IFTA AG
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JBS FUNDAMENT Thermofluor Screen

Cat. No.	Amount
CS-330	1 Kit

For *in vitro* use only.
Quality guaranteed for 3 months.
Store at 4°C.

Application

Screen for thermal stability of proteins **as a function of the FUNDAMENTAL variables pH and ionic strength** without interference from SPECIFIC (undesired buffer-change and additives) effects.

Kit Contents

- 5 x ready-to-use 96-well PCR plates arranged in a pH x NaCl concentration gradient (15 µl per well)
- 1 x 15 µl fluorophore – stock solution (5000x)
- 1 x 25 µl control protein (CP) – stock solution

Additionally required (not in the kit):

- Optically clear PCR strips or 1x 96-well PCR plate
- Standard real-time PCR machine with excitation filter at 483 nm and emission at 568 nm
- Statistical software packages for data analysis such as Origin (http://www.originlab.com/), Grafit (http://www.erithacus.com/grafit/) or Graphpad (http://www.graphpad.com/)

The concept of FUNDAMENTAL and SPECIFIC variables affecting protein stability *in vitro*

Protein stability *in vitro* - greatly dependent on the protein's aqueous buffered environment - is crucial for protein purification, characterization and crystallization [1]. Commonly, this environment is considered to be determined by i) pH ii) ionic strength and iii) additives. This however, has the general weakness of lacking mutual exclusivity since pH, ionic strength and additives are interdependent variables. An environment of identical pH 7.5 may, for example, be obtained by 100 mM TRIS/HCl or 50 mM HEPES/NaOH buffer while ionic strength (100 mM vs. 50 mM buffer) and additives (buffer molecules, Na^+ vs. Cl^-) make up significantly different conditions with unpredictable effect on protein stability - and potentially overlaying/masking the pure pH-effect.

Therefore, the JBS FUNDAMENT and the JBS SPECIFIC Thermofluor Screens base on an alternative approach in which protein environment is categorized into

1. FUNDAMENTAL factors that influence the *whole* protein molecule and
2. SPECIFIC factors that affect energetically important *hot spots* on the protein.

FUNDAMENTAL factors are the proton (H^+) concentration (i.e. the pH) *together* with the ionic strength (i.e. the concentration of all electrolytes). These affect a plethora of sites on the *entire* protein thus altering its fundamental intra- and intermolecular interactions such as coulombic, electrostatic and hydrophobic interactions, salt bridges, hydrogen bonds, and Van der Waals forces.

SPECIFIC factors are additives that, in contrast, affect only one…a few distinct but energetically important sites on the protein (e.g. active site, catalytic cleft, dimerization interface) and include basically any small molecule interacting with the protein such as substrates and their analogs, cations, anions, and many more.

The JBS FUNDAMENT and the JBS SPECIFIC Thermofluor Screen are designed to eliminate the undesired overlay of simultaneous screening of interdependent variables. They strictly categorize stability screening of proteins into

- fundamental (pH and ionic strength) and
- specific (all other)

variables to the maximum possible extent.

JBS FUNDAMENT Thermofluor Screen

The JBS FUNDAMENT Thermofluor Screen for protein stability

A common reporter for protein stability is its melting temperature (Tm) that can easily be determined by a thermal shift assay (Fig. 1). The higher the Tm, the higher is the thermostability of the protein in that specific environment. Therefore, optimization of protein environments became a routine approach to gain stability, particularly prior protein crystallization: It was shown that Tm-increasing environments directly correlate with increased likelihood of protein crystals that yield good diffraction data for structure determination [1, 2].

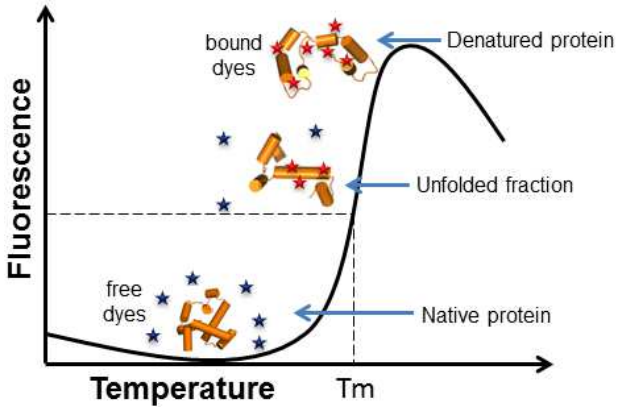

Fig. 1: Thermal shift assay is a rapid and simple denaturation technique that monitors the unfolding of a protein in a temperature-dependent manner. At low temperatures, the protein is properly folded. As temperature increases, the protein is gradually denatured, enabling a present fluorophore to interact with the protein's exposed hydrophobic patches. This results in a fluorescence increase (signal) until the protein is unfolded. From the resulting sigmoidal melting curve the melting temperature Tm can be extracted [3].

