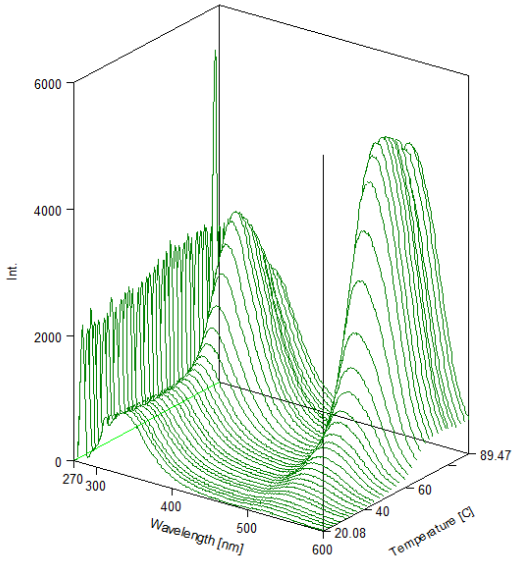
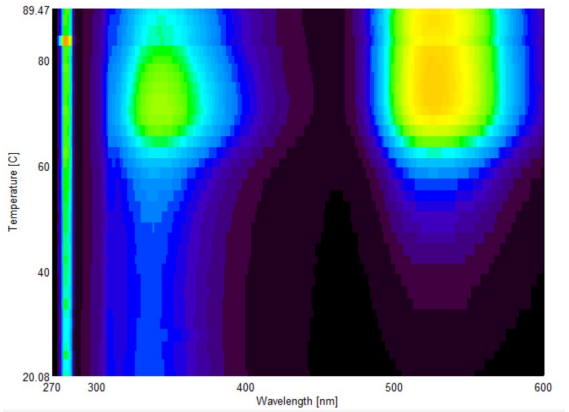
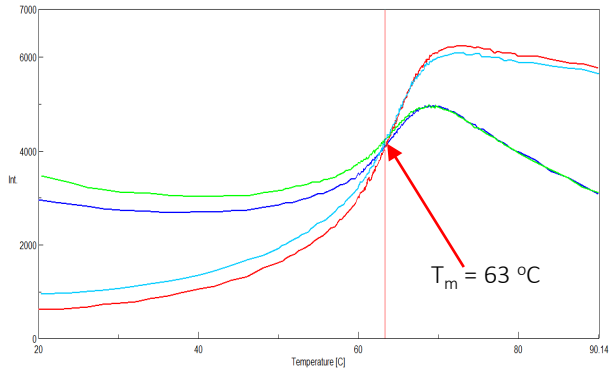






Understanding, Optimizing, and Analyzing Thermal Melts

FOR FLUORESCENCE, UV-VIS, AND CIRCULAR DICHROISM SPECTROSCOPY

BY SHERRY L. HEMMINGSEN, PH.D.
MOLECULAR SPECTROSCOPY PRODUCT SPECIALIST

Seminar Overview

Background

- Fluorescence review
- Thermal melt curves

Experimental design and optimization

- Cuvettes, single vs. multi-cell
- Single-point vs scanning
- Uniform ramping vs. stages

Analysis and data modeling

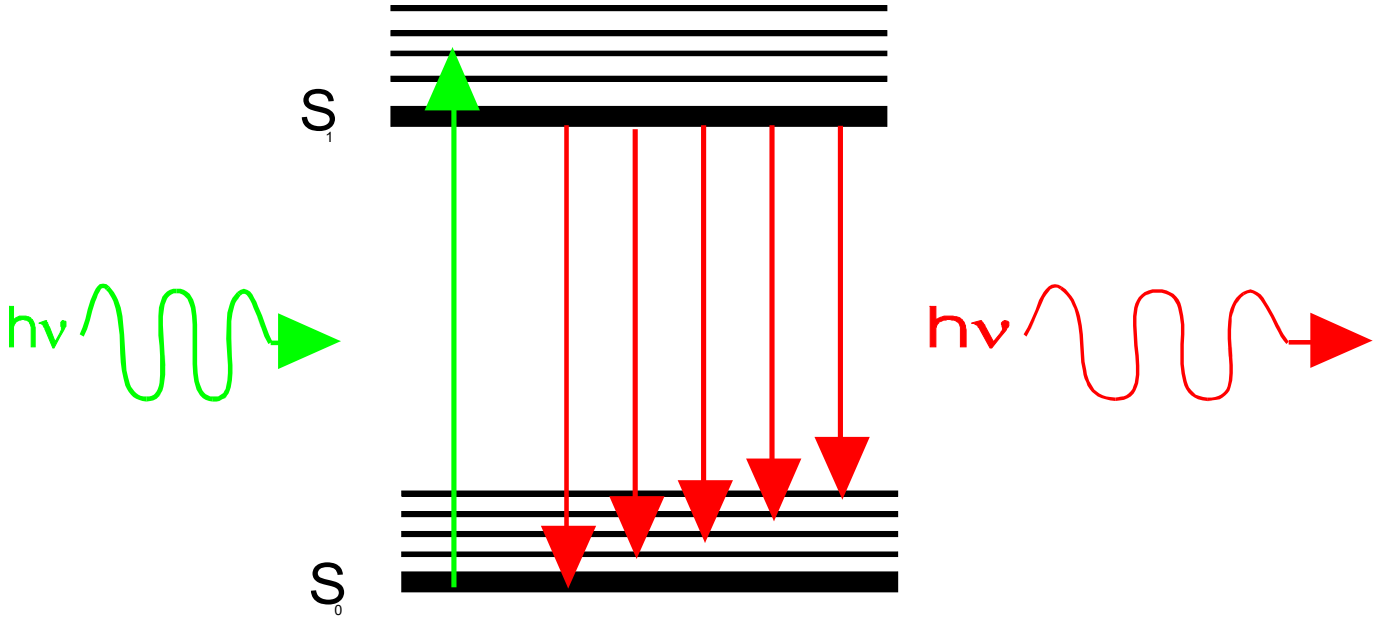
- Traditional methods
- Recommendations for good analyses

JASCO Corporation

JASCO: Our Products

Fluorescence

The radiant transition of the lowest level of the excited state to any sublevel of the ground state.



Fluorescence Advantages

SENSITIVE!

SENSITIVE!

SELECTIVE!

What Factors Affect Fluorescence?

Solution Conditions

- Solvent
- pH
- Ionic Strength
- Temperature

Concentration

Fluorescence is a highly sensitive tool, especially to local microenvironments around the fluorophore

BUT, this requires careful experimental control to ensure that the observed fluorescence changes are due to the experimental conditions that were *intentionally* modified

Fluorescence Applications

Biochemical

- Protein folding, drug delivery, binding interactions, aggregation, imaging

Environmental

- Pollutant detection / tracking, microbial water testing

Materials Science

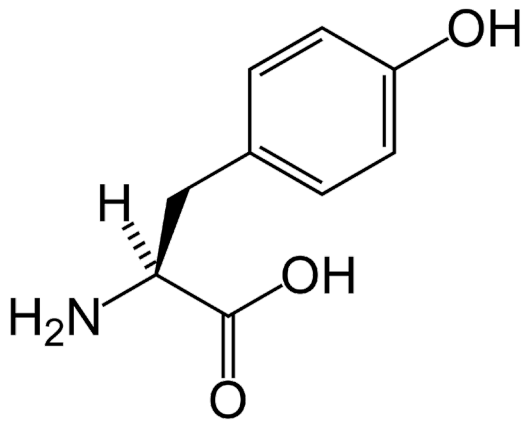
- Nanoparticles-quantum dots, new material development

Food Science

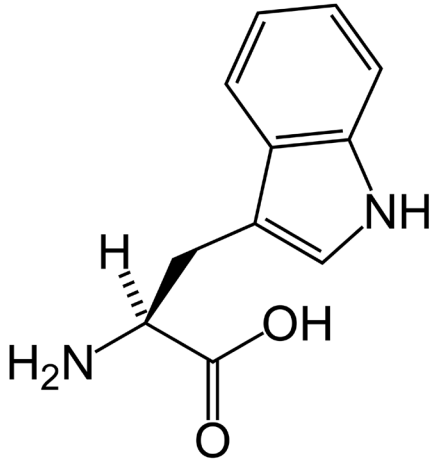
- Ingredient quantitation, antioxidant testing, packaging

And the list goes on…..

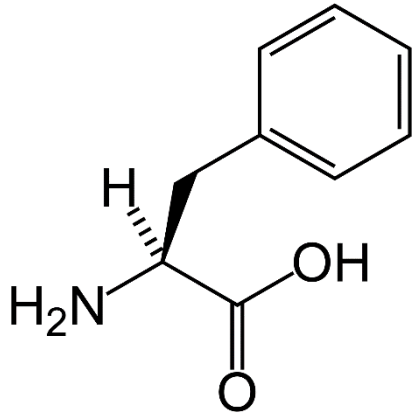
Intrinsic Probes: Aromatic Amino Acids



Tyrosine



Tryptophan



Phenylalanine

Aromatic amino acids provide intrinsic fluorescent probes for studying protein interactions and folding

Extrinsic Probes: Suit Your Needs

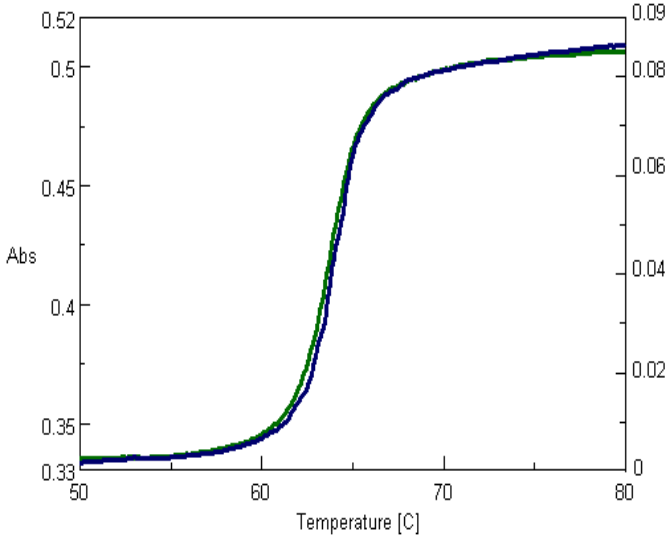
Specific spectral characteristics

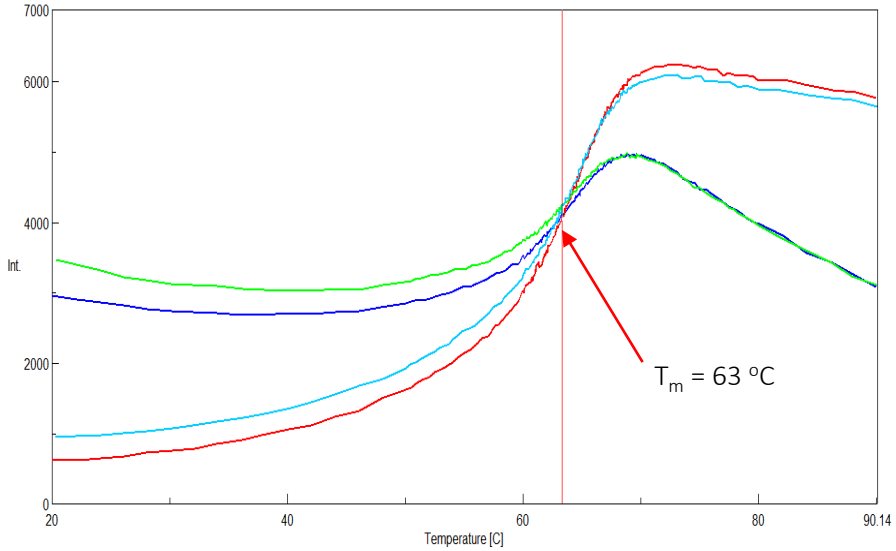
- Emission at longer wavelengths than background fluorescence
- Excite at longer wavelengths than common biological interferences…like the three fluorescent amino acids

Affinity for a particular chemistry

- Hydrophobic vs. hydrophilic (SYPRO Orange, ANS)
- Intercalate in grooves of DNA (SYBR® Green)
- React with a specific functionality, like thiol-reactive (CPM, BFC)

Quantum efficiency, strength of emission





What is Thermal Stability?

Thermal Stability

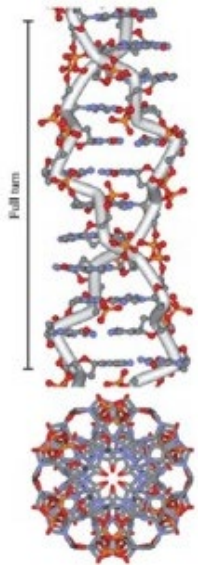
- How a molecule or complex responds to raising and lowering the temperature.
- Greater thermal stability is exhibited by species that maintain their initial state and don't transition to another state until higher temperatures are reached, like
  - A protein going from a native folded state to an unfolded state
  - A protein-protein or protein-ligand interaction dissociating
- Greater thermal stability is also evidenced by a structure's ability to return to its original state after repeatedly raising and the lowering the temperature. The more reversibility, the greater the stability.

Evaluation of Thermal Stability

Two common ways to evaluate thermal stability:

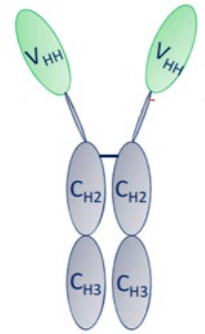
1. **Melt curves** which monitor a signal (like intensity, absorbance, mdeg) at a single wavelength (or wavelength pair) with changes in temperature
2. **Temperature wavelength scans** which collect a series of spectra as temperature is ramped to a target temperature.

