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Jena Bioscience

JBScreen Thermofluor SPECIFIC HTS

TSA for protein stability

Cat. No.	Amount
CS-333	93 solutions (0.5 ml each) Control Protein (1.0 ml)

For *in vitro* use only.
Quality guaranteed for 12 months.
Store at 8-10°C.

Application

Screen for thermal stability of proteins **as a function of the SPECIFIC ions and pH** without interference from undesired buffer-change effects.

Additionally required (not in the kit):

- suitable PCR plates
- JBS Thermofluor Dye (Cat.No. X-TD-70)
- 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. 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 JBScreen Thermofluor FUNDAMENT and the JBScreen Thermofluor SPECIFIC 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 JBScreen Thermofluor FUNDAMENT and the JBScreen Thermofluor SPECIFIC 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.

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The JBScreen Thermofluor SPECIFIC for protein stability

A common reporter for protein stability is its melting temperature (Tm) that can easily be determined by TSA (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].

The JBScreen Thermofluor SPECIFIC uses the TSA methodology for identification of the *specific* variables for protein stability. The screen encompasses inorganic salts of different nature that were selected taking into consideration the number of successful determined structures on the Protein Data Bank, in which they appear as ligands in proteins and protein complexes [4]. The resulting most abundant mono-, di- and trivalent ions have been extracted and arranged in a grid screen against the two different broad-range buffer systems (“*Super-Buffers”*) covering pH 4.0 to 10.0 (Fig. 2) as in the JBS Fundament Thermofluor Screen.

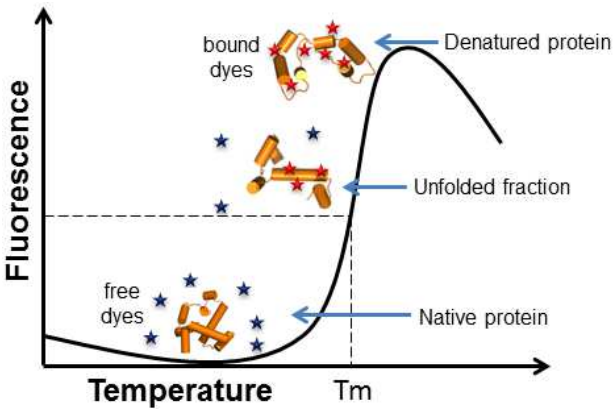

Fig. 1: TSA (Thermal shift assay) is a rapid and simple denaturation technique that monitors the unfolding of a protein in a temperature-dependent manner. At low temperatures, the protein is properly folded. As temperature increases, the protein is gradually denatured, enabling a present fluorophore 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].

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	1	2	3	4	5	6	7	8	9	10	11	12
A	CP control buffer 1	CP control buffer 1	CP control buffer 1	CP control buffer 2	CP control buffer 2	CP control buffer 2	CP control buffer 3	CP control buffer 3	CP control buffer 3	TP empty	TP empty	TP empty
B	4.0	4.0	4.0 33 mM MgSO4	4.0 17 mM FeCl3	4.0 17 mM ZnCl2	4.0 17 mM MnCl2	4.0	4.0	4.0 33 mM MgSO4	4.0 33 mM CaCl2	4.0 17 mM ZnCl2	4.0 17 mM MnCl2
C	5.0	5.0	5.0 33 mM MgSO4	5.0 17 mM FeCl3	5.0 17 mM ZnCl2	5.0 17 mM MnCl2	5.0	5.0	5.0 33 mM MgSO4	5.0 33 mM CaCl2	5.0 17 mM ZnCl2	5.0 17 mM MnCl2
D	6.0	6.0	6.0 33 mM MgSO4	6.0 17 mM FeCl3	6.0 17 mM ZnCl2	6.0 17 mM MnCl2	6.0	6.0	6.0 33 mM MgSO4	6.0 33 mM CaCl2	6.0 17 mM ZnCl2	6.0 17 mM MnCl2
E	7.0	7.0	7.0 33 mM MgSO4	7.0 17 mM FeCl3	7.0 33 mM LiCl	7.0 33 mM KCl	7.0	7.0	7.0 33 mM MgSO4	7.0 33 mM CaCl2	7.0 33 mM LiCl	7.0 33 mM KCl
F	8.0	8.0	8.0 33 mM MgSO4	8.0 17 mM FeCl3	8.0 33 mM LiCl	8.0 33 mM KCl	8.0	8.0	8.0 33 mM MgSO4	8.0 33 mM CaCl2	8.0 33 mM LiCl	8.0 33 mM KCl
G	9.0	9.0	9.0 33 mM MgSO4	9.0 17 mM FeCl3	9.0 33 mM LiCl	9.0 33 mM KCl	9.0	9.0	9.0 33 mM MgSO4	9.0 33 mM CaCl2	9.0 33 mM LiCl	9.0 33 mM KCl
H	10.0	10.0	10.0 33 mM MgSO4	10.0 17 mM FeCl3	10.0 33 mM LiCl	10.0 33 mM KCl	10.0	10.0	10.0 33 mM MgSO4	10.0 33 mM CaCl2	10.0 33 mM LiCl	10.0 33 mM KCl

