Practical 5: Thermofluor Stability Assay

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We will be running a practical to introduce course students to optimisation of experimental buffers by Thermofluor screening[1,2]. A protein is screened against a matrix of buffer (salt and pH) and ligands/additives using a thermal denaturation assay to identify rapidly conditions, additives and ligands offering improved solubility, reduced aggregation and, in some cases, improved crystallisation. Potential applications to protein complexes include identification of working conditions for preparation of individual subunits prior to mixing and reassembly, and identification of complex-stabilising conditions.

The assay is performed in a 96 well format and, during the afternoon, students will be introduced to the various tools available for working in this high-throughput format. The sample plate contains the protein, various buffers, additives and a fluorescent dye: SYPRO orange. This dye interacts with hydrophobic molecules which greatly enhances its fluorescence. Previously, it has been used to stain SDS-PAGE gels for fluorescence imaging of protein samples; here the same effect is exploited to monitor thermal denaturation when the hydrophobic core residues become solvent-exposed upon unfolding. Conveniently, a real-time PCR machine can be used to heat the sample plate gradually whilst detecting fluorescence emissions from SYPRO.

You will need 250 micrograms of your purified protein (crystallisation-grade purity)
Concentration: approx 1-5 mg/ml (approx 20-100 micromolar)
We suggest you flash-freeze your protein in PCR tubes in liquid nitrogen (preferably without glycerol) and ship samples on dry ice.

References

1. Ericsson, U. B., Hallberg, B. M., Detitta, G. T., Dekker, N. & Nordlund, P. *Anal Biochem* **357**, 289-98 (2006).
2. Vedadi, M. et al. *Proc Natl Acad Sci U S A* **103**, 15835-40 (2006).

Thermofluor protocol

Instrument
Mx3005P Q-PCR, Stratagene
Excitation/Emission filters: 492 nm/ 516 nm

Material required

- Clear, non skirted, low profile, optical reading-compatible 96 well plate (96 well polypropylene plates #410088, Stratagene)
- Clear seal for PCR plate (Microseal "B" adhesive seals #MSB-1001, Bio-Rad)
- Centrifuge with microplate compatible rotor
- SYPRO Orange protein gel stain stock solution 5000x (10 × 50 µl #S6651, Invitrogen)
- HEPES screening buffer (100 mM HEPES, 150 mM NaCl, pH 7.5)
- 96 Deep Well stock plate with 1 ml of each buffers at 500 mM (100 mM final conc in assay)
- Protein samples 20-100 µM (final concentration per well 2-10 µM).

Protocol

Preparation steps

1. Turn on the Mx3005P and MxPro software 20 min before use.
2. Thaw protein sample and SYPRO Orange protein on ice

Dispensing reaction components per well

100 mM Buffer (stock 500 mM)	4
NaCl	6 [a]
5 × SYPRO (stock 100 ×)	1 [b]
2-10 µM Protein	2
H_2O (+/- additives)	7
	20 µl.

[a] [NaCl]

Rows C, D, G and H

100 mM NaCl (stock 1 M)	2
H_2O	4

Rows A, B, E and F

500 mM NaCl (stock 2 M)	5
H_2O	1

[b] Dilute SYPRO (stock as supplied is 5000 ×) 50-fold in 100 mM HEPES, 150 mM NaCl, pH 7.5; 120 µl final volume at 100 ×.

Seal the plate and centrifuge for 1 minute at 1000 RPM

Temperature scan experiment

Program

1 °C step increases each minute from 25 °C to 75 °C, i.e. 50 “cycles”.

Output calculations

See Excel Macro.

NaCl	1	2	3	4	5	6	7	8	9	10	11	12
A 500 mM	CitricAc 5,0	NaCitra 5,5	MES 5,8	Kphos 6,0	NaCitra 6,0	MES 6,2	MES 6,5	Naphos 6,6	MES 6	Naphos 7,0	Kphos 7,0	HEPES 7,0
B 500 mM	AmAcet 7,3	Naphos 7,6	Tris 7,5	Imidazo 8,0	HEPES 8,0	Tris 8,0	Tricin 8,0	Bicine 8,5	Bicine 8,0	Tris 8,5	Bicine 9,0	Nothing
C 100 mM	CitricAc 5,0	NaCitra 5,5	MES 5,8	Kphos 6,0	NaCitra 6,0	MES 6,2	MES 6,5	Naphos 6,6	MES 6	Naphos 7,0	Kphos 7,0	HEPES 7,0
D 100 mM	AmAcet 7,3	Naphos 7,6	Tris 7,5	Imidazo 8,0	HEPES 8,0	Tris 8,0	Tricin 8,0	Bicine 8,5	Bicine 8,0	Tris 8,5	Bicine 9,0	Nothing
E 500 mM	CitricAc 5,0	NaCitra 5,5	MES 5,8	Kphos 6,0	NaCitra 6,0	MES 6,2	MES 6,5	Naphos 6,6	MES 6	Naphos 7,0	Kphos 7,0	HEPES 7,0
F 500 mM	AmAcet 7,3	Naphos 7,6	Tris 7,5	Imidazo 8,0	HEPES 8,0	Tris 8,0	Tricin 8,0	Bicine 8,5	Bicine 8,0	Tris 8,5	Bicine 9,0	Nothing
G 100 mM	CitricAc 5,0	NaCitra 5,5	MES 5,8	Kphos 6,0	NaCitra 6,0	MES 6,2	MES 6,5	Naphos 6,6	MES 6	Naphos 7,0	Kphos 7,0	HEPES 7,0
H 100 mM	AmAcet 7,3	Naphos 7,6	Tris 7,5	Imidazo 8,0	HEPES 8,0	Tris 8,0	Tricin 8,0	Bicine 8,5	Bicine 8,0	Tris 8,5	Bicine 9,0	Nothing

Positive control

Protein to test