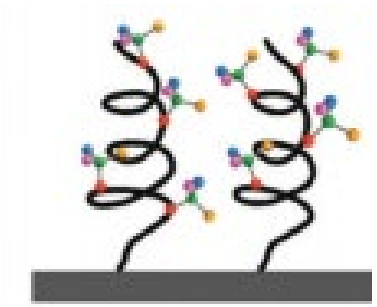
Thermal Stability Applications

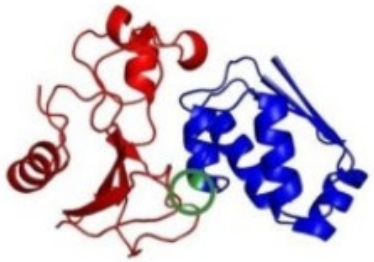


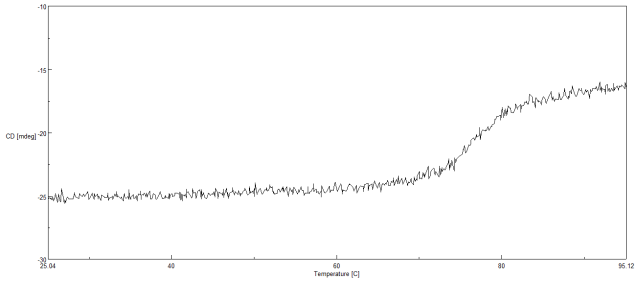
- Protein structure
- Antibody structure
- DNA/RNA structure
- Protein - Protein interactions
- Protein - Nucleic Acid interactions
- Ligand binding

- Pharmaceutical formulation and storage
- Drug discovery
- Enzymatic robustness



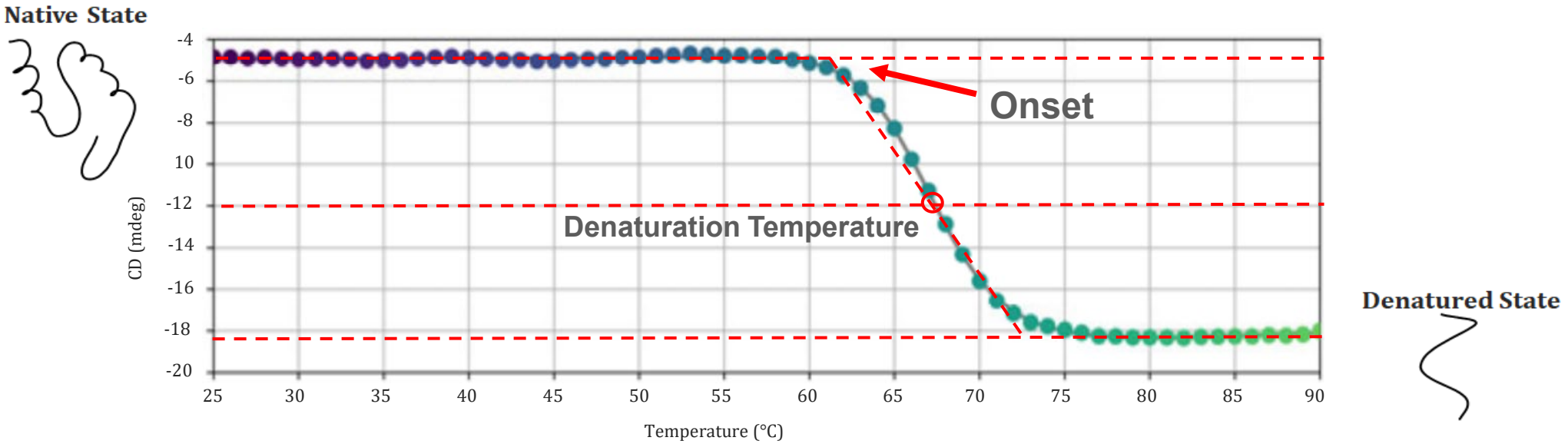






M. M. Gromiha, et al. *Current Opinion in Structural Biology* 44 (2017).
M. Zhang, et al. *Chemical Science Reviews*. 41 (2011)..
Holm, et al. *Physical Chemistry Chemical Physics* 10. 33 (2012).

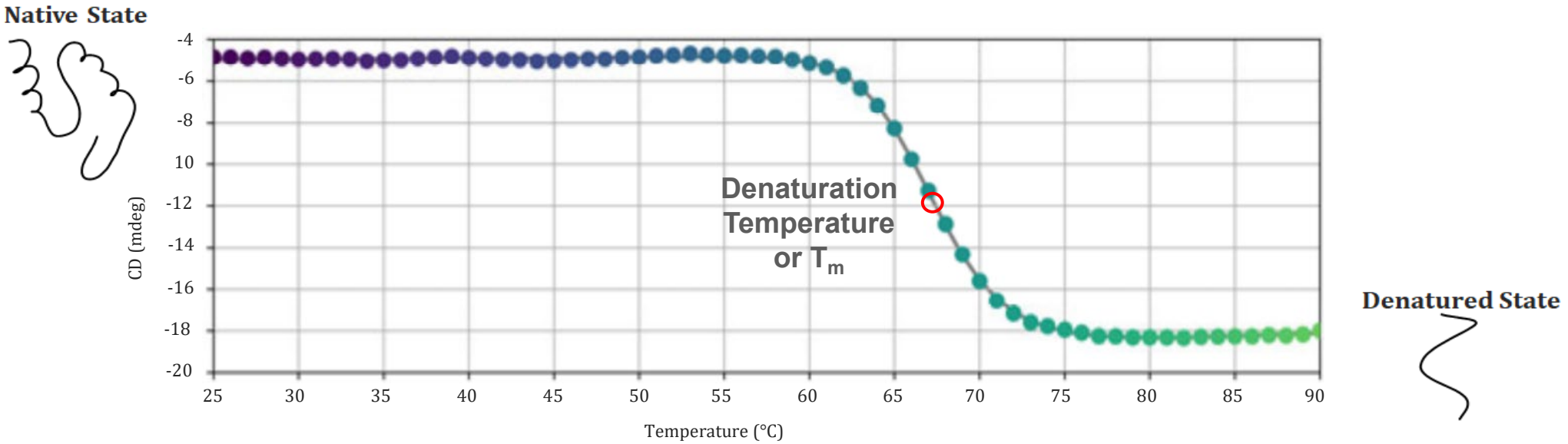
Evaluating Thermal Stability of Proteins



Denaturation Temperature: The point of intersection of the straight line midway between the CD value before and after denaturation, and the straight line fitted to the region of change.

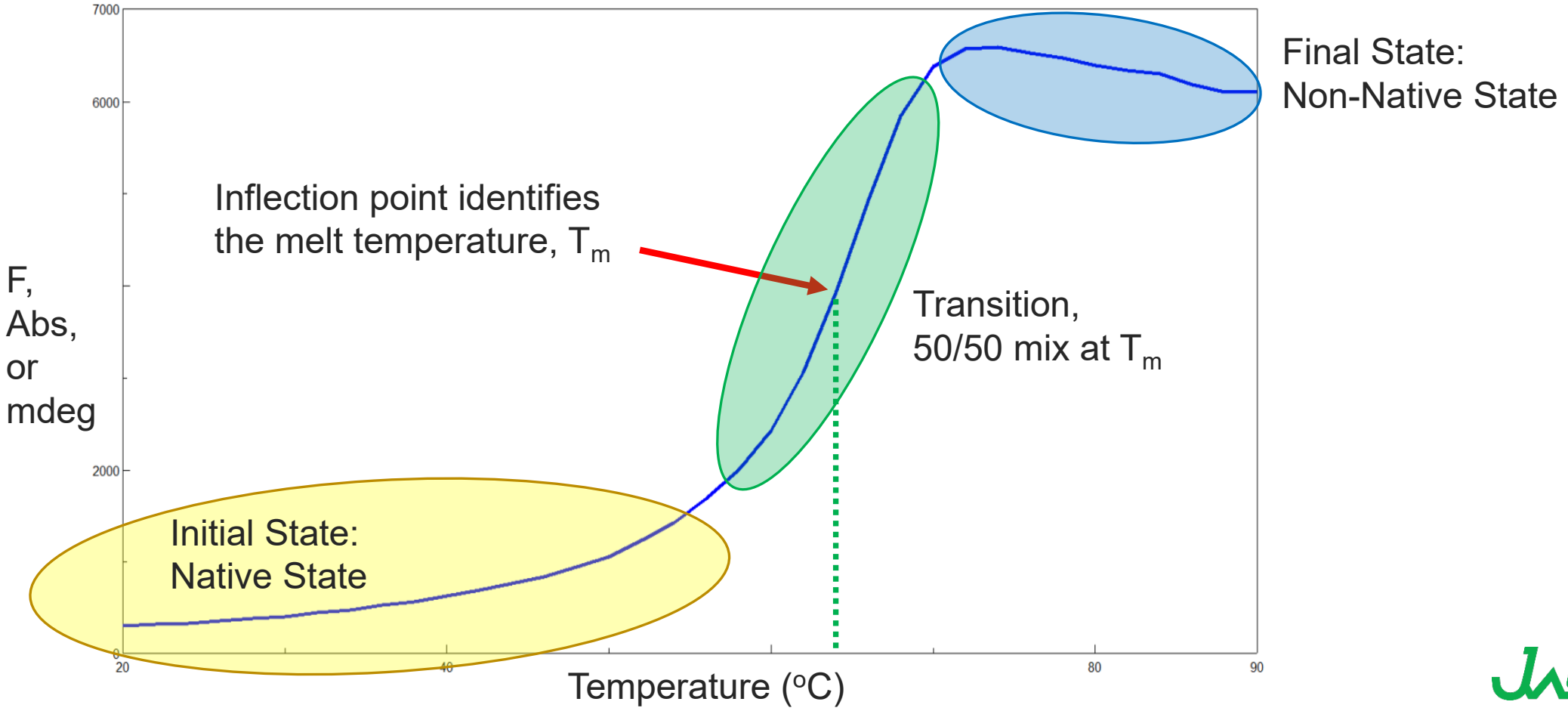
Onset: The temperature at which the protein begins to change from its native state. The higher this temperature, the more stable the protein.

Evaluating Thermal Stability of Proteins

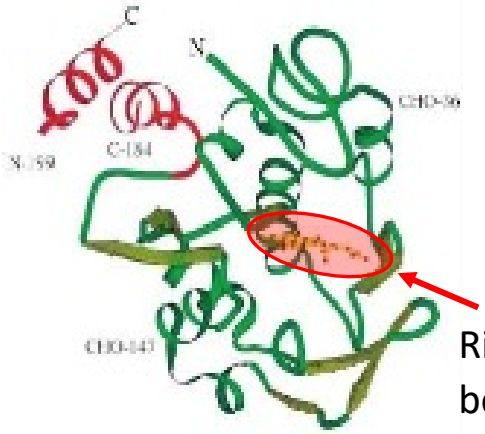


	T_m (°C)	ΔH (kJ/mol)	ΔS (J/mol·K)
Sample 1	47.40 ± 0.067629	820.558 ± 44.389	2559.83 ± 138.477
Sample 2	47.61 ± 0.065995	782.463 ± 40.3946	2439.37 ± 125.932

Classic Thermal Melt Curve: Sigmoidal



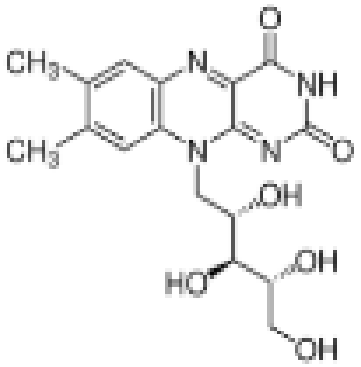
Riboflavin Binding Protein (RBP)



