

ThermoFluor Assay

By Jennifer Keeffe

April 2013

Background:

The ThermoFluor assay is a quick, temperature-based assay to assess the stability of proteins. It can be used as an alternative to CD melts to determine melting temperatures. Jan Kostecki in Steve Mayo's lab has been using this assay to assess the thermal stability of designed proteins.

The method takes a small amount of protein (45 uL of 5 uM) and adds SYPRO Orange dye. The dye is similar to ANS and binds to hydrophobic patches/denatured protein/molten globules and fluoresces. As you increase the temperature and the protein unfolds, you can monitor the increase in fluorescence and determine a melting temp. The temperature and fluorescence monitoring are done using a qPCR machine (real time PCR).

References:

Lavinder et al. High-throughput thermal scanning: a general, rapid dye-binding thermal shift screen for protein engineering. *J. Am Chem. Soc.* 2009: 3794-3795.

Ericsson et al. Thermofluor-based high-throughput stability optimization of proteins for structural studies. *Analytical Biochemistry* 2006: 289-298.

Phillips and Hernandez de la Pena. The Combined Use of the Thermofluor Assay and ThermoQ Analytical Software for the Determination of Protein Stability and Buffer Optimization as an Aid in Protein Crystallization. *Current Protocols in Mol. Biol.* 2011: 10.28.1-10.28.15.

Reagents:

Optically clear PCR strips: [USA Scientific Optically Clear 8-Tube Strips Item #1402-2500 \(\\$79/125 strips\)](#) or [USA Scientific Optically Clear 12-Tube Strips Item #1402-2600 \(\\$106.50/80 strips\)](#)

SYPRO orange: 5000X concentration in DMSO; [Life Technologies S-6650 \(\\$189/500uL\)](#)

Samples:

3 x 45 uL of 5 uM in buffer of choice -- run each sample in triplicate

Include buffer + dye (no protein) as a control. This signal should be subtracted from the signal from the proteins

Reactions:

Add 45uL of 5uM protein to PCR tubes (or plates) in triplicate

Make fresh 200X SYPRO orange solution in the buffer of choice (from 5000X stock)

Add 5uL of 200X SYPRO orange solution to each sample

Instrument: Elowitz Lab qPCR machine

File --> Open --> Protocol --> Jennifer K --> Fast Melt (method takes about 40 minutes)

25 °C for 30 sec

Hold at temperature for 30 sec, take a reading, increment up 0.5°

Go from 25° to 99° then return to 25°

Click on the plate

Select the wells and choose "Unknown" sample

Select Scan mode "FRET"

Save as --> plate_template

Start run

Close lid (automated)

Click on start run and save result file

Data Analysis:

The qPCR machine exports data (in Excel format) for both the raw fluorescence signal and the derivative of the fluorescence signal as a function of the temperature.

Take the average of the three replicates (either fluorescence signal or derivative). Subtract the average of the three replicates of buffer+dye (no protein control).

Plot the corrected average of the fluorescence signal or derivative as a function of temperature.

Fluorescence Signal plots: The significant increase in slope corresponds to the melting of the protein. The T_m is at the inflection point of this slope. As the protein unfolds, more dye binds and the signal increases. In some cases, two transitions can be seen--this may be from different Ig domains with the Fabs and IgGs. The starting fluorescence also indicates something about the starting state of the protein. Higher values (~4000+) indicate the protein is "less happy," partially unfolded, or completely unfolded.

Derivative plots: The temperature at which the derivative is the lowest (most negative) is the melting temperature.

Sample Data:

1. The high initial fluorescence signal of the Molten Globule and the Conalbumin samples indicate that the proteins are not well behaved under these conditions (e.g., partially unfolded). Typically, samples will have an initial fluorescence of <4000 units at low temperature if they are well behaved.
2. The lack of a transition in the molten globule samples indicates that the protein is not well folded.
3. The Conalbumin pH 8, Cellulase pH 8, and Cellulase pH 3 samples all exhibit transitions of unfolding (~50°C, ~55°C, and ~70°C respectively).
4. The change in pH from 8 (purple curve) to 3 (dark green curve) significantly improves the apparent stability of the cellulase: from an apparent T_m of 50 to 70°C. Therefore buffer conditions can significantly alter the apparent T_m of the protein and that effect can be seen with this method.

