing the two reagents. Top left is at 20 °C, top right is 40 °C, bottom left is 60 °C and bottom right is 80°C.

A Fast Method of Studying the Impact of Temperature on Chemical Reactions. Agilent Application Note. Oct. 2018. 5994-0385EN

Hydrolysis Reactions

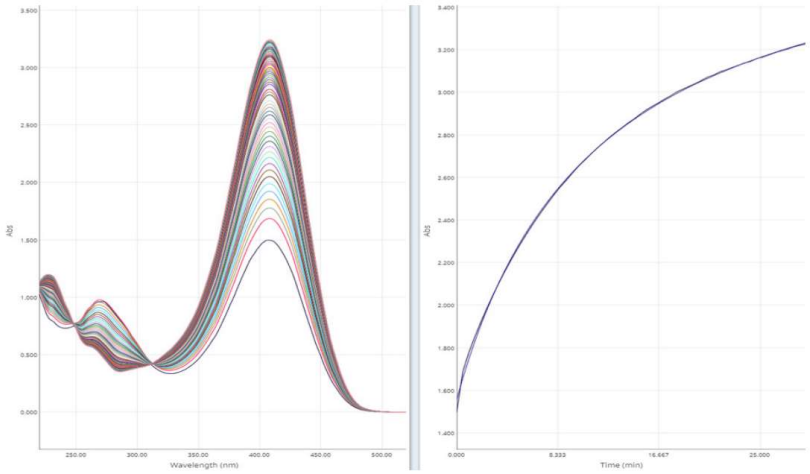
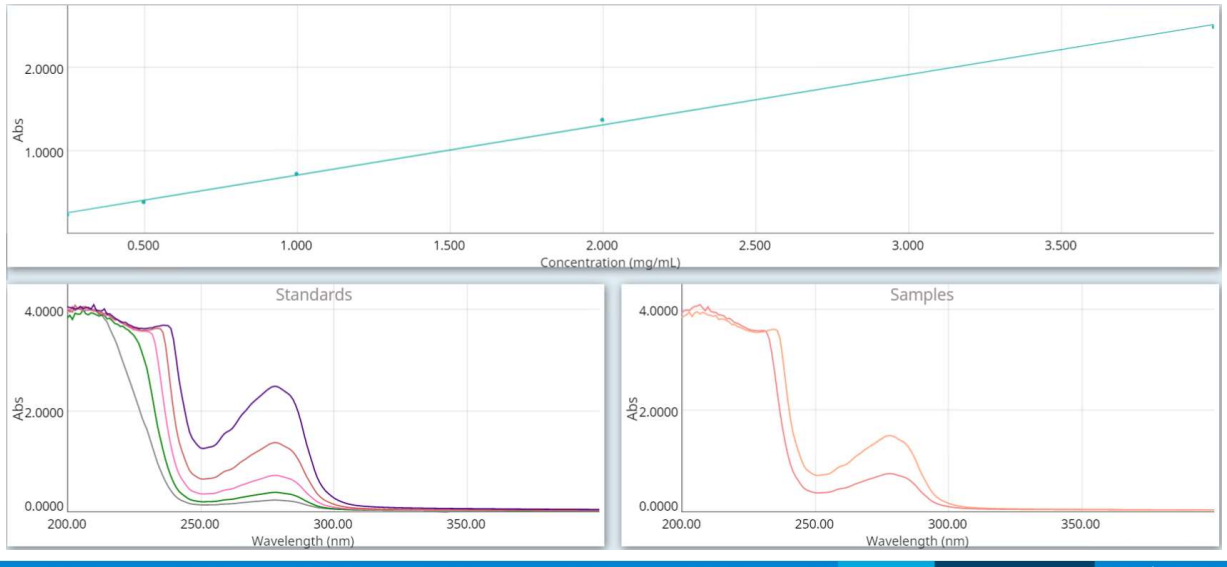


Figure 3. The spectra for the reaction performed at 80 °C with characteristic isosbestic points (left). The change in absorbance over time at 408 nm (right) was plotted within the Cary UV Workstation software and used to determine the reaction rate.