The JBS FUNDAMENT Thermofluor Screen uses the thermal shift assay methodology for identification of the *fundamental* variables for protein stability. It is composed of two different broad-range buffer systems ("*Super-Buffers*") covering pH 4.0 to 10.0 as a function of ionic strength (0 to 1M NaCl). The designed FUNDAMENT formulation therefore allows screening of pH and ionic strength while maintaining the chemical nature of the protein's environment (Fig.2).

JBS FUNDAMENT Thermofluor Screen comes with pre-dispensed solutions providing a fast read-out of the protein melting profile with minimum sample handling. The integrated sealed plates are DNase/RNase free and optically transparent - ideal for fluorescent-based measurements. Moreover, the unskirted low plate profile is compatible with most commercial qPCR machines.

JBS FUNDAMENT Thermofluor Screen

	1	2	3	4	5	6	7	8	9	10	11	12
A	CP + Dye CB-1	CP + Dye CB-1	CP + Dye CB-1	CP + Dye CB-2	CP + Dye CB-2	CP + Dye CB-2	CP + Dye CB-3	CP + Dye CB-3	CP + Dye CB-3	TP + Dye Original Buffer	TP + Dye Original Buffer	TP + Dye Original Buffer
B	4.0	4.0 50 mM NaCl	4.0 125 mM NaCl	4.0 250 mM NaCl	4.0 500 mM NaCl	4.0 1 M NaCl	4.0	4.0 50 mM NaCl	4.0 125 mM NaCl	4.0 250 mM NaCl	4.0 500 mM NaCl	4.0 1 M NaCl
C	5.0	5.0 50 mM NaCl	5.0 125 mM NaCl	5.0 250 mM NaCl	5.0 500 mM NaCl	5.0 1 M NaCl	5.0	5.0 50 mM NaCl	5.0 125 mM NaCl	5.0 250 mM NaCl	5.0 500 mM NaCl	5.0 1 M NaCl
D	6.0	6.0 50 mM NaCl	6.0 125 mM NaCl	6.0 250 mM NaCl	6.0 500 mM NaCl	6.0 1 M NaCl	6.0	6.0 50 mM NaCl	6.0 125 mM NaCl	6.0 250 mM NaCl	6.0 500 mM NaCl	6.0 1 M NaCl
E	7.0	7.0 50 mM NaCl	7.0 125 mM NaCl	7.0 250 mM NaCl	7.0 500 mM NaCl	7.0 1 M NaCl	7.0	7.0 50 mM NaCl	7.0 125 mM NaCl	7.0 250 mM NaCl	7.0 500 mM NaCl	7.0 1 M NaCl
F	8.0	8.0 50 mM NaCl	8.0 125 mM NaCl	8.0 250 mM NaCl	8.0 500 mM NaCl	8.0 1 M NaCl	8.0	8.0 50 mM NaCl	8.0 125 mM NaCl	8.0 250 mM NaCl	8.0 500 mM NaCl	8.0 1 M NaCl
G	9.0	9.0 50 mM NaCl	9.0 125 mM NaCl	9.0 250 mM NaCl	9.0 500 mM NaCl	9.0 1 M NaCl	9.0	9.0 50 mM NaCl	9.0 125 mM NaCl	9.0 250 mM NaCl	9.0 500 mM NaCl	9.0 1 M NaCl
H	10.0	10.0 50 mM NaCl	10.0 125 mM NaCl	10.0 250 mM NaCl	10.0 500 mM NaCl	10.0 1 M NaCl	10.0	10.0 50 mM NaCl	10.0 125 mM NaCl	10.0 250 mM NaCl	10.0 500 mM NaCl	10.0 1 M NaCl