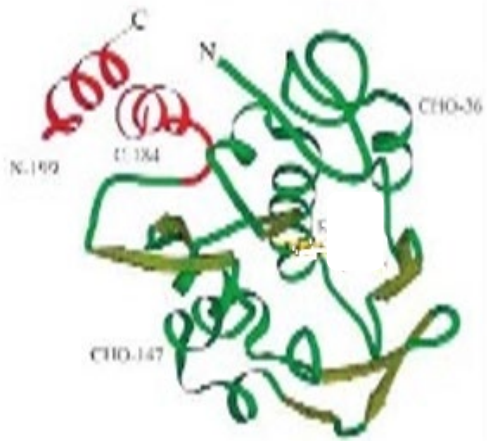
Riboflavin bound in pocket

Holo-RBP

Native Folded State



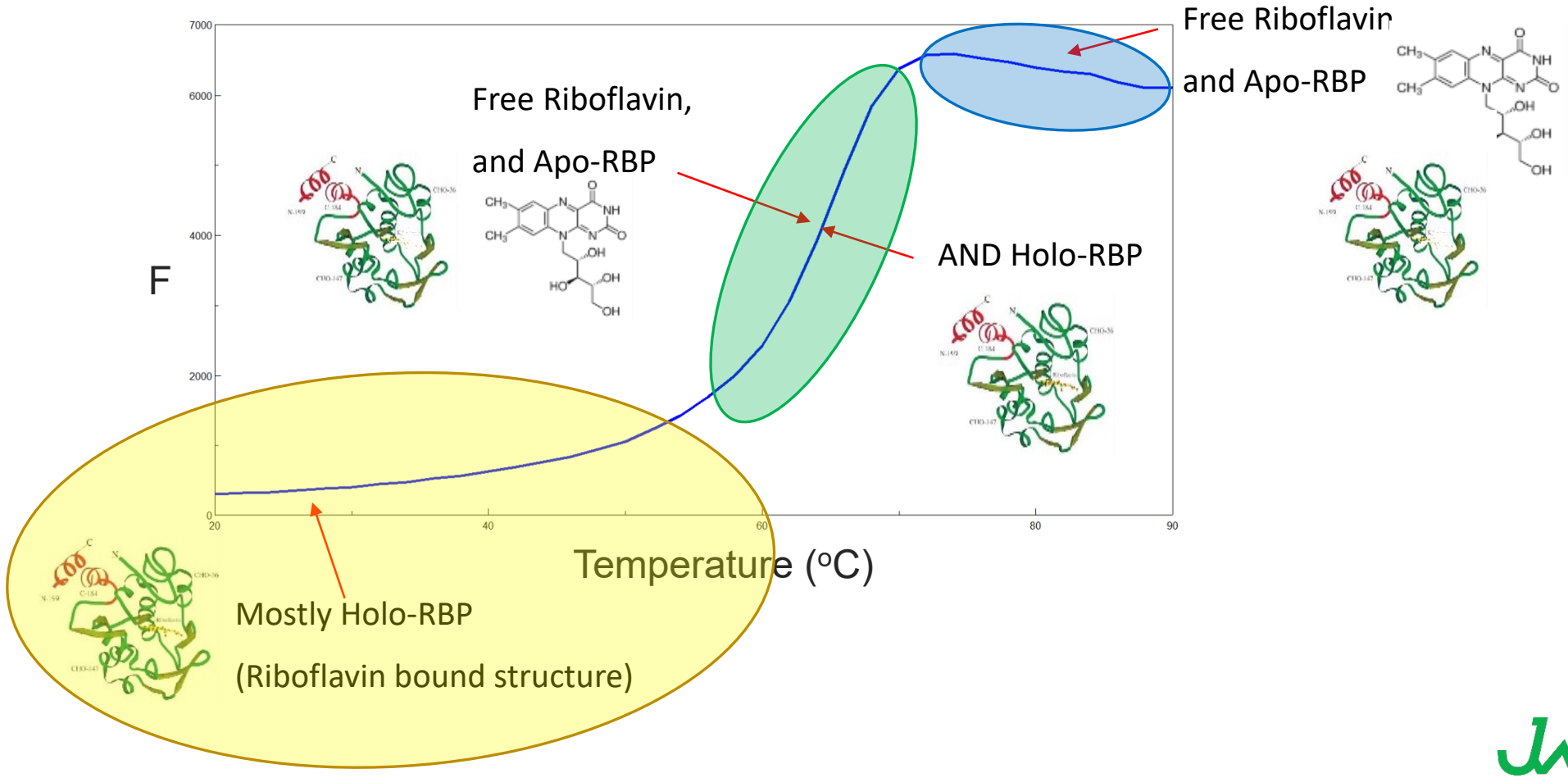
Free Riboflavin



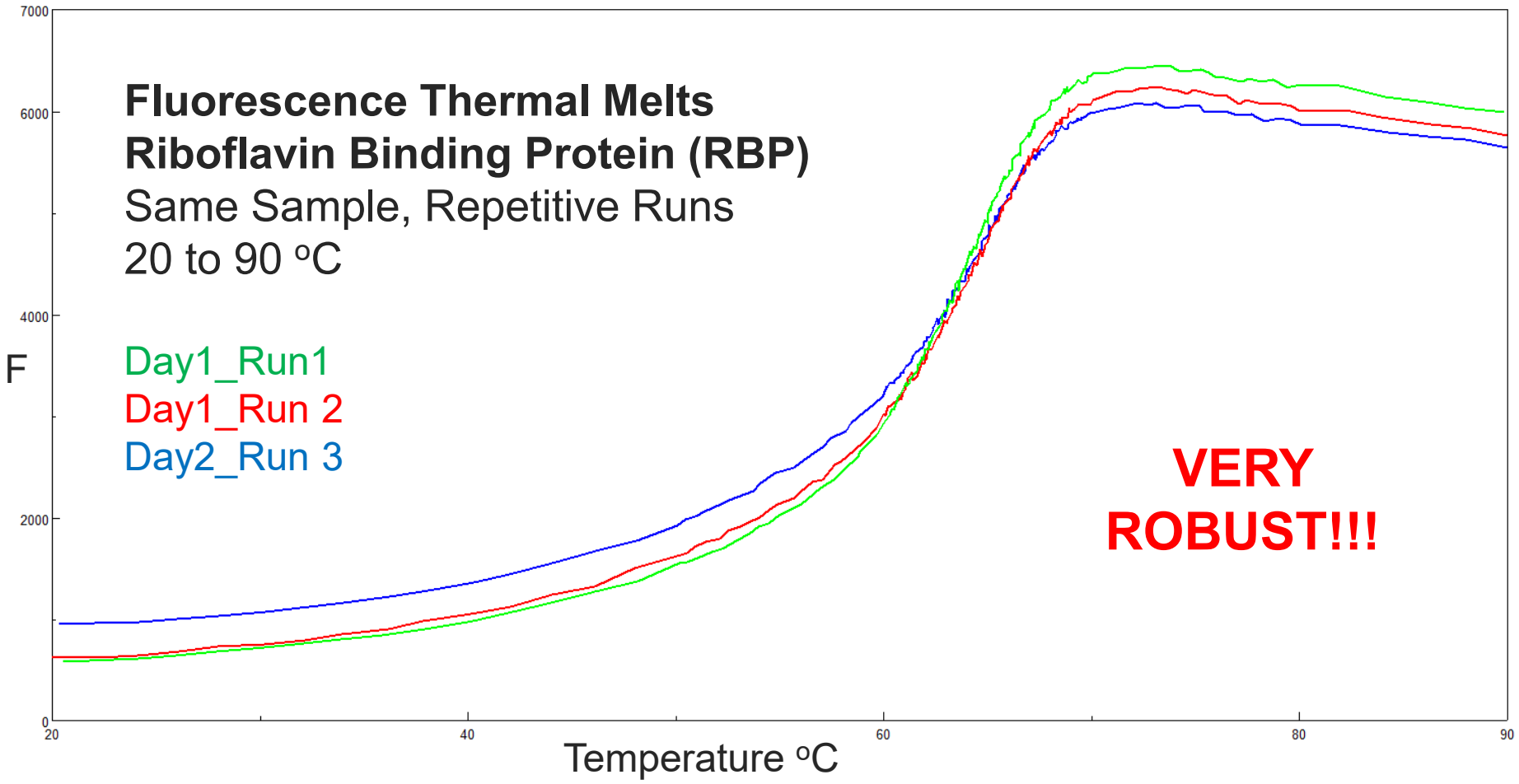
Apo-RBP

Unfolded Non-Native State

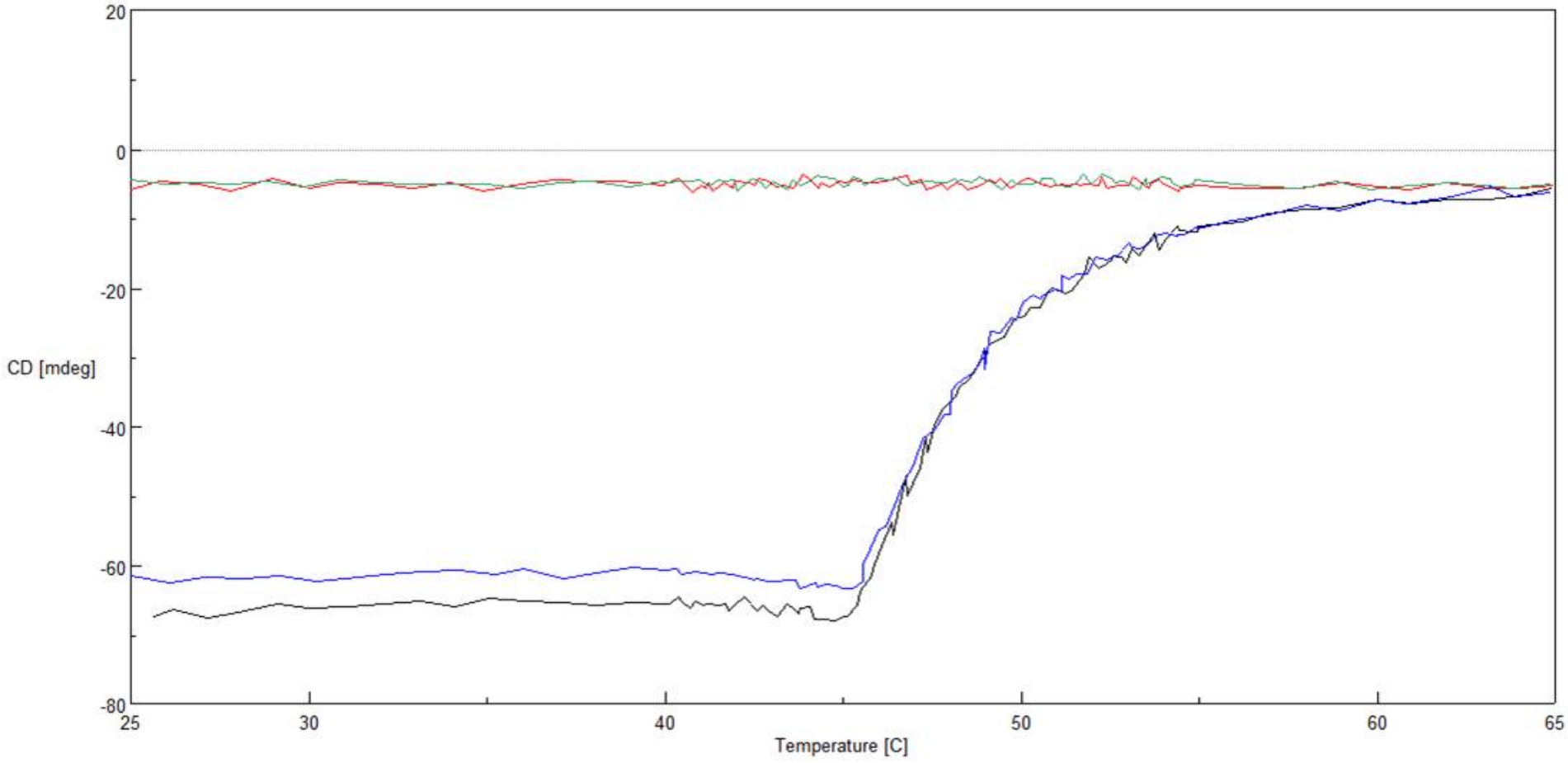
Riboflavin Binding Protein (RBP)



Repeatable: Implied Reversibility

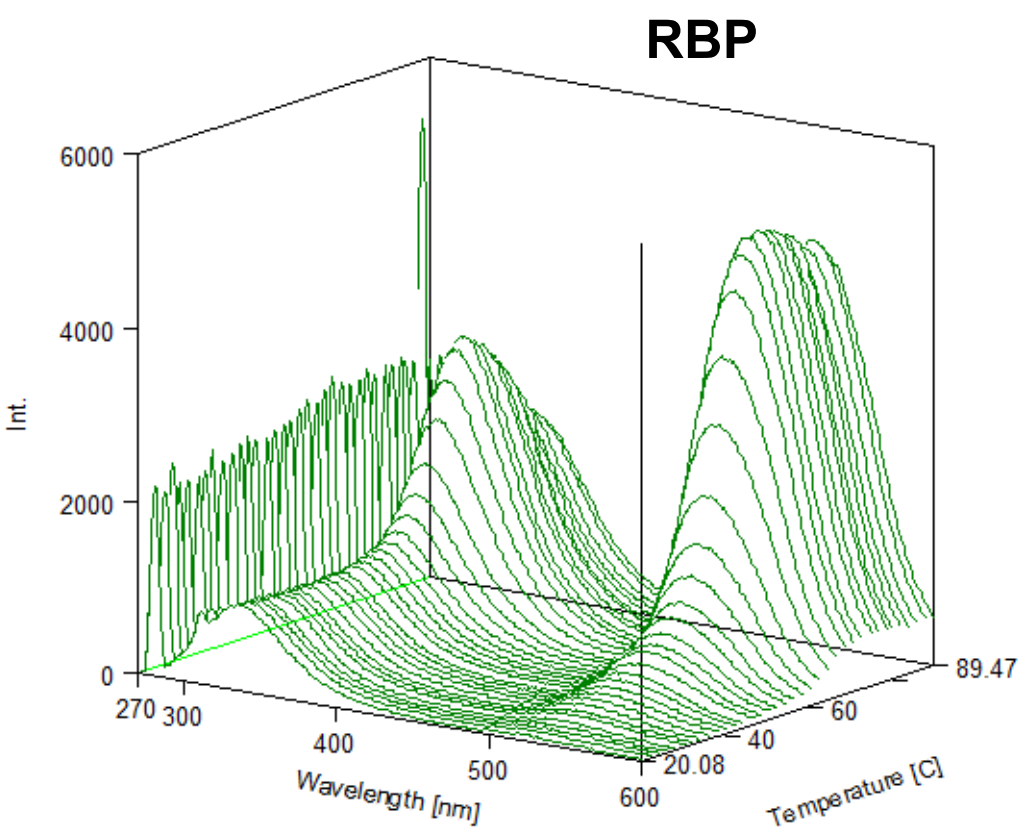


Irreversible



CD Thermal Melts
20 to 65 °C
65 to 20 °C
Duplicate Runs

Scanning Wavelength Thermal Melts



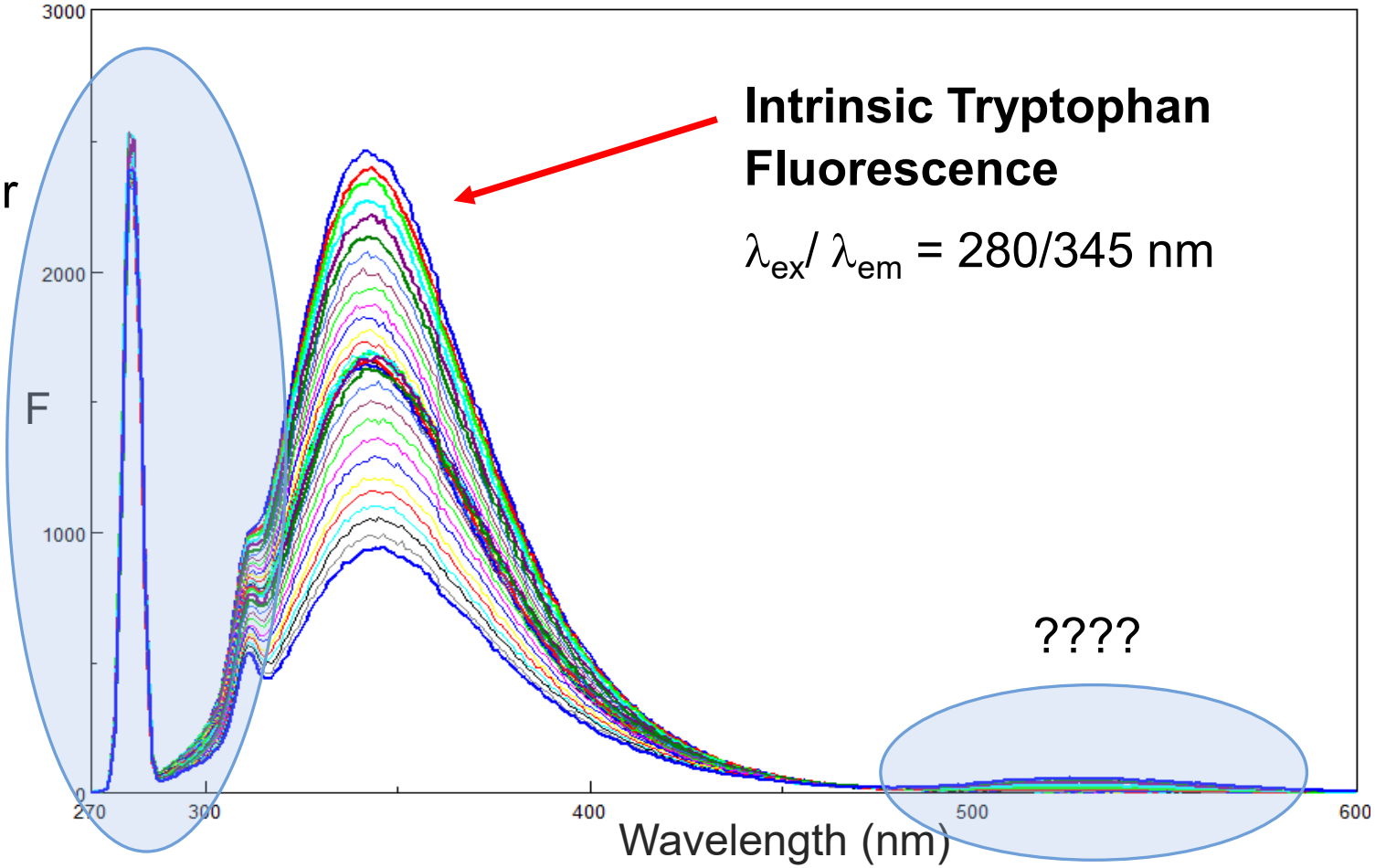
Format offers a wealth of information, including the ability to:

- Capture a bigger picture of what is going on with sample
- Monitor scatter contributions and aggregation
- Observe presence of unexpected species
- Extract thermal melt curves at multiple wavelengths of interest

Advantages apply to fluorescence, UV-Vis and CD thermal melts.

