innovative medicines initiative

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Thermal Shift Assay for screening inhibitors

Version: 1.0

Version Date: October 2020

1. Rationale/Aim

Thermal shift assay (or Differential Scanning Fluorimetry, DSF) is a quick and uncomplicated assay for screening inhibitors for proteins of interest without a need of prior knowledge of their substrates or activities. The assay measures unfolding of the protein under investigation in a temperature range of 25-95°C through an increase in fluorescence signal of the dye (SYPRO^{TM} Orange), which interacts with hydrophobic parts of the unfolded protein. Binding of inhibitors can enhance protein stability, resulting in an increase in their melting temperature (Tm). Usually higher Tm shifts (ΔTm), calculated from the difference between Tm of native and inhibitor-bound form, correlate with greater stabilization effects of the tested inhibitors, which is in turn an indicative of their higher affinity. Several studies have shown that this assay can be used for detecting inhibitor binding for many protein classes, such as kinases and bromodomains, and the affinities of inhibitors have been shown to correlate well with ΔTm values.[1]

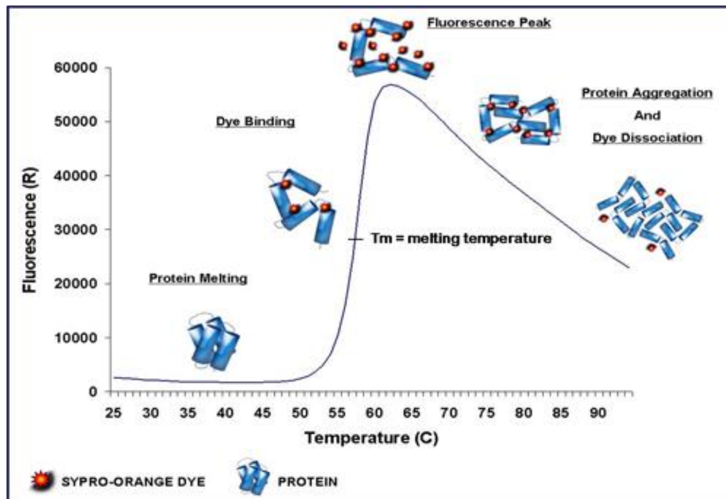


Fig. 1: Thermal shift experiment principle (source Wikipedia)

2. Experimental conditions

2.1 Key Requirement:

The assay is conducted in a 384-well format, which requires the following:

- QuantStudio 5 Real-Time-PCR-System (Thermo Fisher)

- Multi-channel pipette (recommended 16 channels or substituted 8 channels).

- Plate centrifuge.
- Echo acoustic dispenser.

2.2 Key Resources Table:

Reagents (items)	**Suppliers**	**Cat. No.**
SYPRO Orange	Invitrogen	S6650
Protein of interest (>80% purity).	In-house	
384-well armadillo plate	Thermo Fisher	AB3384
qPCR-compatible adhesive plate seal *	Starlab	E2796-9795
Compound library	In-house	

** adhesive seal (other suppliers possible)*

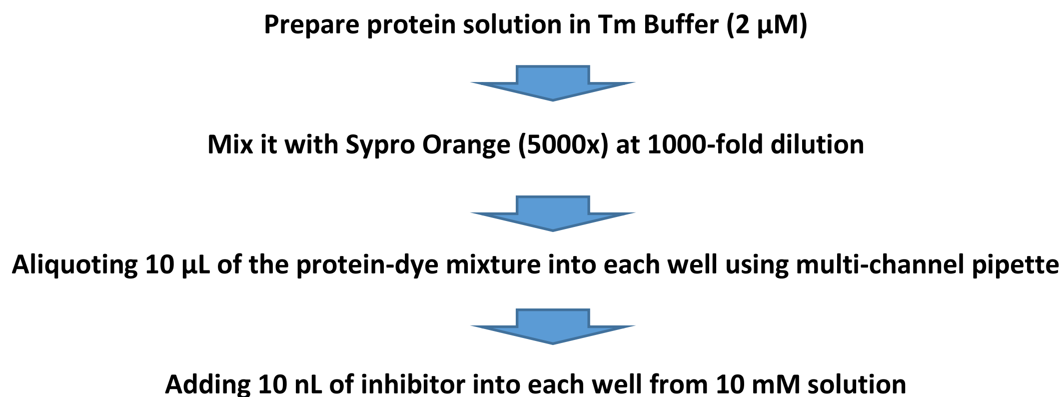
3. Protocol

Note 1: Standard Tm buffer: 25 mM Tris-HCl, pH 8.0, 500 mM NaCl*

** Modifications are possible (pH, NaCl concentration, other ions, reducing agents, etc.). Hydrophobic substances should be avoided or at least kept to minimum.*

