

Prometheus NT.Plex: CMI Getting Started Guide to Differential Scanning fluorimetry using Intrinsic Protein Fluorescence (NanoDSF)

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Introduction

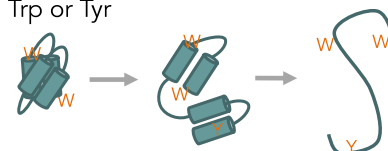
NanoDSF is a modified differential scanning fluorimetry method that monitors intrinsic tryptophan and tyrosine fluorescence as a function of temperature, time, or denaturant concentration. Tryptophan and tyrosine fluorescence intensity and wavelength maximum will vary as the local chemical environment changes. Significant changes occur when buried or packed aromatic side chains become solvent exposed upon unfolding. NanoDSF measures fluorescence intensity at 350 nm and 330 nm, and compares the ratio as a function of temperature or denaturant concentration. Free energies of folding and temperatures of unfolding measured using NanoDSF are comparable to values determined by differential scanning calorimetry (DSC) for a range of sample types, and NanoDSF is faster, easier, and uses less material. One limitation of NanoDSF is that the *protein must contain aromatic amino acids (tryptophan or tyrosine)* that change their local chemical environment upon denaturation.

Instrument Overview

The CMI has a **Prometheus NT.Plex instrument** from NanoTemper Technologies with aggregation optics. The CMI has these Data collection and analysis software packages:

- **PR.ThermControl** for thermal stability data collection
- **PR.ChemControl** for chemical stability data collection
- **PR.TimeControl** for time interval data collection
- **PR.Stability Analysis** for advanced data analysis

folded protein
with Trp or Tyr



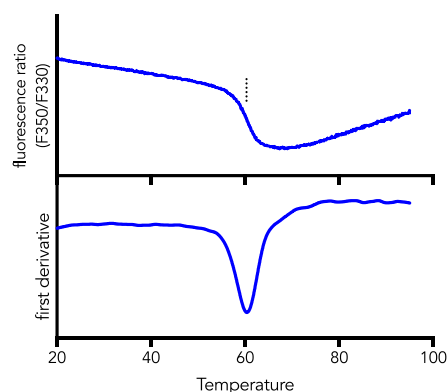
temperature

Applications

- Unfolding temperature (T_m and T_{onset})
- Critical denaturant concentrations (c_m)
- Free energy of folding (ΔG , $\Delta\Delta G$)
- Aggregation analysis (T_{agg})

Key Features

- Up to 24 measurements/experiment
- Chemical stability measurements in minutes
- Thermal stability measurements in 10 -120 min
- Sample volume 20 μ l
- Dye-free, detergent compatible
- Wide concentration range: 5 μ g/ml to 250 mg/ml protein
- Temperature range from 25 $^{\circ}$ C to 95 $^{\circ}$ C
- Simultaneous detection of protein unfolding and protein aggregation



Required Supplies

- NanoTemper NT.Plex Capillary Chips
 - 2x 8 Standard 24-Capillary Chips, NanoTemper Catalog # PR-AC002
 - 2x 8 High Sensitivity 24-Capillary Chips, NanoTemper Catalog # PR-AC006
- 384-well plates for loading capillaries (any type, V-bottom for minimal sample)

Sample Preparation

Assay Buffers

- DSF is a good tool for comparing the effect of different buffers on protein stability.
- Differences in buffer, salt and additive composition, concentrations of components, or pH can result in variability in protein thermal stability.
- When comparing thermal stability of mutant proteins or protein/ligand complexes, it is important to match buffers between samples.
- Don't use buffers that absorb or fluoresce in the UV range (e.g. Triton-X100 and Imidazole).

Samples

- 20 µl protein per measurement
- Perform replicate measurements.
- Minimal protein concentration depends on the number of fluorescent residues (Trp and Tyr).
 - Concentration of protein may need to be optimized for best results.
 - 0.2 mg/ml recommended for most proteins with tryptophan residues
 - For tyrosine-only proteins, use 5-10X more protein (1-2 mg/ml).
 - BSA at 2 mg/mL typically requires 20% excitation power.
- Protein aggregates can interfere with the NanoDSF signal.
 - Assess protein heterogeneity via light scattering.
 - Purify protein samples with soluble aggregates by size-exclusion chromatography.

Getting started

Resources

Additional resources are available at the instrument, including: the Prometheus Manual, and the PR.ThermControl, PR.ChemControl, and PR.TimeControl Software Manuals.

Experimental Tips

- Use high sensitivity capillaries when fluorescence signal is low.
- Membrane proteins may require higher concentrations and/or high-sensitivity capillaries.
- Minimize bubbles by incubating samples at RT (or starting temperature) and/or by spinning down the setup plates before filling the capillaries.
- Wells need to be filled completely.
- Chemical denaturation experiments require a long incubation period (typically at least 8-10 hours at the experimental temperature) prior to measurement but are fast to collect (discovery scan only that takes a couple of minutes).
- Use capillary sealant for experiments exceeding 3 hr.