pH

100 mM Super Buffer 1

100 mM Super Buffer 2

Ionic Strength

Ionic Strength

Fig. 2: The JBS FUNDAMENT Thermofluor Screen is composed of 15 µl screening solution per well (B1-H12). The concentrations given are the final concentrations after adding target protein (TP) and dye (25 µl total assay volume). Two different broad-range buffer systems (*"Super-Buffers"*) covering pH 4.0 to 10.0 are arranged in a grid screen against ionic strength (0 to 1M NaCl).
A1-A9 allow for reference assay with control protein (CP) in control buffer (CB); A10-A12 for internal control with target protein (TP) in original buffer.
Super Buffer 1 = CHC buffer, produced by mixing Citric acid:HEPES:CHES in the molar ratios 2:3:4;
Super Buffer 2 = MIB buffer, produced by mixing Malonic acid:Imidazole:Boric acid in the molar ratios 2:3:3.

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Experimental Protocol

I. Dilution of the dye

- 1st dilution to **100x** stock: Add 735 µl of H_2O to the 15 µl dye stock solution.
- 2nd dilution to **20x** stock: Add 40 µl of H_2O to 10 µl of 100x dye solution.

Note: The 100x stock solution is stable at -20°C for 12 weeks. The 20x stock solution is stable at -20°C for 4 weeks.

II. Pre-Assay for determination of optimal target protein (TP) and dye concentration

a)

target protein (TP) concentration (mg/ml)

dye concentration

	column 1-3	column 4-6	column 7-9	column 10-12
row A	1x Dye 0 TP	1x Dye 0.1 TP	1x Dye 0.5 TP	1x Dye 1 TP
row B	2x Dye 0 TP	2x Dye 0.1 TP	2x Dye 0.5 TP	2x Dye 1 TP
row C	4x Dye 0 TP	4x Dye 0.1 TP	4x Dye 0.5 TP	4x Dye 1 TP

b)

TP [µl]	row A	0	1.25	6.25	12.5
20x dye [µl]		1.25	1.25	1.25	1.25
TP Buffer [µl]		23.75	22.5	17.5	11.25
TP [µl]	row B	0	1.25	6.25	12.5
20x dye [µl]		2.5	2.5	2.5	2.5
TP Buffer [µl]		22.5	21.25	16.25	10.0
TP [µl]	row C	0	1.25	6.25	12.5
20x dye [µl]		5.0	5.0	5.0	5.0
TP Buffer [µl]		20.0	18.75	13.75	7.5

Fig. 3: Suggested thermal shift pre-assay experimental grid for the determination of the optimal concentration of dye and TP in original buffer. The condition that best approaches the characteristic protein melting curve (Fig. 1) is further used for the JBS FUNDAMENT Thermofluor Screen. Please note that the specified grid limitations might require adjustments depending on the obtained melting curves of the target protein.
a) Plate layout. b) Corresponding pipetting scheme considering a TP stock solution of 2 mg/ml.

(1) Before starting the JBS FUNDAMENT Thermofluor Screen, the best-performing ratio between TP and dye needs to be identified by a pre-assay. Perform the pre-assay in triplicate in a PCR plate (not provided in this kit) according to Fig. 3.

(2) Seal the plate and incubate in a plate mixer (3 min at 1500 rpm).

(3) Spin down (30 s at 2500 rpm).

(4) Use any real-time thermocycler (excitation 483 nm / emission 568 nm) for fluorescent measurements; recommended parameters:
- 2 min initial incubation at 25 °C
- melting temperature range from 25 °C to 99 °C in 1 °C/min
- 2 min final incubation at 99 °C

(5) Determine the optimal concentrations of TP and dye by visual inspection of the melting curves.

III. JBS FUNDAMENT Thermofluor Screen

(6) Calculate the required stock solutions and prepare a pipetting scheme according to the results obtained by the pre-assay.
Note: The assay is performed in triplicate and the background analysis in duplicate. The final assay volume is 25 µl/well.

Background Plates (performed in duplicate)

(7) Spin down the plate (30 s at 2500 rpm).

(8) Add dye to wells A1-A9 and B1-H12 to achieve the optimal dye concentration determined by the pre-assay.

(9) Re-use seal for sealing the plate and incubate in a plate mixer (3 min at 1500 rpm).