Note 2*:* Inhibitors should be prepared at 10 mM concentration (giving 5-fold molar excess) in a source plate compatible for Echo acoustic dispenser, which contains also some control wells (DMSO).

3.1 General Workflow



using Echo acoustic liquid dispenser or pipette (final conc. Is 10 µM)

Seal the plate and spin down shortly

Place the plate in the qPCR machine and use standard program for continuous heating from 25-95°C while measuring fluorescence signals (filter465 nm and 590 nm)

Analyze data with 'Protein Thermal Shift™ (Software Version 1.4, Thermo Fisher)

3.2 Step by Step Protocol:

This following protocol is based on screening a commercial Library (e.g. L1200) for CAMK1D in a 384-well format using QuantStudio5 (Thermo Fisher) and internal Analyzing 'Protein Thermal Shift™ software Version 1.4'

3.2.1 Setting up measurement

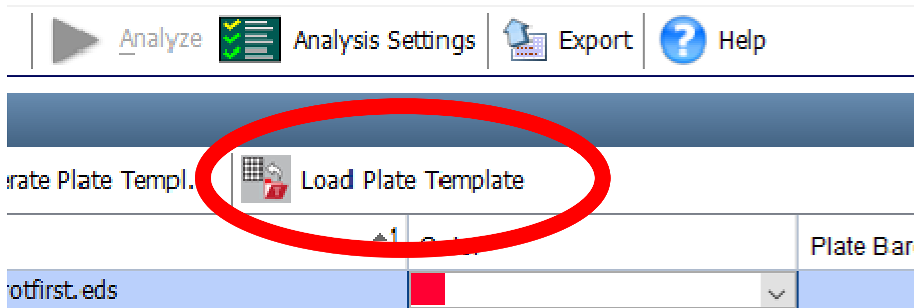
- Prepare 4 ml of protein solution at 2 µM concentration in Tm buffer (e.g. dilute 400 µL of 20 µM CAMK1D in 4 mL of the buffer).
- Add 4 µL of SYPRO™ orange (1:1000 dilution)
- Aliquot 10 µL into each well of a 384-well armadillo plate using 16-channel pipette.
- Transfer 10 nl inhibitors (final concentration 10 µM) from the 10 mM source plate to the 384-well armadillo plate pre-filled with the protein-dye mixture.
- Seal plate with the qPCR-compatible adhesive plate seal.
- Spin the plate briefly using a table-top plate centrifuge (1000 rpm, 30s)
- Place the plate in the real-time qPCR QauntStudio5, and run the standard DSF program with a ramp-up of temperature from 25-95 °C at the rate of 0.05 degree per second. Fluorescence signals are recorded for every 0.05 degree increment step.
- For each plate, measurement time is ~35-40 min