Scanning Wavelength Thermal Melts

Rayleigh and Raman scatter



Apo-RBP

λ_{ex}=280 nm

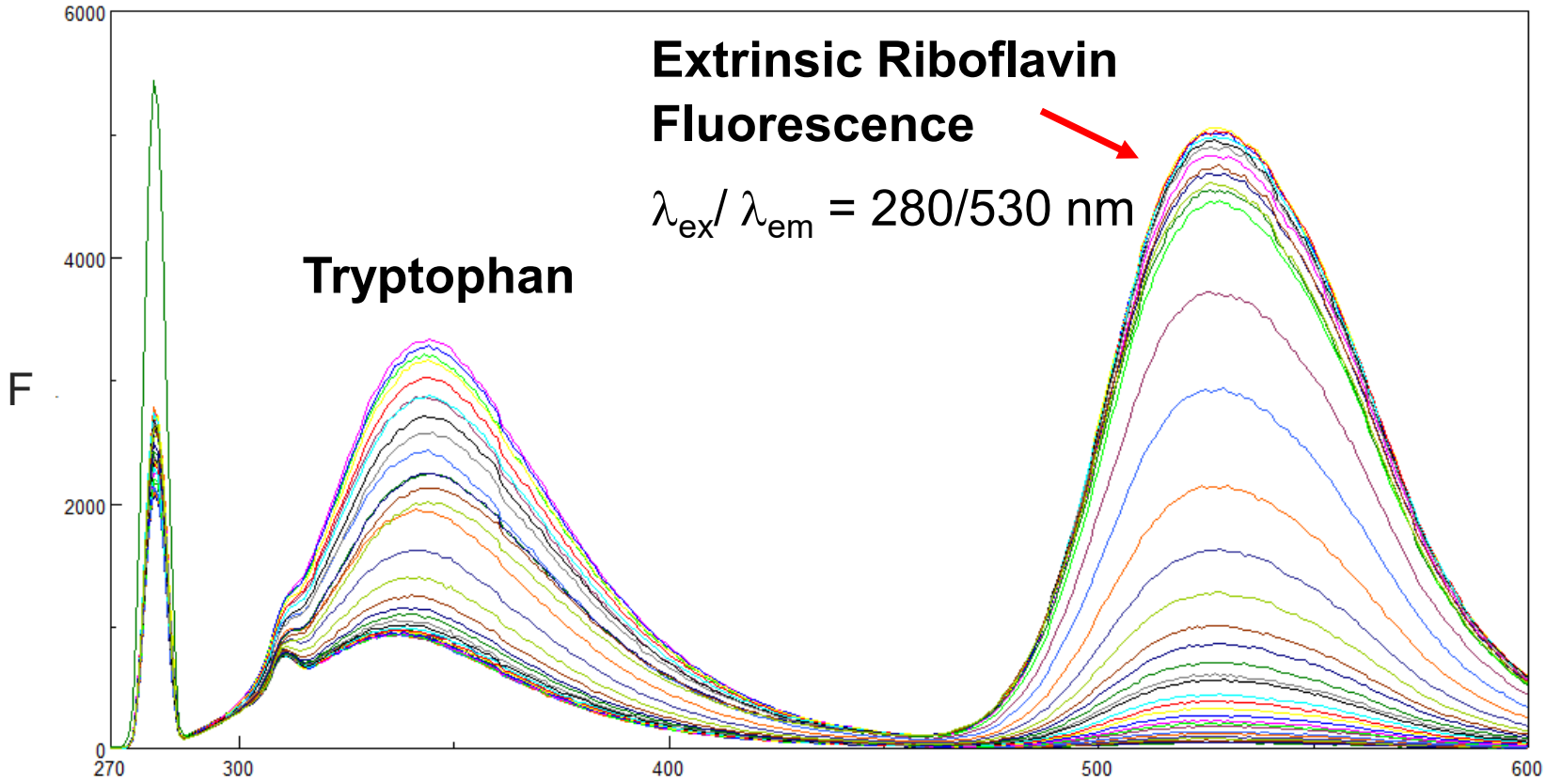
λ_{em}=270 – 600 nm

T range = 25 – 90 °C

Data Interval = 2 °C

Ramp Rate = 2 °C/min

Scanning Wavelength Thermal Melts



Holo-RBP

λ_{ex}=280 nm

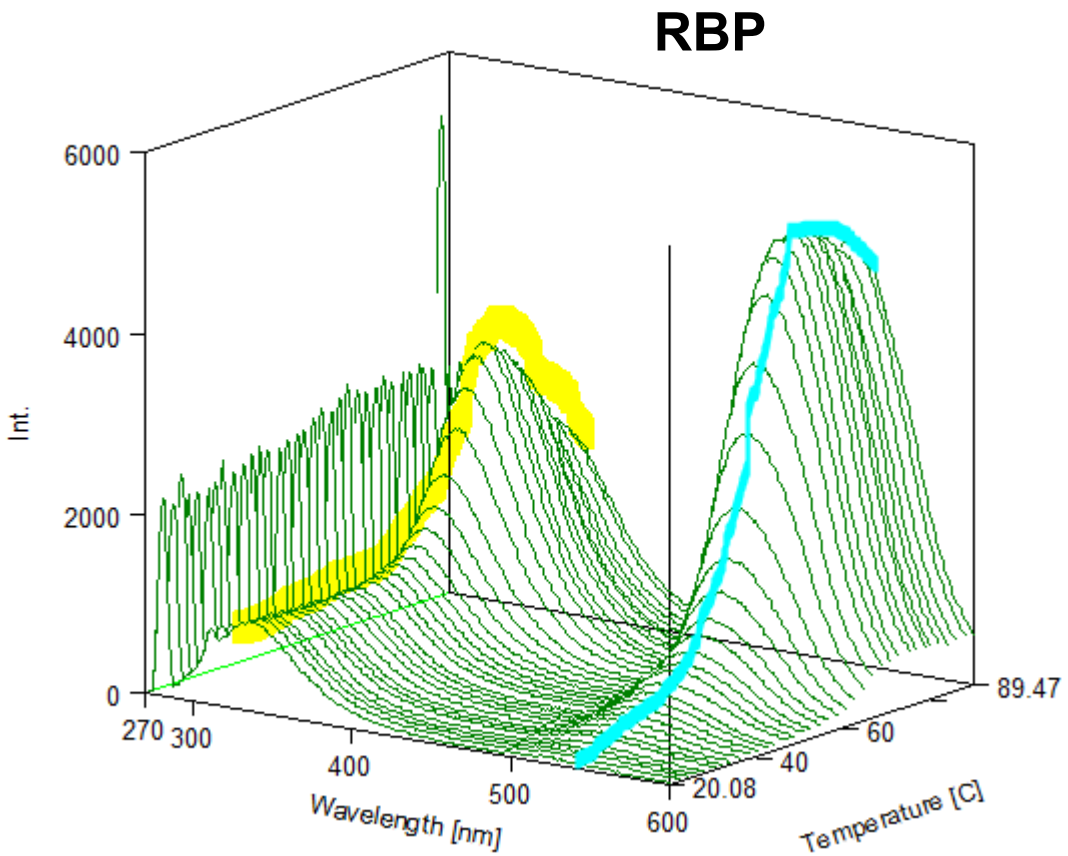
λ_{em}=270 – 600 nm

T range = 25 – 90 °C

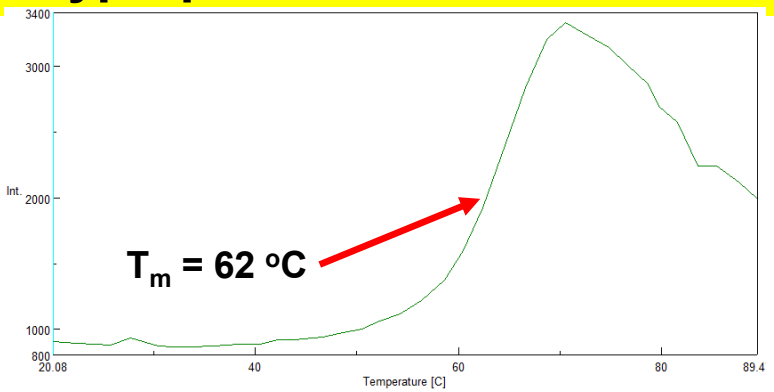
Data Interval = 2 °C

Ramp Rate = 2 °C/min

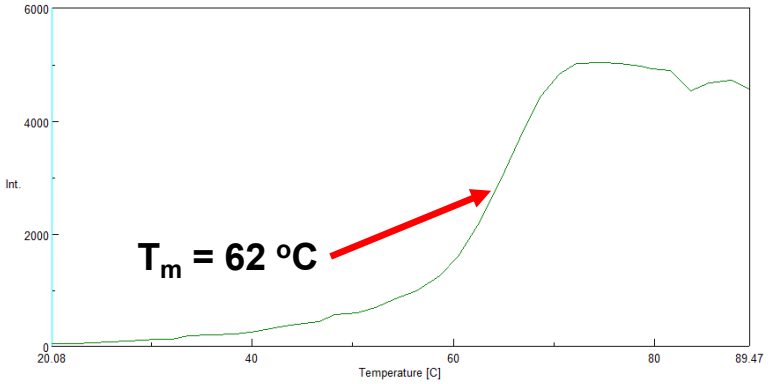
Extracting Single λ Melt Curves from Scanning Thermal Melts



Tryptophan 345 nm Em Slice



Riboflavin 530 nm Em Slice

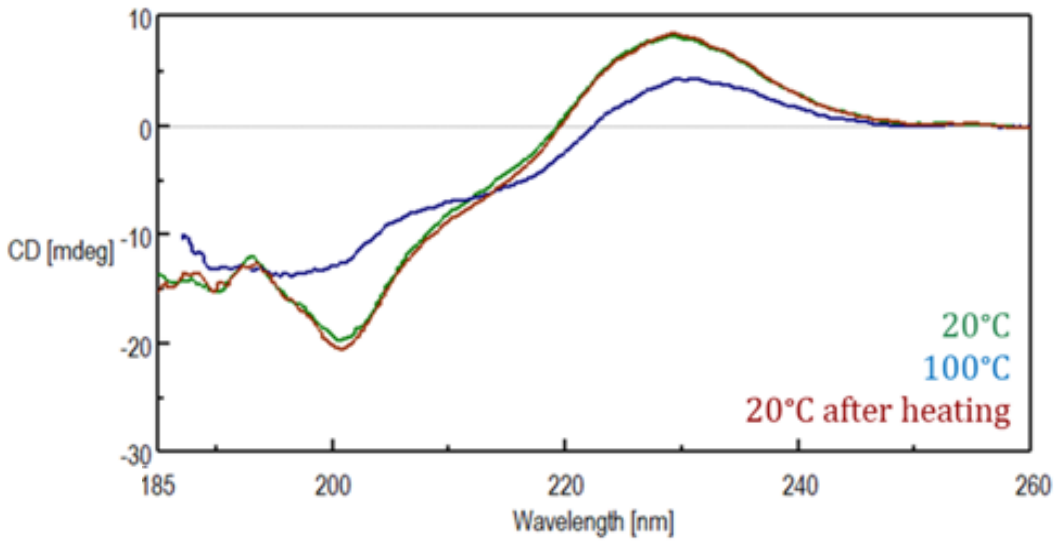


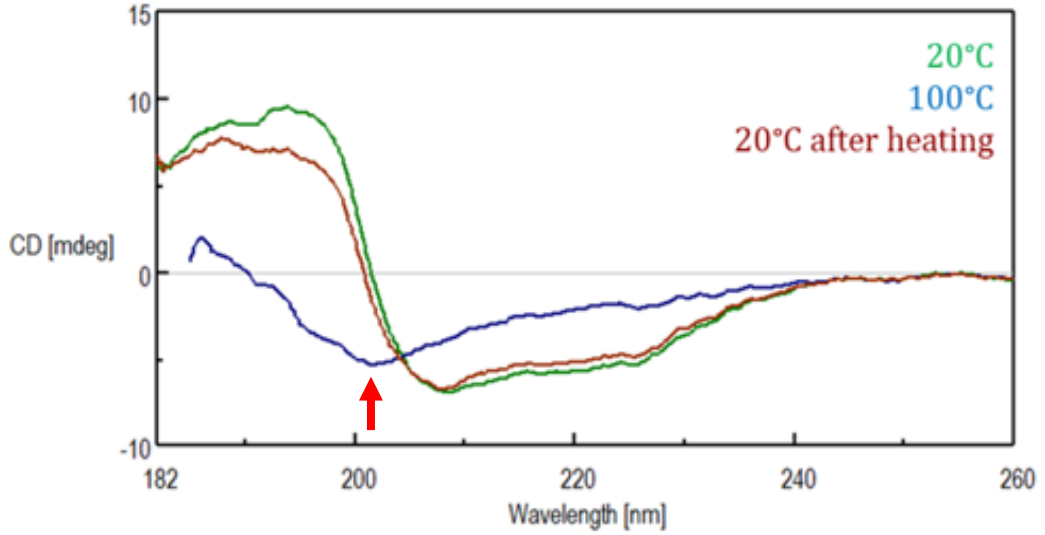
Scanning Wavelength Thermal Melts

Getting the most from scanning wavelength thermal melt curves: fluorescence, UV-Vis, and CD

- Start with these as a survey to determine best wavelengths to monitor in single-point melt curves
- Often run faster with less temperature resolution
- Use extracted melt curves to identify T range of greatest signal change
- Build an efficient single point thermal melt multi-stage profile which slows ramp rate and increases T points collected in this T range

Reversible Processes: Wavelength Temperature Scans for CD





- Protein folding appears to be reversible in both plots
- On Left plot: Always more information when looking at wavelength scans, but not really missing anything if only do a single point thermal melt
- On Right plot: Would miss the shift to shorter λ's at 100 deg C and not realize that change in secondary structure, if don't look at the full spectra data scans