- Non-aggregating protein samples will typically have scattering intensity ~90-110, which will increase 10-20X on aggregation.

General Care and Maintenance

- Keep the mirror clean.
 - Avoid touching the surface.
 - Wipe gently with water and with alcohol to clean.
- Always return the capillary chip cover to the instrument after use.

Startup

1. Book time on the PPMS calendar before you start.
2. Login to the computer using your PPMS credentials (eCommons ID and password).
3. Turn on Prometheus NT.Plex instrument (back, left).
4. Setup a NanoDSF experiment.
 - a. Prepare samples in a 384-well plate (at least 20 μ l/well).
 - b. Load the NT.Plex capillary chips with sample, making sure to fill capillaries completely. Use the Capillary Filling Station for convenient loading from a 384-well plate.
 - i) Pay attention to the chip orientation when filling.
 - (1) Two arrows mark the end of the capillary chip (closest to capillary 24).
 - (2) Generally, position 1 should be on the left of a 384-well plate.
5. Open Door on Prometheus.
6. Remove the metal capillary chip tray cover.
7. Place capillary chip on the mirrored surface being careful to avoid fingerprints and spills.
 - a. Position 1 at the front of the platform, Position 24 (closest to the arrows) at the back.
8. Cover the capillary chip with the metal capillary chip tray cover.
9. Close Door on Prometheus.

Data Collection

Thermal Stability Measurement

1. Open PR.ThermControl Software.
2. Create New Project (or open existing project).
3. Discovery Scan (Tab 1):
 - a. Discovery scan is used to detect fluorescence intensity and to position each capillary
 - b. Set Excitation Power to 20 % and click Start Discovery Scan.
 - c. Adjust the power from 1-100 % as needed until the fluorescence intensity is within the upper and lower limits of the detector, marked by red dashed lines. The Optimal Detection Range is 2,000-15,000 counts.
4. Melting Scan (Tab 2):
 - a. Capillaries within the dynamic detection range are auto-selected. You may select additional capillaries that are below the detection limit.
 - b. Set the Temperature Ramp.
 - i) Temperature Ramp of 1 C/min – 2 C/min is recommended.

- ii) 1 C/min (or slower) will most closely mimic DSC data.
 - iii) 7 C/min for a quick sample assessment (T_m may deviate significantly).
 - iv) Remember to use the same temperature ramp when comparing data.
 - c. Set the Start and End Temperatures.
 - i) Start Temperature: 20 C (15 C minimum).
 - ii) End Temperature: 95 C (maximum).
 - d. Start Measurement.
 - i) Plot 350/330 ratio, 350 nm, 330 nm data as raw, or raw and first derivative, for stability data.
 - ii) Plot scattering for aggregation analysis.
 - iii) Export as Chart (image file) or Processed Data (Excel or CSV).
- 5. Annotation and Results (Tab 3).
 - a. Annotations may be made before, during, or after measurement.
 - b. Multi-selection is possible with click and drag (Ctrl+click or Shift+click).
 - c. Annotate a selected group by typing in one of the selected fields.
 - d. Use Create New Column to add annotation fields.
 - e. Use Assign Colors to change colors of selected capillaries.
 - i) Single Color: assigns selected capillaries to one color.
 - ii) Categories Colors: colors according to unique annotation by selecting on fields in a column to be used for coloring (e.g. Sample ID).
 - iii) Gradient Colors: colors in gradient by selecting on fields in a column to be used for coloring (e.g. capillary number)..
 - f. Proceed to PR.Stability Analysis software for additional data analysis options.

Chemical Stability Measurement

1. Open PR.ChemControl Software.
2. Plan:
 - a. Name, Target, and Denaturant.
 - b. Delta dilution values must be constant between capillaries (e.g. 0.25 M).
 - c. Enter Highest Concentration and either Delta Dilution or End Concentration.
3. Results:
 - a. Detector - select which detectors to fit: e.g. ratio 350 nm/330 nm.
 - b. Fit – choose two-state or three-state model.
 - c. Export Chart or Raw data.
4. Proceed to PR.Stability Analysis software for additional data analysis options.

Time Stability Measurement

1. Open the PR.TimeControl Software.
2. Due to the variability in experimental design and more qualitative nature of these experiments, there is no data analysis package for Time Control experiments. You may export the processed data for analysis in 3rd party software packages.
3. See the instructions in PR.TimeControl Software Manual for the following options:
 - a. Isothermal.
 - b. Two-Temperature Cycle.

- c. Incremental Cycle.
- d. Temperature Stepping.