pH

167 mM Super Buffer 1 | 167 mM Super Buffer 2

250 mM NaCl | 250 mM NaCl

Fig. 2: The JBScreen Thermofluor SPECIFIC is designed for TSA in a 96-well PCR plate with a final volume of 25 µl per well: 15 µl screening solution + protein sample and fluorescent dye to a maximum volume of 10 µl. This dilution yields a final buffer concentration of 100 mM, the final concentrations of the mono-, di- and trivalent salts are given in brackets (150 mM NaCl, 20 mM MgSO4, 10 mM FeCl3, 10 mM ZnCl2, 20 mM LiCl, 10 mM MnCl2, 20 mM KCl, 20 mM CaCl2).
A1-A9 allow for control assay with the supplied control protein (CP) in control buffer; A10-A12 are empty wells for the reference assay 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.

Experimental Protocol

A pre-assay for determination of the optimal target protein and dye concentration is recommended. Screening should be performed in triplicate, background plates (containing buffer without protein) in duplicate.

Apply 15 µl screening solution in 25 µl final assay volume and 2.5 µl control protein in wells A1-A9. 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

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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 control assay

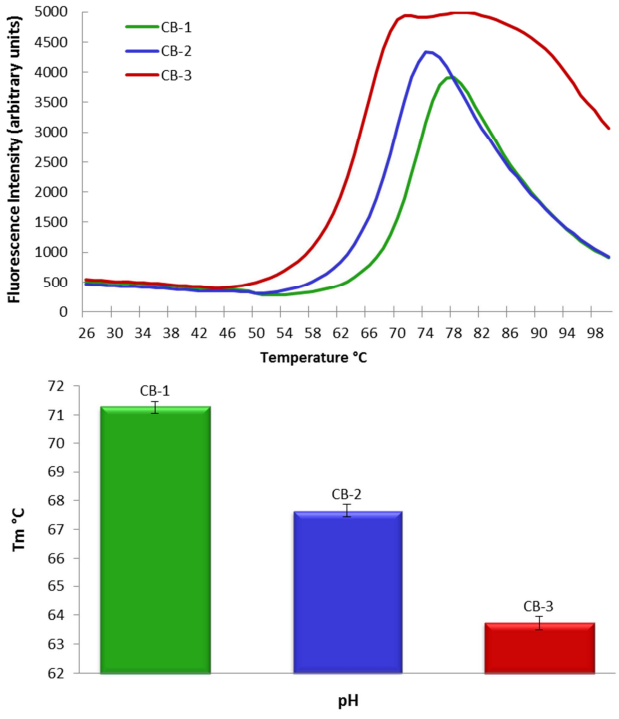

Fig. 3: Control assay: Averaged melting curves and calculated melting temperatures (Tm) of the CP in control-buffer 1, 2 and 3 (CB-1, CB-2 and CB-3

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

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.

[4] http://www.rcsb.org/pdb/home/home.do