QuantStudio 6/7: CMI Getting Started Guide to Differential Scanning Fluorimetry

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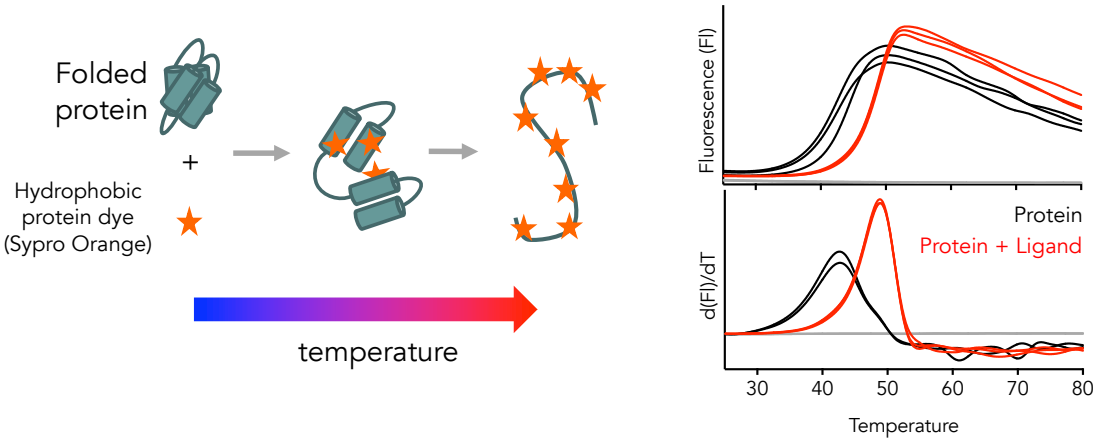
Introduction

Differential Scanning Fluorimetry (DSF) uses a real-time PCR instrument to monitor thermally induced protein denaturation by measuring changes in fluorescence of a dye that binds preferentially to unfolded protein (such as Sypro Orange, which binds to hydrophobic regions of proteins exposed by unfolding). This experiment is also known as a Protein Thermal Shift Assay, because shifts in the apparent melting temperature can be measured upon the addition of stabilizing or destabilizing binding partners or buffer components.



Instrument Overview

The CMI has a modified Quant Studio 6/7 from Life Technologies for conventional DSF. It is a hybrid between a QuantStudio 6 and 7, with decoupled excitation and emission filters.

Applications

- Unfolding temperature (T_m)
- High-throughput buffer optimization
- Small molecule binding screen

Key Features

- Rapid data collection (~30 min/96-well plate)
- Low sample consumption (typically 1-5 µg/well)
- Proprietary LifeTechnologies Protein Thermal Shift Analysis Software

Required Supplies

- 96-well FAST-block optical plate, e.g. LifeTechnologies MicroAmp FAST optical 96-well reaction plate, 0.1 mL, 4346907
- Optical adhesive film, e.g. LifeTechnologies MicroAmp Optical Adhesive Film, 4360954
- DSF compatible dye, e.g. LifeTechnologies Protein Thermal Shift Dye Kit, 4461146 (Sypro Orange)

Sample Preparation

Assay Buffers

- Differences in buffering agent, salt, additive composition, concentrations of components, or pH can result in variability in protein thermal stability.
    - DSF is a good tool for comparing the effect of different buffers on protein stability
- When comparing thermal stability of mutant proteins or protein/ligand complexes, it is important to *match buffers between samples*.
- Hydrophobic buffers, including those with detergent, will bind to Sypro Orange and lead to high background staining.
- Assay buffer should never include protein, as any protein will bind to Sypro Orange and the unfolding of the buffer protein will likely drown out the signal of your protein of interest. This includes BSA, which has very high background staining with Sypro Orange.

Fluorescent Dye

- Concentration of fluorescent dye should be optimized for best results
- Sypro Orange, a hydrophobic dye that binds protein upon denaturation, is the most common DSF dye. Hydrophobic dyes are incompatible with detergent and other hydrophobic buffer components.
- Sypro Orange dyes are sold as either 1000X Protein Thermal Shift dye kit or 5000X Sypro Orange Protein Stain. *These reagents have the same concentration,* and both be used dilutions of 1/200 – 1/1000:
    - 1000X Protein Thermal Shift dye kit, final concentration 0.2X-5X (1X most common)
    - 5000X Sypro Orange Protein Stain, final concentration 1X-20X (5X most common)
- Cysteine-reactive dyes, such as Bodipy-cystine, which can be used with membrane proteins in detergent if the protein has a buried free cysteine.

Protein Samples

- Concentration of protein should be optimized for best results
- Protein: 0.05-10 µg/well (1-5 µg)
- Total volume: 10-30 µL/well (20 µl)

Getting Started

Resources

Additional resources are available at the instrument, including control and analysis software.

Experimental Design Tips

- Perform 3-4 replicates
- Perform a No-Protein Control (NPC) with buffer and fluorescent dye to test for reaction of buffer components with the dye and/or background fluorescence

General Care and Maintenance

- Keep the instrument door (on the right side) free of obstruction.
- If the control software won't connect to the instrument, restart the software. If the control software still won't connect, cycle the power on both the instrument and the computer.