Experimental Design and Optimization

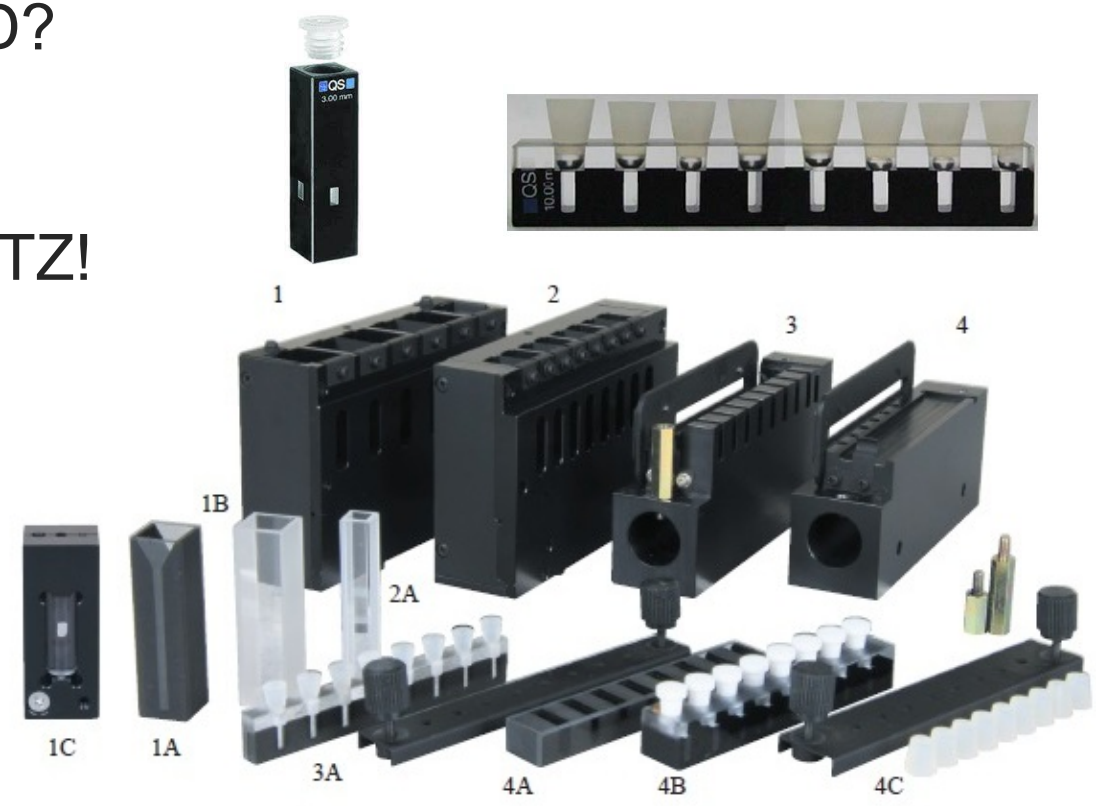
THERMAL MELT EXPERIMENTS

Cuvettes: What to ask…

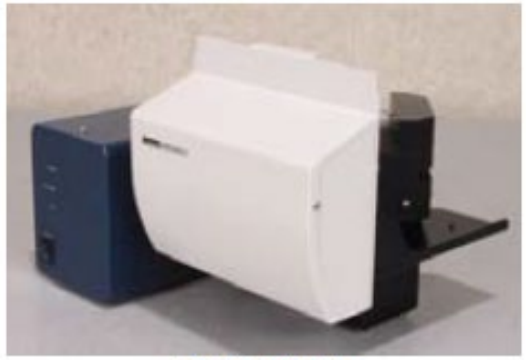
What techniques: UV, Fluorescence, CD?
How much sample volume?
Is stirring important? Is it possible?
What material: plastic or quartz? QUARTZ!
Black-masked? Z-height?

3x3 and 5x5 mm cuvettes with jacket

Low head space cuvette



Single-Cell vs. Multi-Cell Peltier



PCT-818

FP-8300 + PCT-818

Single-Cell vs. Multi-Cell Peltier

Single-Cell Peltier

- No moving parts
- Minimal alignment concerns
- Temperature Consistency
- Lower Cost

Multi-Cell Peltier

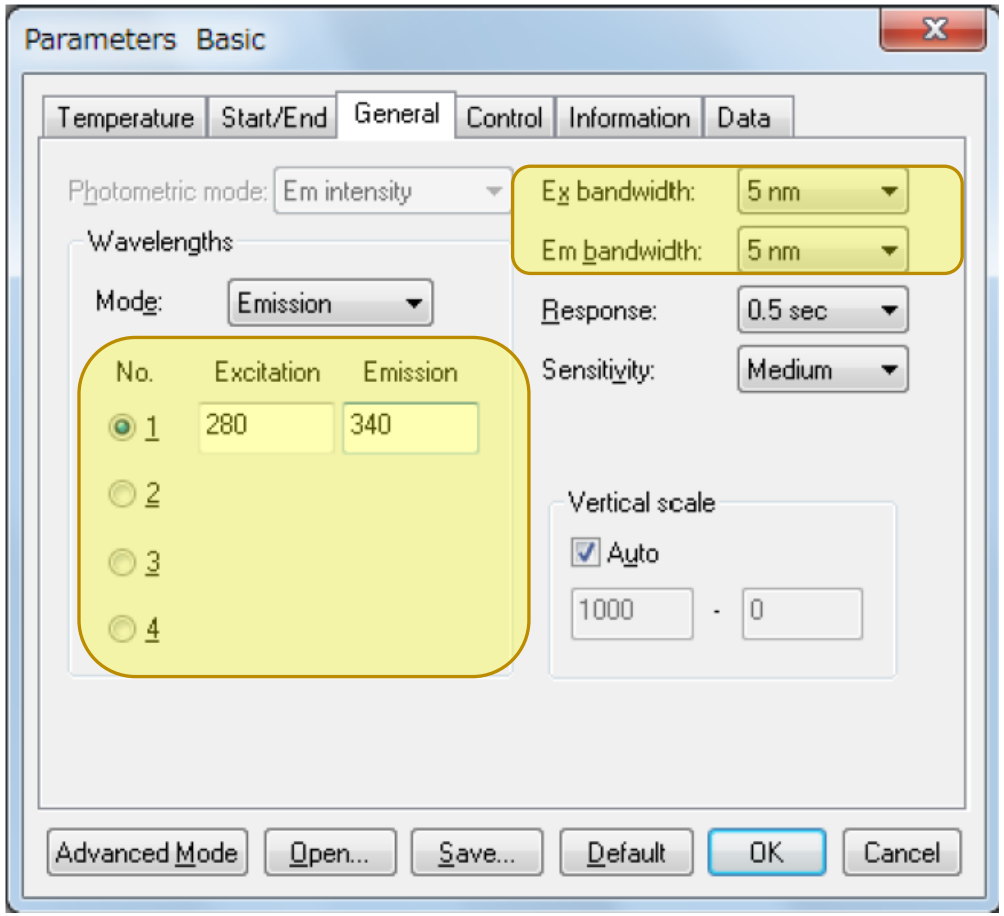
- Multiple samples in a single experiment
  - Replicates
  - Different conditions
- Especially valuable when running more time consuming experiments like thermal melts
- Can sacrifice a cell position to monitor solution temperature in cuvette with a T probe

Single/Multi-Point Thermal Melts Fluorescence: Basic Parameters

Up to 4 λ_{Ex} / λ_{Em} Pairs

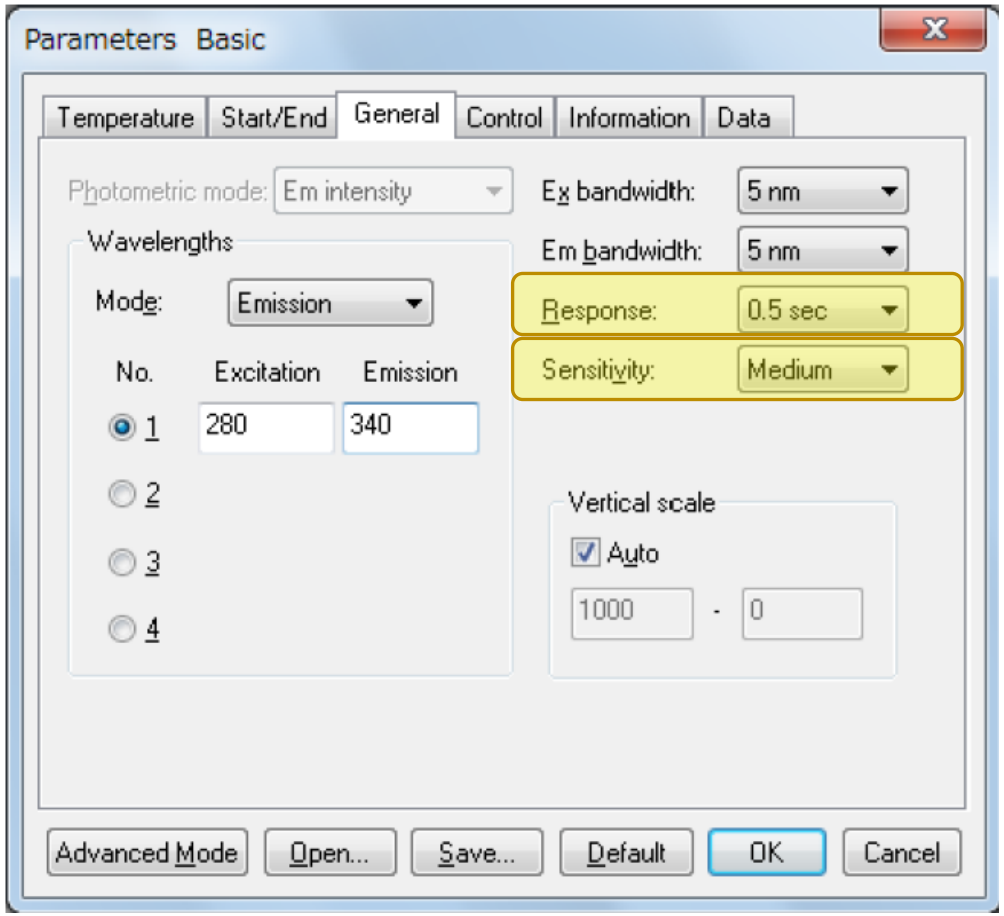
- Monitor several points of interest
- Can monitor scatter and aggregation

For UV and CD slightly different parameters and up to 8 wavelengths



SBW adjust for needed sensitivity and discrimination if multiple emitting species

Single/Multi-Point Thermal Melts Fluorescence: Basic Parameters



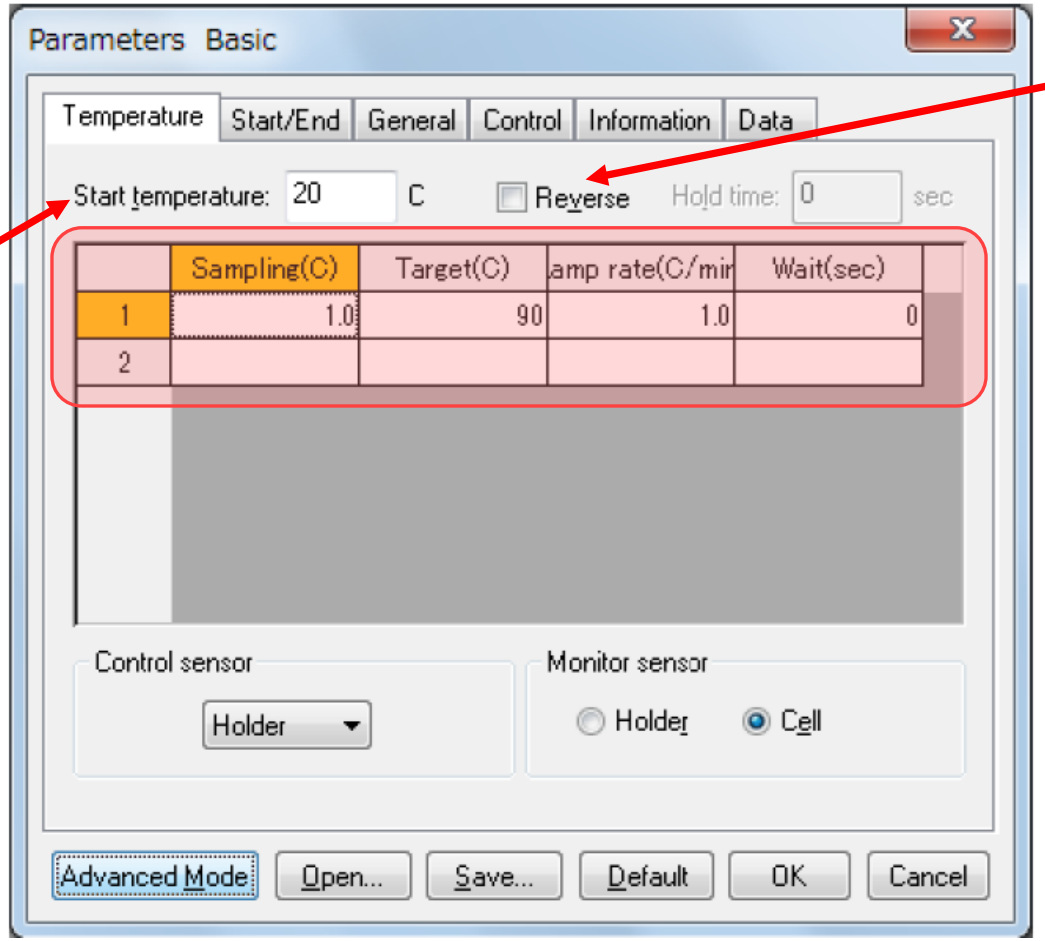
Response Single point collections allow for longer signal averaging times

Detector HV

- Set for maximized sensitivity to change in fluorescence
- Must consider what signal will max out at if fluorescence starts low and increases as T increases.