A Fast Method of Studying the Impact of Temperature on Chemical Reactions. Agilent Application Note. Oct. 2018. 5994-0385EN

Identification and Quantification



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Summary

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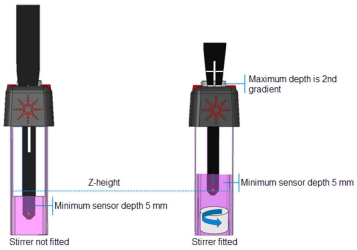
Summary

- A **high level of reproducibility** can be achieved for faster ramp rate thermal melts
  - Standard protocols of 0.5C/min (or less) can be exceeded
  - The time needed to perform these measurements can be reduced without compromising results
- There is an opportunity for **time savings** with the right equipment to support UV-VIS temperature measurements
  - An experiment that has historically taken hours could be reduced to minutes
  - The UV-VIS instrument does not have to be a bottle-neck in the lab
- Other experiments benefit as well from **precise** temperature control and **fast** data collection
  - Fast data collection improves workflow and allows for more measurements to be processed
  - Strong temperature control provides confidence in the data

Cary 3500

Unique Design Concept

Or



Cary 3500 Compact UV-Vis.

Cary 3500 Multicell UV-Vis

Cary 3500: Improved Thermal UV-VIS

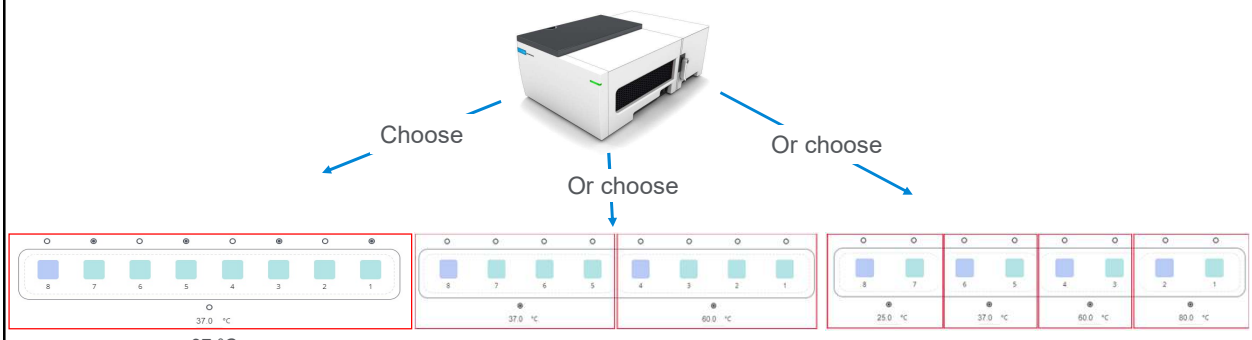
Powerful, integrated and **fully air-cooled** thermal control

Heat and cool samples from 0 to 110 °C and back in <20 minutes

- **Neat:** no noisy water re-circulator, no pipes, no cables, no plumbing
- **Fast and efficient:** measure all samples simultaneously
- **Reliable:**
  - no multiple moving parts
  - permanent optical alignment
  - accurate/reproducible data from microcells

Configure experiments like you've never been able to before

Innovative: Use software to configure up to four temperature zones within an experiment



Single zone

Simultaneously characterize up to seven samples (or the same sample in seven chemical buffers)

Two-zone

Test the stability of three formulations at two temperatures

Four zone

Simultaneously characterize the behavior of a formulation at four different temperatures

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Agilent Molecular Spectroscopy

Cary 60 UV-VIS

Cary 3500 UV-VIS

Cary 630 FTIR

Vaya Handheld Raman Spectrometer

TRS–100 Raman System

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