Data Analysis

For PR.ThermControl (Thermal Stability) and PR.ChemControl (Chemical Stability) Measurements:

1. Open the PR.Stability Analysis Software.
2. Select New Analysis PR.ThermControl or PR.ChemControl

PR.ThermControl Data Analysis

1. Open PR.ThermControl Data file.
2. Merge Data (optional):
 - a. Software should auto-merge data based on annotations made in the ThermControl software.
 - b. Add additional data files (optional):
 - i) Expand Analysis Data tab (Sidebar).
 - ii) Click Add Files and choose additional files to add.
 - c. Edit annotations (optional).
 - d. Merge Sets via the Menu bar after adding additional data or changing annotations.
3. Assign Reference (optional).
4. Select a sample (a single capillary or a set) in the data table and then click assign reference (Menu Bar).
5. A reference is a sample to which all others are compared to and is used to measure similarity between samples and ΔT_m and ΔIP .
6. Data Table summarizes the calculated data.
7. Inflection Points (IP#1, IP#2) are calculated automatically and represent peaks in the first derivative of the ratio (350/330), 350 nm, 330nm, or scattering data.
8. Expand Merge Sets to see individual data for each capillary in the set.
9. Exclude data by clicking the checkbox.
 - a. Warning signs indicate an air bubble was detected (exclude sample).
10. Charts and Display Options.
11. Choose the data you wish to view, such as: Ratio 350/330, First derivative, or Scattering.
12. Thermal Stability Fit:
 - a. Click Add Region of Interest above the graphs and choose Two-State Fit or Three-State Fit.
 - b. Click on the graph to select the region of analysis (select a low temperature before the inflection point and a high temperature after the inflection point).
 - c. Evaluate all or some of the data using these criteria.
 - d. More than one analysis can be performed with different ranges or samples.
13. Choose Key Parameters from the Menu Bar to summarize and compare data.
14. Export:
 - a. Batch Export (left panel) allows selection of batch export options.
 - b. Graphs and Tables can be exported with export button above.

PR.ChemControl Data Analysis

1. Open the PR.ChemControl Data file.
2. Merge Data:
 - a. Software should auto-merge data based on annotations made in the Control software.
 - b. Add additional data files (optional):
 - i) Expand Analysis Data tab (Sidebar).
 - ii) Click Add Files and choose additional files to add.
 - c. Edit annotations (optional).
 - d. Merge Sets via the Menu bar after adding additional data or changing annotations.
3. Assign Reference (optional):
 - a. Select a sample dataset in the data table and the click assign reference (Menu Bar).
 - b. A reference is a sample to which all others are compared and is used to measure similarity between samples and ΔT_m and ΔIP .
4. Data Table summarizes the calculated data:
 - a. Select dataset(s) to view Charts and fit data.
 - b. Labels:
 - i) ΔG : Free Energy of Unfolding.
 - ii) C50: Concentration at which 50 % is unfolded.
 - iii) D_0 : Fraction denatured at 0 M denaturant.
 - c. Exclude data by clicking the checkbox.
 - i) Warning signs indicate an air bubble was detected (sample should be excluded).
5. Charts and Display Options and Chemical Stability Fit:
 - a. Choose the data you wish to view, such as Ratio 350/330, First derivative, or Scattering.
 - b. Choose Selected Fit Model: Two-State or Three-State to best fit your data.
6. Choose Key Parameters from the Menu Bar to summarize and compare data.
7. Export:
 - a. Batch Export (left panel) allows selection of batch export options.
 - b. Graphs and Tables can be exported with export button above.

Shutdown

1. Clean up in and around the instrument.
2. Discard capillaries in the sharps container. You can break away individual capillaries if you have not used an entire cassette, then save the cassette for a future experiment.
3. Return capillary chip tray cover to the instrument and close the door.
4. Close the control and analysis software.
5. Turn off the instrument power.
6. **Logoff from PPMS!**

Data Management

Technology	Nano Differential Scanning Fluorimetry (nanoDSF)
Instrument	NanoTemper Prometheus NT.Plex
Recommended Repository	Generalist Repository

Data Collection (Thermal Denaturation) Software

Current Version	PR.ThermControl, Version 2.3.1
Data Files (Type, ~size)	experiment file (therm control) .prc 10-30 MB/project
	raw data (therm control) .xlsx 2 MB/project

Data Analysis Software

Current Version	PR.Stability Analysis, Version 1.1
Data Files (Type, ~size)	analysis file .pra 2-6 MB/project
Readable Exports	processed data .xlsx ~500 KB/sample
	results table .xlsx ~30 KB/project

Data Collection (Chemical Denaturation) Software

Current Version	PR.ChemControl, Version 1.4.3
Data Files (Type, ~size)	experiment file (chem control) .prcc 10 MB/project
Readable Exports	raw data (chem control) .xlsx 6 KB/sample

Data Collection (Time Control) Software

Current Version	PR.TimeControl, Version 1.0.2
Data Files (Type, ~size)	experiment file (time control) .prtime 2 MB/project
Readable Exports	raw data (time control) .xlsx 60 KB/project

Book time and Report Problems through the PPMS system: <https://ppms.us/hms-cmi>

- rates are based on *booked and real-time usage*.

Contact cmi@hms.harvard.edu with questions.

last edited: 2024-12-10