Thermal Parameters: Setting T Profile

Start Temperature
For T < ~15 °C use dry air or N_2 in sample compartment; prevents condensation on cuvettes and optics

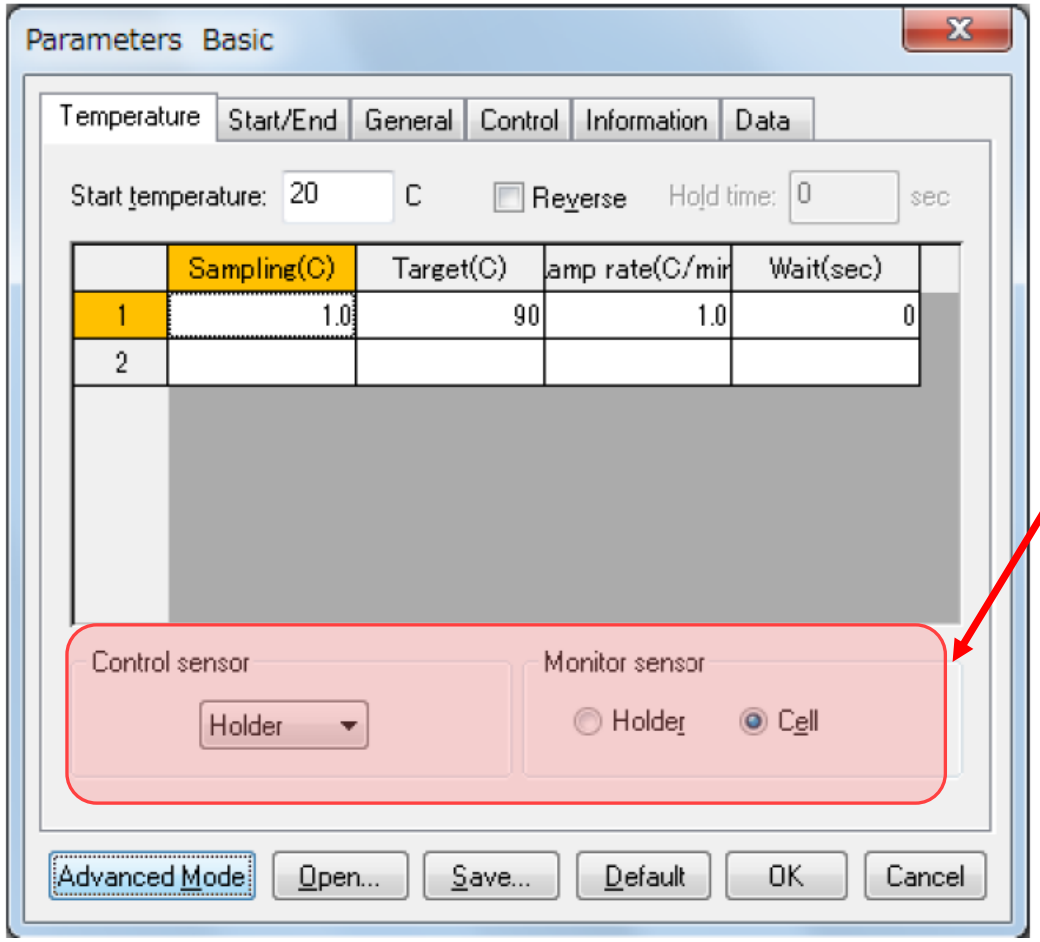


Reverse Run
Run melt back down to low temperature to check reversibility of process

Temperature Profile
Set melt conditions for

- **Data interval**
  - IMPORTANT!!
  - Controls data density
- **Final T** of the stage or run
- **Ramp Rate** for T change
- **Wait time** before taking measurement after reaching target temperature

Thermal Parameters: Monitoring vs. Controlling T



Temperature Control

Set how temperature is controlled and monitored

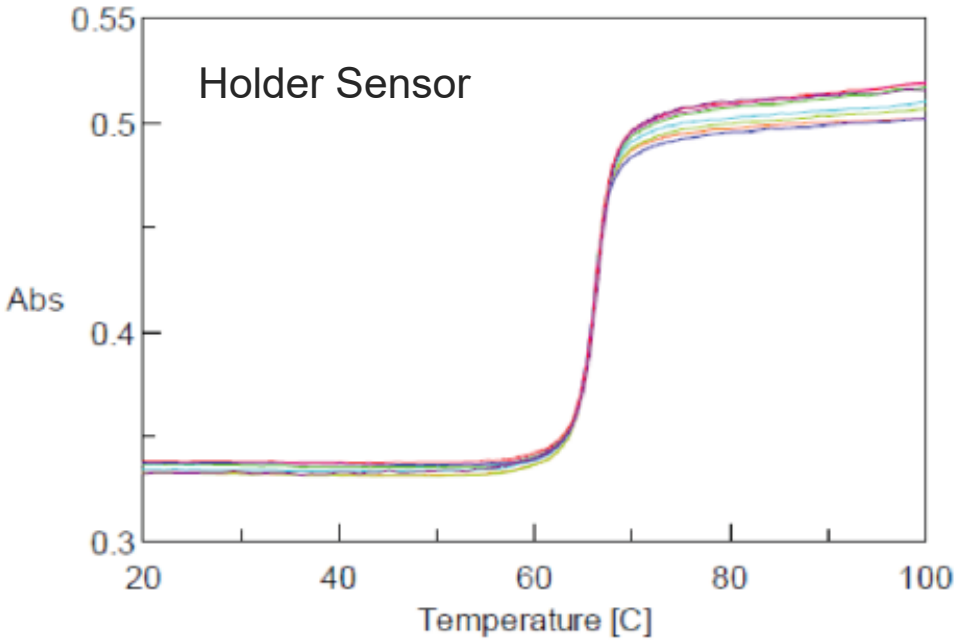
- **Control sensor** drives the Peltier to the target temperatures set in the profile
  - Data collection is triggered based on T of holder (Peltier block) or in-cell sensor
- **Monitor sensor** is what is plotted as temperature
  - Select whether use T data from Peltier block or in-cell sensor

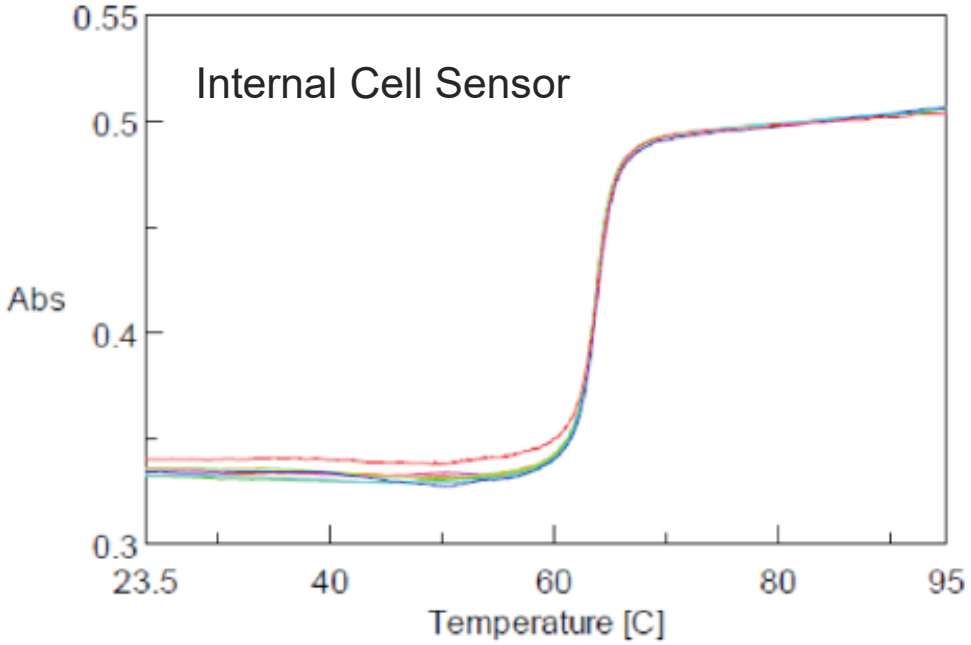
Monitoring the Holder vs. Inside Cell: A Micro UV Thermal Melt

Instrument: JASCO V-630 spectrophotometer with a 6 cell position Peltier
Sample: 20 ug/mL solution of poly (dA-dT)-Poly (dA-dT)
Cell: 8-channel micro cuvette, 7 were used for samples; 1 to monitor T

Measurement Conditions			
Wavelength	260 nm	Response	Fast
Ramp Rate	2°C/min	Start Condition	±0.01°C for 3 seconds
Data Interval	1°C (20-50°C), 0.1°C (50-70°C), 1°C (70-100°C),		

Monitoring the Holder vs. Inside Cell: A Micro UV Thermal Melt





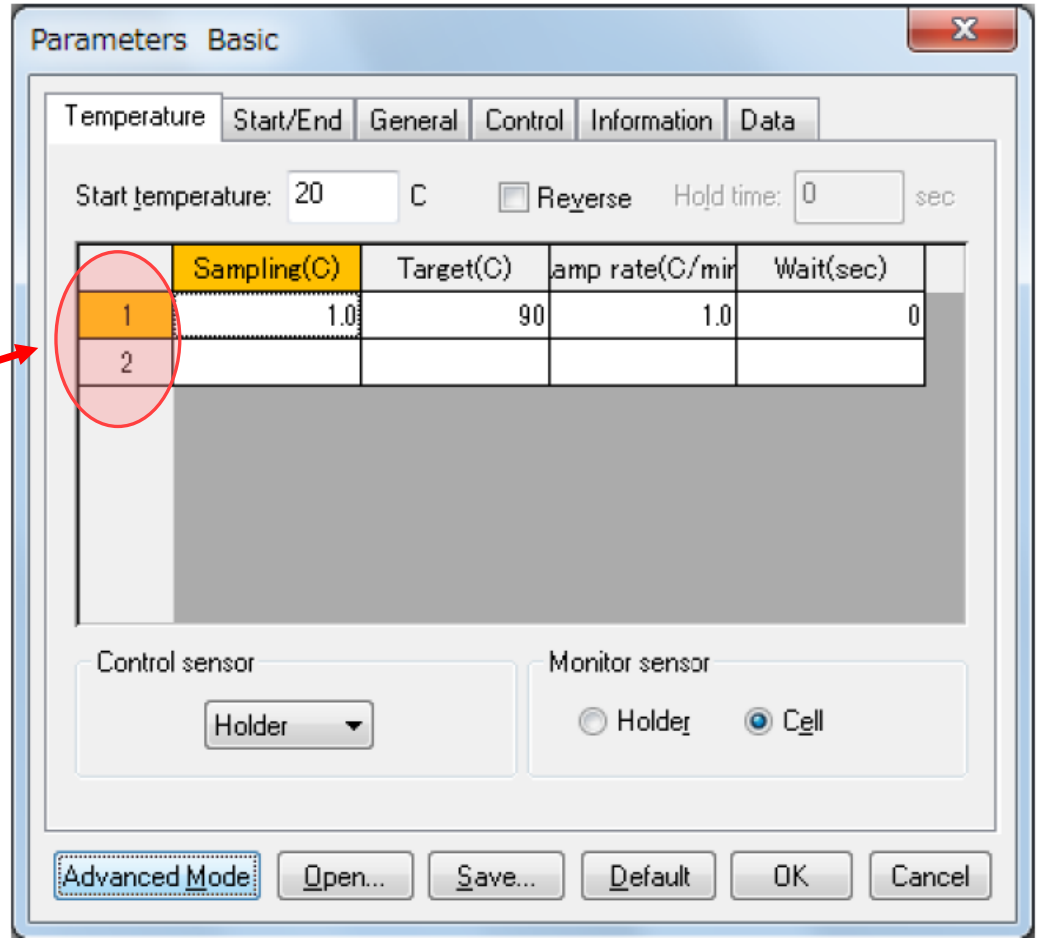
Monitoring the Holder vs. Inside Cell: A Micro UV Thermal Melt

	Temperature °C	
	Holder Sensor	Internal Cell Sensor
Cell 1	66.1	63.6
Cell 2	66.0	63.6
Cell 3	66.0	63.6
Cell 4	66.1	63.6
Cell 5	66.1	63.7
Cell 6	66.0	63.7
Cell 7	66.2	63.7
Cell 8	66.2	63.7
Average	66.1	–
Standard Deviation	0.08	0.08
C.V.	0.13	0.12

- T_m was about 2.5 °C higher for data collected monitoring holder sensor
- Both data sets were very reproducible
- An in-cell temperature sensor is strongly recommended for the most accurate T_m's

Thermal Parameters: Monitoring vs. Controlling T

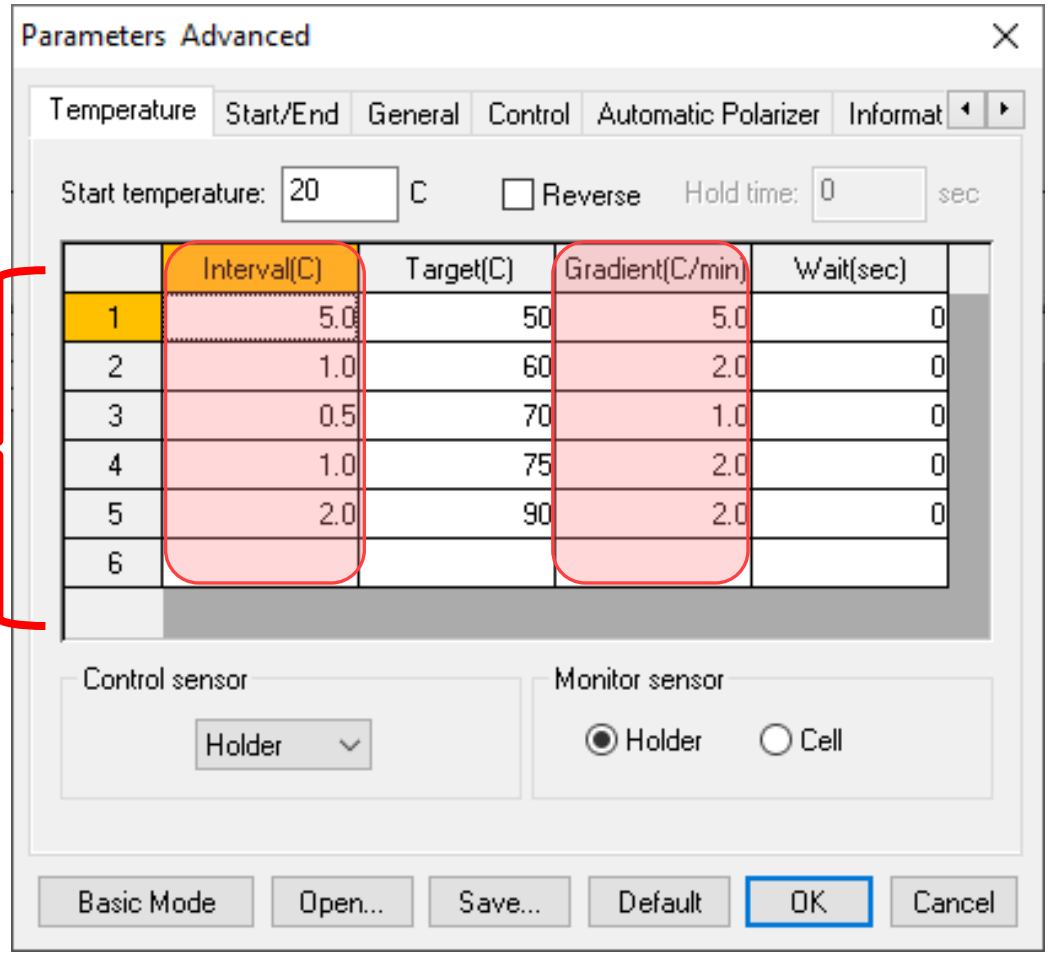
????