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. Set up the DSF plate
   a. Mix protein, buffer and fluorescent dye (added last).
   b. Load 96-well FAST block optical plate. Include replicates and No-Protein Controls.
   c. Cover the plate with optical film
4. Tap the touchscreen to wake the instrument from sleep by pressing the power button.
5. Open the instrument door.
6. Place the plate in the instrument and close the door.

Data Collection

1. Open **Quant Studio Real-Time PCR Software**.
2. On the Setup tab, select Template
   a. Open the file Standard_Protein_Thermal_Shift_template_x1m3.edt (located on the desktop).
3. Setup Tab.
   a. Experiment Properties.
      i) Experiment name: provide a name
      ii) Instrument: **Quant Studio 7**
      iii) Block: FAST 96
      iv) Type: Melt Curve
      v) Reagents: Other
      vi) Properties: Standard
   b. Define.
      i) Targets: Target 1
         (1) Reporter: ROX
         (2) Quencher: NONE
      ii) Passive Reference: NONE
   c. Assign
      i) Select entire plate (even if only partially filled)
         (1) this will avoid losing data from human error in well selection
         (2) The entire plate is read no matter which wells are selected
      ii) Do not label wells at this time (labels are not transferred to the analysis software).
   d. Run Method.
      i) Set reaction volume: 20 µL.
      ii) Graphical View (set ramp parameters)
         (1) select continuous collection.
         (2) 25 ˚C 2 min, 1.6˚ C/s.
         (3) 0.05 ˚C/s ramp.

(4) 99 ˚C 2 min (approximate run time 30 min).

e. Optical Filters: select x1-m3 for **melt curve filter**; deselect others (x4-m4 is the standard ROX set, which works but has a lower S/N).

4. Save experiment to your folder.
5. Run Tab: click Start Run and select the instrument number from the pulldown
6. (x4-m4 is the standard ROX set. If you select x1-m3, then you will receive an error message stating that the ROX filter set has not been selected… run it anyway).
7. After data collection, **you must press the Analyze button before proceeding to data analysis**.
8. Save experiment.

Data Analysis

The Protein Thermal Shift Software is proprietary and can only be used to analyze data collected on Life Technologies instruments. It can be used to merge and compare data, and to fit thermal inflection points using a first-derivative model or Boltzmann model.

The Protein Thermal Shift software determines the Tm in two ways. The first method calculates the first derivative of the melting curve and uses the peak center to define the inflection point(s) in the thermal transition. The first derivative method works when there is a single transition or if there are multiple transitions). The second method is to fit a region of analysis to a Boltzmann 2-state equilibrium model (a type of sigmoid fit), where the Tm is the midpoint in the unfolding equilibrium (the melting temperature). For many samples with a single thermal transition, these methods will typically give results that are similar but not identical. If so, either method can be used, just make it clear in your method description, which method you are using. For samples with multiple transitions, you'll need to use the first derivative. When comparing samples, you should apply a consistent method to all.

1. Open **Protein Thermal Shift Software**.
2. Create Study
3. Setup Tab
   a. Study Properties.
      i) Define study name.
      ii) Select instrument type: Quant Studio 6/7.
   b. Conditions. Add conditions to define well content, eg:
      i) Protein
      ii) Ligand
      iii) Buffer
      iv) Analysis Group (each analysis group can have only one reference sample type)
   c. Experiment Files
      i) Import data saved from QuantStudio Real-Time PCR software.
      ii) Assign wells.
         (1) Task
            (a) Samples: protein and dye-containing regular samples.

(b) Reference: the sample to which all other samples in the analysis group are compared, and from which ΔTm is calculated.
(c) NPC (no protein control) is used to examine background buffer interference with dye fluorescence (should be minimal).
(2) Analysis group –all samples you wish to be compared.
(3) Well components.

4. Analysis Tab.
   a. Go to **Well Results** to examine traces and descriptions, then process data with **Analyze** button.
      i) Review the results of each experiment paying attention to agreement between replicates.
      ii) Examine the results table for error flags and adjust your analysis settings or omit samples, if needed.
      iii) The shape of the first derivative (dFl/dT) can tell you a lot about your sample and can indicate the presence of multiple peaks.
         (1) Many peaks can indicate a low signal strength, and therefor the results should be omitted or interpreted very carefully.
         (2) 2 or more well-defined peaks in the first derivative indicate that you should change the analysis setting to multiple peak (which will identify more than one derivative peak, and disable the Boltzmann fit).
   b. Go to **Replicate Results** to get statistics on replicate groups and to compare Tm values for all samples.
5. Export Raw or processed data.

Shutdown

1. Remove your plate from the instrument.
2. Clean up in and around the instrument.
3. Close the control and analysis software.
4. Logoff from PPMS!

Data Management

Technology	Differential Scanning Fluorimetry (DSF)
Instrument	Life Technologies Quant Studio 6/7
Recommended Repository	Generalist Repository

Software Type	**Data Collection**		
Current Version	QS Real-Time PCR Software, version 1.7.1		
Data Files (Type, ~size)	experiment file	.eds	2-10 MB/plate

Software Type	Data Analysis		
Current Version	Applied Biosystems Protein Thermal Shift, version 1.2		
Data Files (Type, ~size)	experiment file	.eds	2-10 MB/plate
Readable Exports	raw data	.csv	2 MB/plate
	analyzed data	.csv	12 KB/project
	analyzed data	.txt	29 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.

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