(10) Spin down (30 s at 2500 rpm).

(11) Use any real-time thermocycler (excitation 483 nm / emission 568 nm) for fluorescent measurements; recommended parameters:
- 2 min initial incubation at 25 °C
- melting temperature range from 25 °C to 99 °C in 1 °C/min
- 2 min final incubation at 99 °C

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Assay Plates (performed in triplicate)

Note: Use the wells A10-A12 for the internal control assay in the optimum sample + dye concentration determined by the pre-assay.

(12) Spin down the plate (30 s at 2500 rpm).

(13) ***Reference wells:*** Dilute 25 µl control protein (CP) stock solution by adding 25 µl of H_2O and pipet 5 µl into well A1-A9, respectively.

(14) ***Internal control wells:*** Add the target protein (TP) in its original buffer to A10-A12 to achieve the optimal concentration determined by the pre-assay.

(15) Add TP into wells B1-H12 to achieve the optimal concentration determined by the pre-assay.

(16) Add dye to wells A1-H12 to achieve the optimal dye concentration determined by the pre-assay.

(17) Re-use seal for sealing the plate and incubate in a plate mixer (3 min at 1500 rpm).

(18) Spin down (30 s at 2500 rpm).

(19) Use any real-time thermocycler (excitation 483 nm / emission 568 nm) for fluorescent measurements; recommended parameters:
- 2 min initial incubation at 25 °C
- melting temperature range from 25 °C to 99 °C in 1 °C/min
- 2 min final incubation at 99 °C

Data Analysis

Export the raw fluorescence data of the assay plate (AP) triplicates and the background plate (BP) duplicates. Calculate the average of the AP triplicates and subtract the average of the BP duplicates to obtain the averaged melting curve (AMC) for every corresponding well:

$$AMC = \frac{AP1 + AP2 + AP3}{3} - \frac{BP1 + BP2}{2}$$

Use the Boltzmann sigmoid for AMC model fitting [2]. The slope at its inflection point corresponds to the protein melting temperature in each of the different well conditions.

Data Analysis of the CP reference

The CP reference shows the pure pH effect on the thermostability of the CP independent of the chemical buffer composition. The CP reference assay is performed in each plate in wells A1-A9.

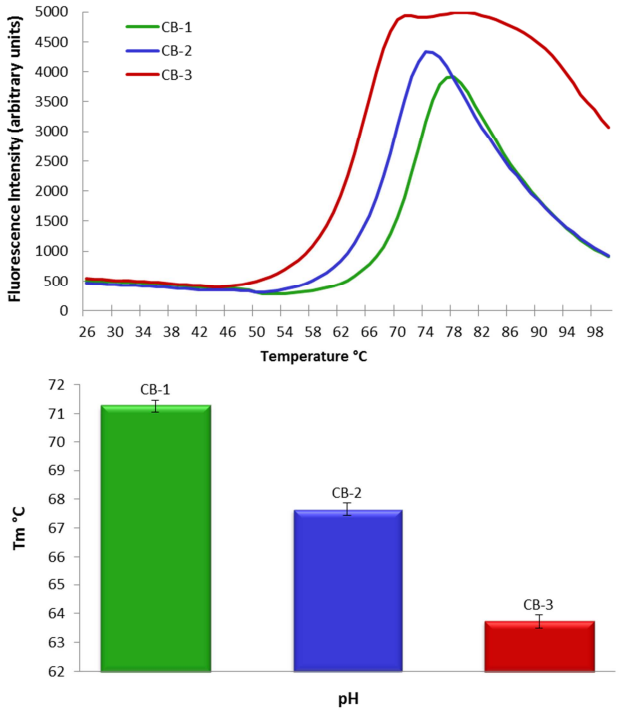

Fig. 4: Reference assay: Averaged melting curves and calculated melting temperatures (Tm) of the CP in control-buffer 1, 2 and 3 (CB-1, CB-2 and CB-3).

References

[1] Ericsson et al. (2006) Thermofluor-based high-throughput stability optimization of proteins for structural studies. *Anal Biochem.* **357(2)**:289

[2] Reinhard et al. (2013) Optimization of protein buffer cocktails using Thermofluor. *Acta Cryst. F* **69**:209

[3] Niesen et al. (2007).The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protoc.* **2(9)**: 2212