Thermal Parameters: Stages for Data Interval and Temperature Ramp Rate

Stages
allow user to optimize
- frequency of data collection and
- temperature ramp rate

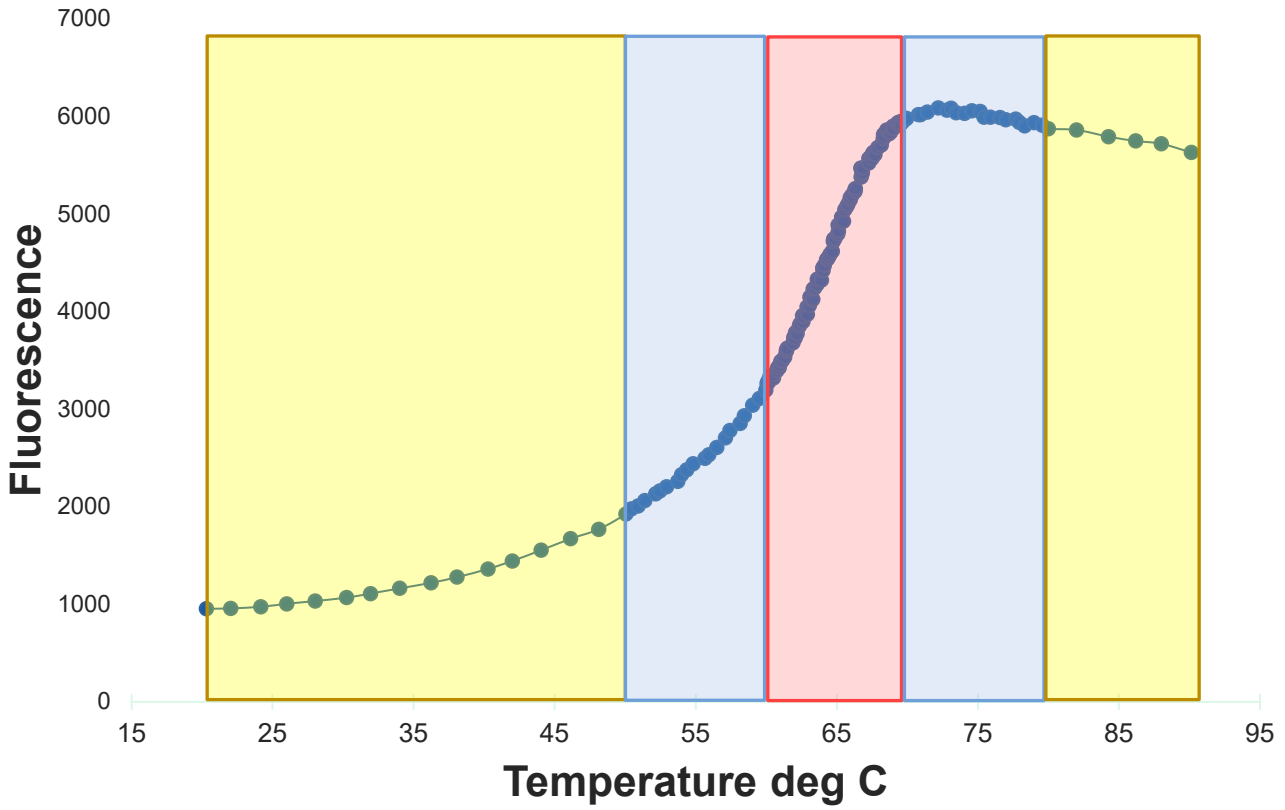
over desired temperature ranges



Thermal Parameters: Stages for Efficiency and Accuracy

Start T = 20 °C

Stage	Interval (°C)	Target T	Ramp °C / min
1	2.0	50.0	5.0
2	0.5	60.0	2.0
3	0.1	70.0	1.0
4	0.5	80.0	2.0
5	2.0	90.0	5.0

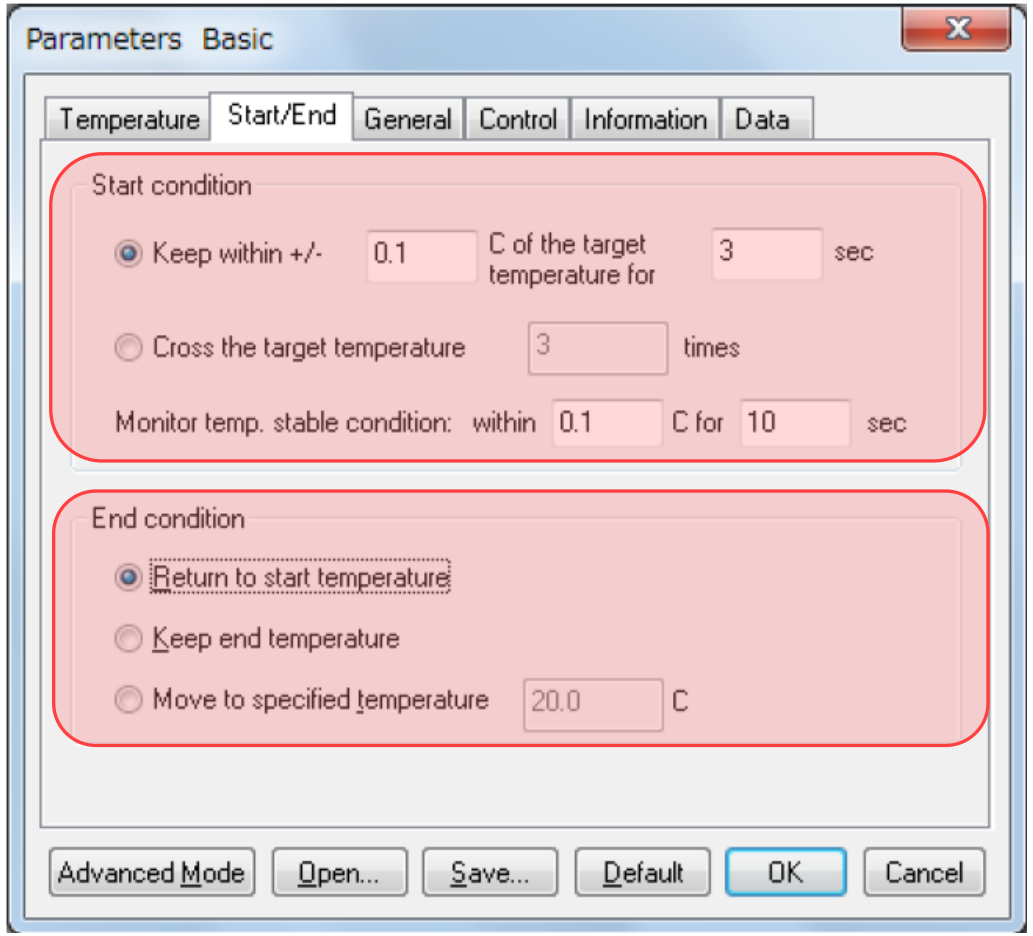


1. Efficiency of collection time
2. Accuracy of T_m calculation
3. Preservation of sample integrity

Thermal Parameters: Start and Stop Conditions

Control how to reach T!
Set conditions for how temperature is reached.

- Keep within a certain T
- Cross target temperature x times
- Stay within a fixed range of target temperature for a set time



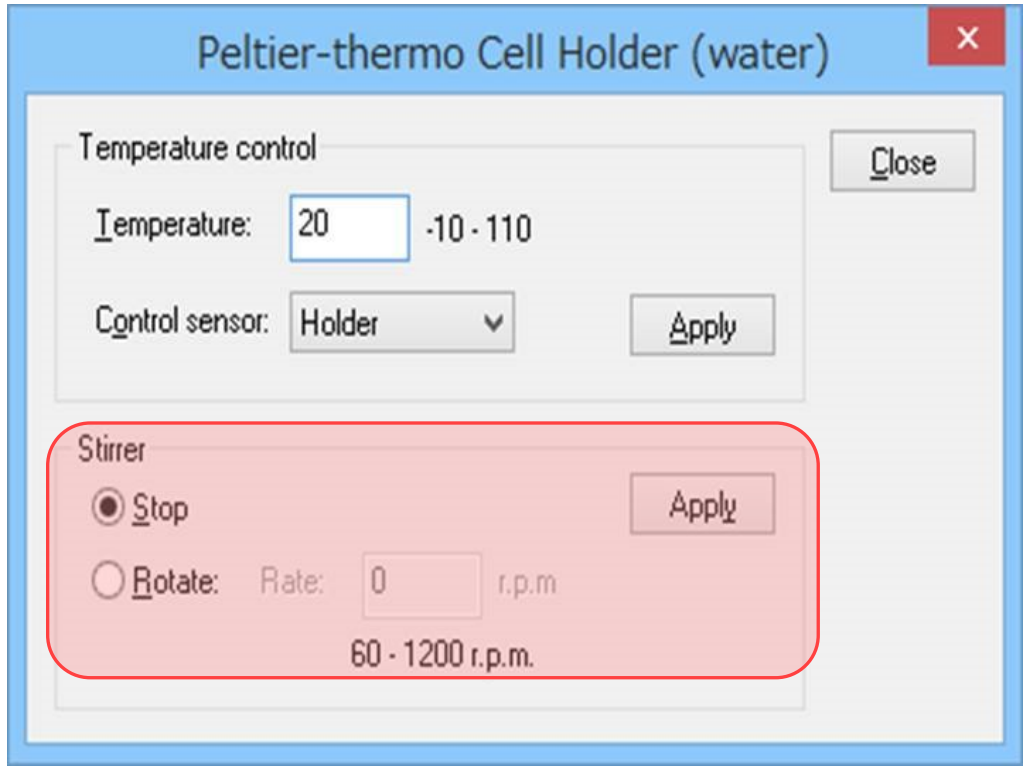
End T Conditions
Set T for end of experiment to:

- stay at current T
- return to start T or,
- move to a specified T

Thermal Parameters: Stirring

Stirrer

- Software control for consistency
- Faster and uniform thermal mixing



Data Analysis and Modeling: Good Practices

- Devote more time to data analysis
- Learn about the programs and try different ones
- Understand the calculations/models employed
- Be aware of limitations
- Determine when each is best to use
- Vary analysis parameters to see how sensitive results are to the changes
- Analyze replicate data

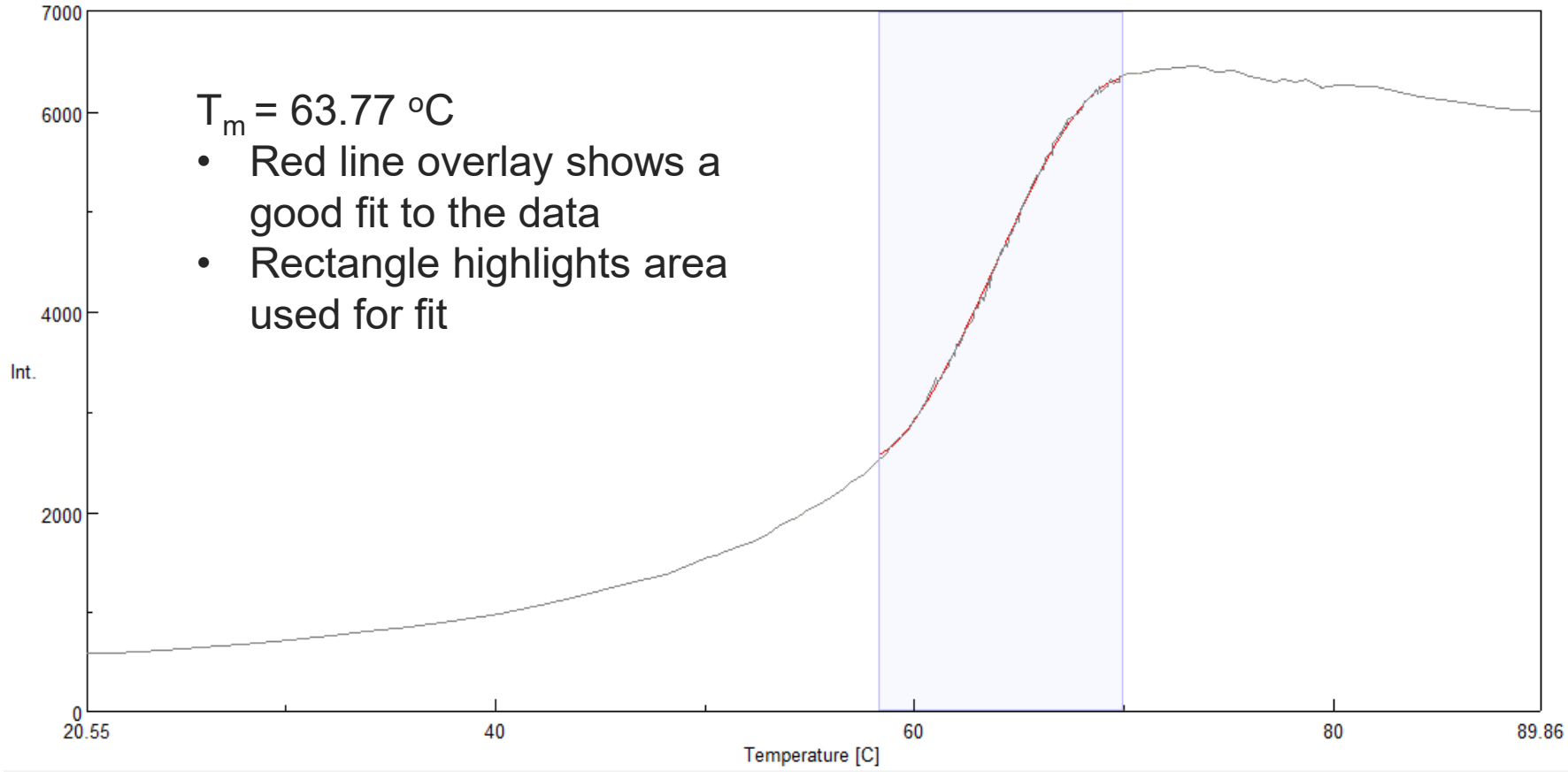
Data Analysis for T_m Calculations

Recall that T_m's are the inflection point on the sigmoidal shaped melt curve.

There are two main ways to calculate thermal melt temperatures from single-point and scanning thermal melt data

1. **2nd Derivative:** This equals zero at the inflection point due to the change in concavity or change in slope
2. **Least Squares:** This fits a line to the area of greatest change. It uses lines drawn though the initial and final states data to determine a bisecting line…as we viewed in the melt curve definition.