3.2.2 Analysis of ΔTm

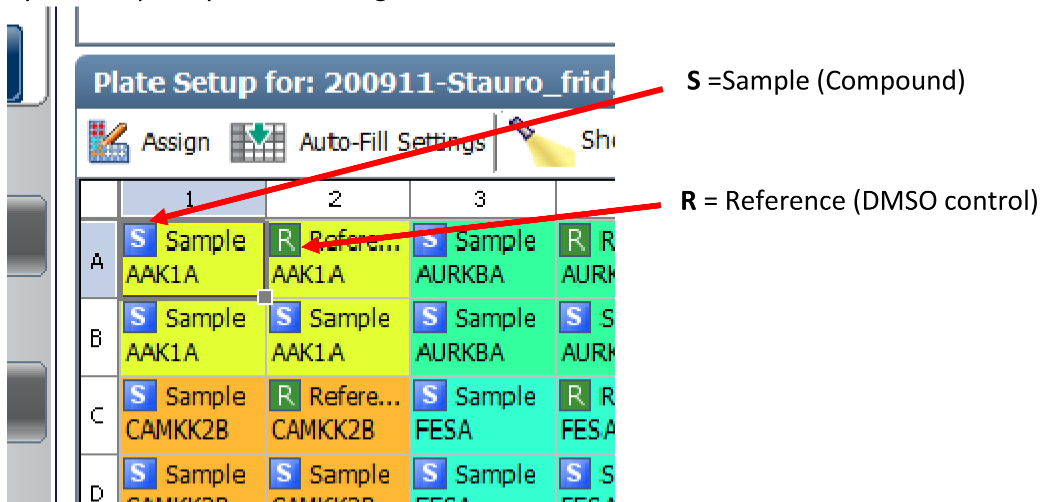
- Open the results in the 'Protein Thermal Shift™ software Version 1.4' provided together with QuantStudio5.

Protein Thermal Shift™ Software Version 1.4

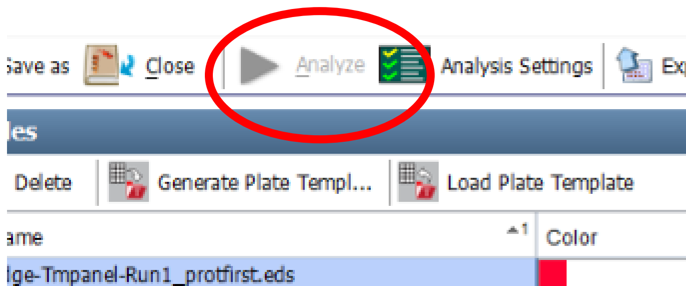
- Load in the inhibitor plate layout

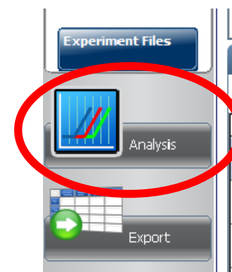


- Assign sample and control wells (DMSO) by right clicking on well. Usually this is already assigned by the template you are loading.



- Press 'analyze' button. Automated data analyses will perform fitting of the melting curves with Boltzmann equation, from which the Tm and Tm shift values will be calculated and displayed under 'analysis' tab.





Tips: It is recommended to inspect the data and analyses manually for abnormality in curves of fluorescence measurement, e.g. high initial intensity or multiple transition or low signals. Typically, these are flagged by the analysis software automatically. You can try to fit the data manually if you are not happy with the fit. To do that use the manual integrate tool in the main tool list. If the curve it so bad that it can't be analyzed at all, it is best to exclude this curve/Tm result. Therefore press on 'omit'.

Manual fitting tools

DMSO control curve

Curve of Active compound

Task	Tm B	ΔTm B	B Fit	Tm B Start	Tm B End	⚠	Single/Multiple	Omit	Analysis Mode	Tm D	ΔTm D	RFU	Fit?	Fit		RFU	NPC		RPL	REF
S	62.77	14.30	1.62	42.06	69.07	2	Single	☐	Auto	63.99	14.45									
S	62.70	14.23	1.56	40.20	69.51	2	Single	☐	Auto	64.27	14.73									
S	48.48	0.01	1.31	34.95	55.18	0	Single	☐	Auto	49.55	0.00									
R	48.47	0.00	1.31	34.62	55.08	0	Single	☐	Auto	49.55	0.00									

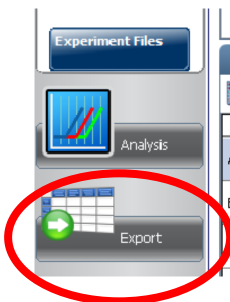
Boltzmann Fit

omit

Standard fit

Flag indicator

- The analyzed results can be exported in an appropriate format, such as a spreadsheet or text, by pressing 'export' button and select the information you want to export (i.e. Tm value, Tm Shift, inhibitor name etc.). Usually the Tm calculated by the Boltzmann Fit is more precise than the 'standard fit'.



Literature

1. Fedorov, O.; Niesen, F. H.; Knapp, S., Kinase inhibitor selectivity profiling using differential scanning fluorimetry. *Methods Mol Biol* **2012,** *795*, 109-18.