Data Analysis: 2nd Derivative Holo-RBP

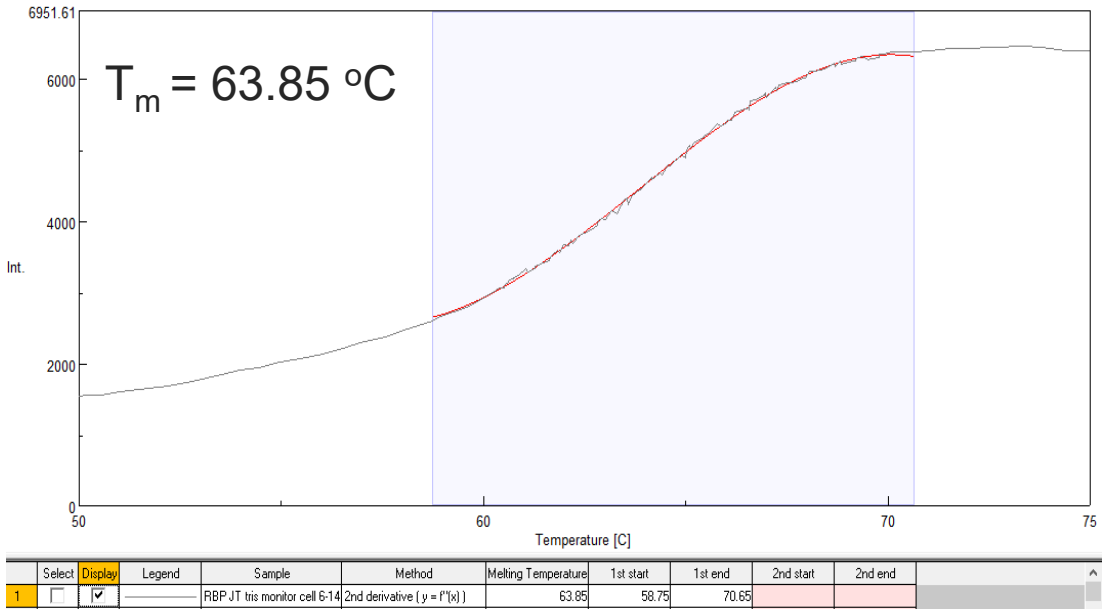


T_m = 63.77 °C

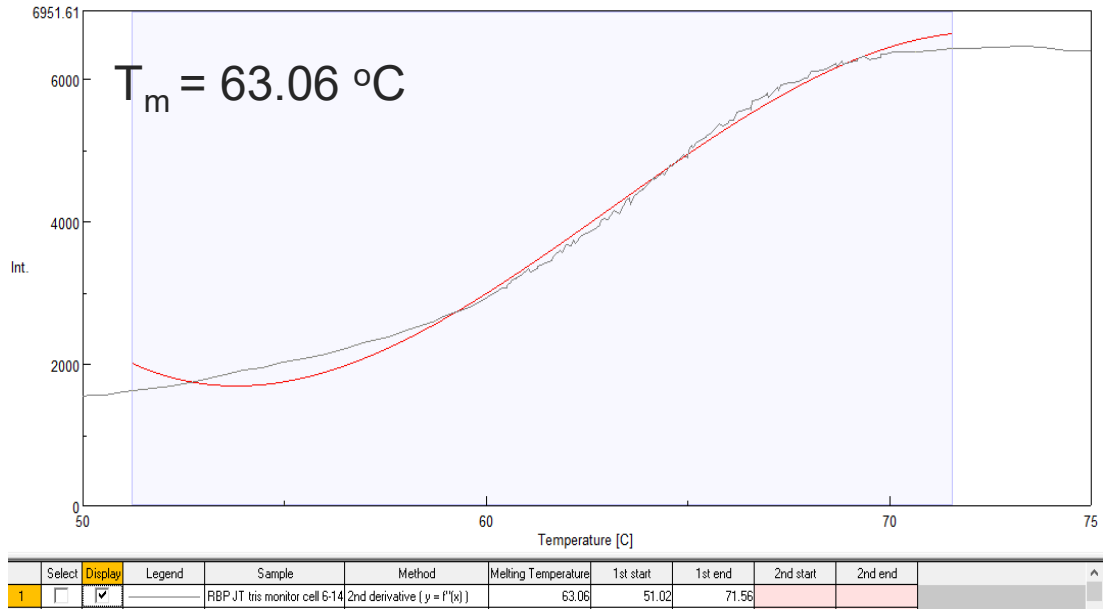
- Red line overlay shows a good fit to the data
- Rectangle highlights area used for fit

	Select	Display	Legend	Sample	Method	Melting Temperature	1st start	1st end	2nd start	2nd end
1	☐	☑	———	RBP JT tris monitor cell 6-14	2nd derivative (y = f''(x))	63.77	58.03	69.89		

Data Analysis: 2nd Derivative and a Broader Range for Analysis for Holo-RBP



	Select	Display	Legend	Sample	Method	Melting Temperature	1st start	1st end	2nd start	2nd end
1	☐	☑		RBP JT tris monitor cell 6-14	2nd derivative ($y = I''(x)$)	63.85	58.75	70.65		

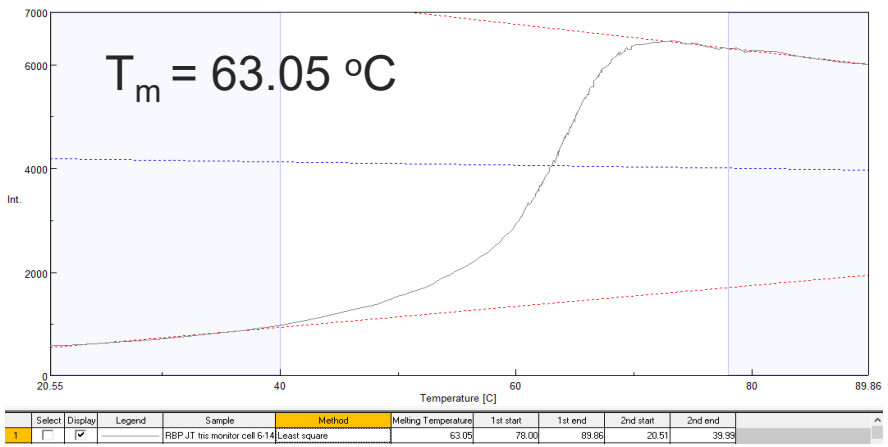


	Select	Display	Legend	Sample	Method	Melting Temperature	1st start	1st end	2nd start	2nd end
1	☐	☑		RBP JT tris monitor cell 6-14	2nd derivative ($y = I''(x)$)	63.06	51.02	71.56		

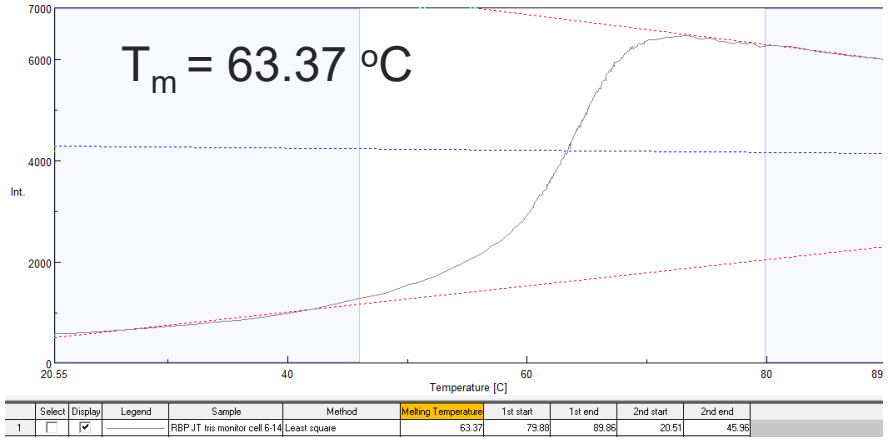
T_m °C
63.77
63.85
63.06

- Red line overlay visibly shows an increasingly poor fit to the data as T range for analysis is increased
- Varied area used for fit (temperature range to use)
- Same data set produced values that varied almost 0.8 degrees C depending on parameters used

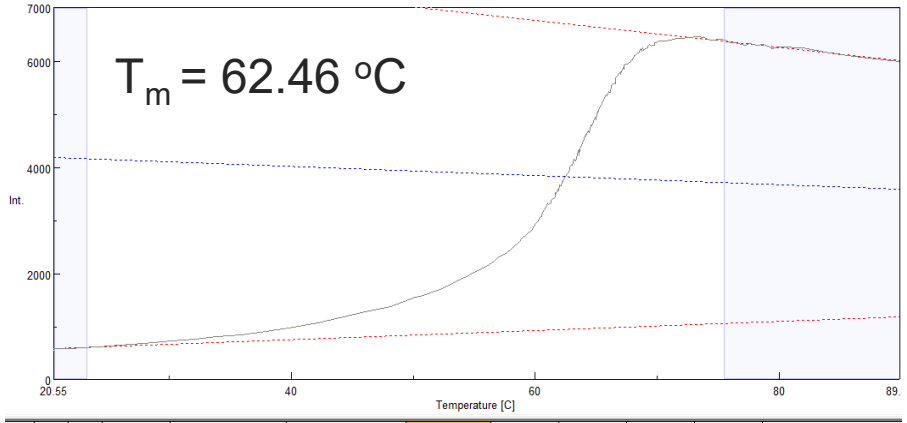
Data Analysis: Least Squares Holo-RBP



	Select	Display	Legend	Sample	Method	Melting Temperature	1st start	1st end	2nd start	2nd end
1	☐	☑		RBP JT tris monitor cell 6-14	Least square	63.05	78.00	89.86	20.51	39.99



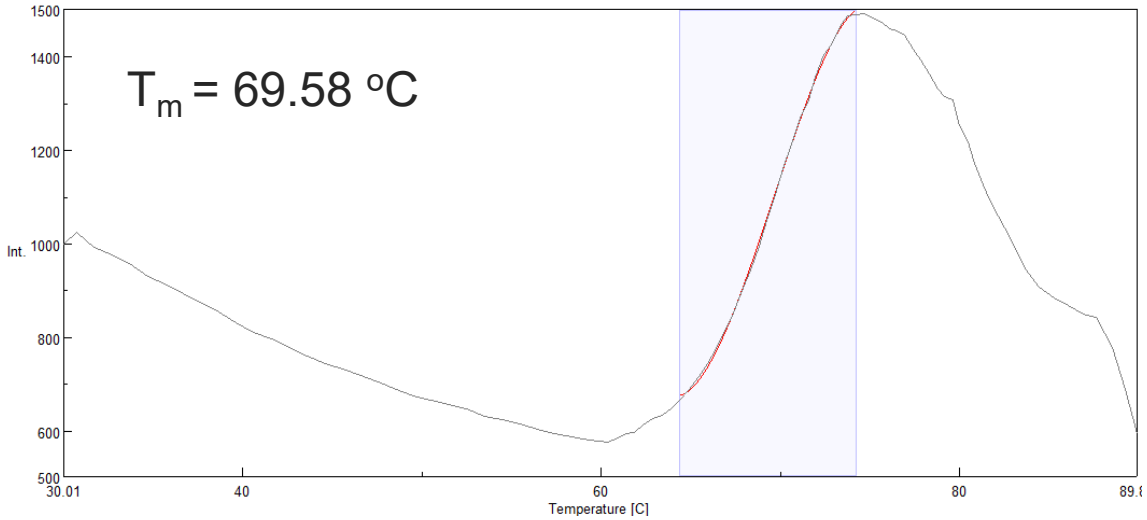
	Select	Display	Legend	Sample	Method	Melting Temperature	1st start	1st end	2nd start	2nd end
1	☐	☑		RBP JT tris monitor cell 6-14	Least square	63.37	79.88	89.86	20.51	45.96

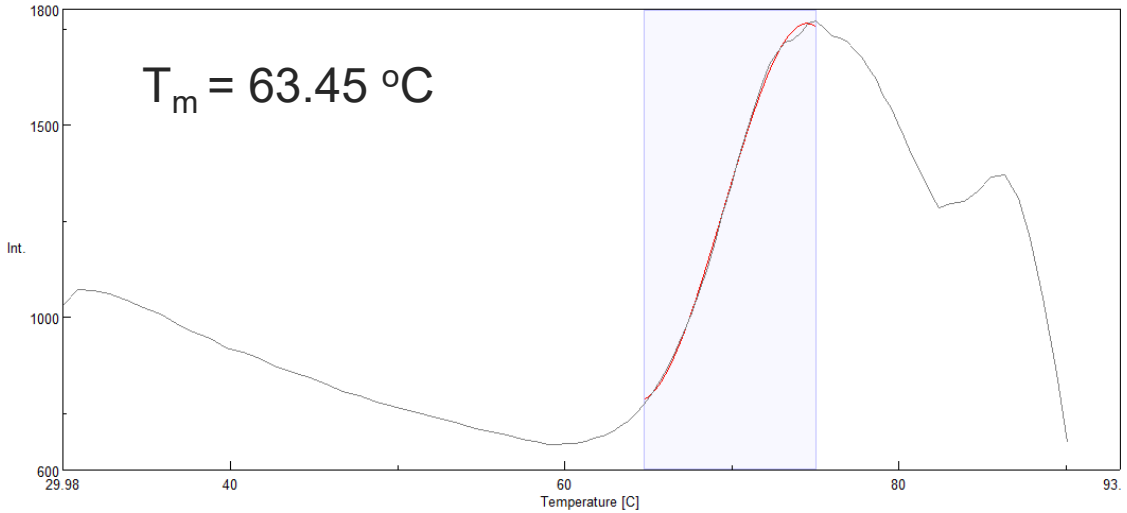


	Select	Display	Legend	Sample	Method	Melting Temperature	1st start	1st end	2nd start	2nd end
1	☐	☑		RBP JT tris monitor cell 6-14	Least square	62.46	75.50	90.07	20.51	23.23

- Lines are drawn for the initial and final states using the regions highlighted by the rectangles
- Varied temperature range to use for each region
- Same data set produced values that varied about 0.9 degrees C for LS analysis and was on the whole lower than the 2nd derivative method.

Data Analysis: Replicates BGG





Best to use 2nd derivative method!

Summary: Take-Home Messages

- Thermal melt studies can provide valuable stability data to better understand protein folding, binding events, nucleic acids, etc. and assist in identification of molecular candidates with desired properties
- Use Single-point and scanning wavelength thermal melts can be in combination to construct a more complete view of the changing chemistry/structure
- Combine multiple techniques like fluorescence, UV-Vis, and CD to get additional supporting information
- Set collection parameters carefully to ensure that the temperature being recorded is the same as the temperature inside the cell…don't make assumptions
- Use multiple temperature stages to ensure efficient data collection and highest quality data in the region of greatest change where it is needed the most
- Put more time into conducting and understanding data analysis. *Change analysis parameters* and note how it impacts the calculated value

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BGG thermal melt data using GloMelt

FP-8300 with ETC-815 Single Cell Peltier

Capital University, Chemistry Department

Joshua Tomsich and Dr. Tracey Murray

RBP thermal melt data

FP-8500 with PCT-818 Multi-Cell Peltier

J-1500 CD with Multi-Cell Peltier

V-730 and V-770 UV-Vis-NIR with PAC-743 6-position Peltier

JASCO Educational Resources

Webinars: https://jascoinc.com/learning-center/webinars/

- Vibrational Circular Dichroism
- Fluorescence Spectroscopy
- FTIR Theory, Instrumentation, and Techniques
- FTIR Microscopy
- Circular Dichroism Theory and Applications
- Circular Dichroism Measurement Optimization
- Raman Microscopy and Imaging
- SFC Theory and Applications

E-books and Tips and Tricks Posters

- Raman
- Fluorescence
- FTIR
- CD

KnowledgeBase

WE'RE TAKING A BREAK FROM WEBINARS IN JULY, *BUT* WE WILL START-UP AGAIN IN AUGUST WITH TWO EXCITING SPEAKERS….

Upcoming Webinars

Our Series will start up again in August with these two exciting guest speakers:

August 4th, 2020, 2 pm EDT

Dr. Luis Rodriguez-Saona

The Ohio State University, Department of Food Science and Technology

Metabolic fingerprinting for diagnosis of fibromyalgia and other rheumatology disorders using Raman microscopy analysis of bloodspots

August 11th, 2020, 2 pm EDT

Dr. Andrew Jacque

Water Quality Investigations, https://wqinvestigations.com/

Excitation-Emission Matrices as fluorescence fingerprints of biofilms: A unique approach to assessing, remediating and troubleshooting water quality and biological treatment processes

Thanks for joining us!!
Questions?