CrossMark

Optimization of protein buffer cocktails using Thermofluor

Linda Reinhard,‡§ Hubert Mayerhofer,¶ Arie Geerlof,‡‡ Jochen Mueller-Dieckmann§§ and Manfred S. Weiss¶¶

EMBL Hamburg Outstation, c/o DESY, Notkestrasse 85, D-22603 Hamburg, Germany

‡ Previously known as Linda Schuldt.
§ Present address: PUMPKIN – Centre for Membrane Pumps in Cells and Disease, Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10c, DK-8000 Aarhus C, Denmark.
¶ Present address: Institut de Biologie Structurale J.-P. Ebel, 41 Rue Jules Horowitz, F-38027 Grenoble CEDEX 1, France.
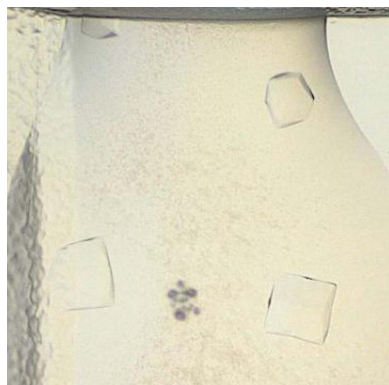‡‡ Present address: Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstädter Landstrasse 1, D-85764 Neuherberg, Germany.
§§ Present address: Biocenter Klein Flottbek, University of Hamburg, Ohnhorststrasse 18, D-22609 Hamburg, Germany.
¶¶ Present address: Helmholtz-Zentrum Berlin für Materialien und Energie, Macromolecular Crystallography (HZB-MX), Albert-Einstein-Strasse 15, D-12489 Berlin, Germany.

The stability and homogeneity of a protein sample is strongly influenced by the composition of the buffer that the protein is in. A quick and easy approach to identify a buffer composition which increases the stability and possibly the conformational homogeneity of a protein sample is the fluorescence-based thermal-shift assay (Thermofluor). Here, a novel 96-condition screen for Thermofluor experiments is presented which consists of buffer and additive parts. The buffer screen comprises 23 different buffers and the additive screen includes small-molecule additives such as salts and nucleotide analogues. The utilization of small-molecule components which increase the thermal stability of a protein sample frequently results in a protein preparation of higher quality and quantity and ultimately also increases the chances of the protein crystallizing.

1. Introduction

Although many biological macromolecules have been crystallized over the past few decades, the crystallization of a new macromolecule usually requires screening against a large number and a large variety of crystallization cocktails (Rupp, 2009). The chances of identifying successful crystallization conditions are usually higher when the macromolecule exhibits a higher level of purity, chemical and conformational homogeneity and stability. In order to assess the stability and monodispersity of a sample, various quality-control techniques are available such as size-exclusion chromatography, mass spectrometry, static and dynamic light scattering, circular-dichroism spectroscopy and analytical ultracentrifugation (Geerlof *et al.*, 2006). Another high-throughput approach is the fluorescence-based thermal-shift assay (Thermofluor), which is also referred to as differential scanning fluorimetry (DSF; Niesen *et al.*, 2007). The Thermofluor assay was originally developed for drug-discovery applications since it allows the rapid screening of molecules from compound libraries that bind to and therefore stabilize the protein of interest (Pantoliano *et al.*, 2001; Senisterra *et al.*, 2006; Niesen *et al.*, 2007). In addition, the Thermofluor method is often used in everyday laboratory work in order to screen for optimized buffer conditions by varying the pH, buffer molecules and small-molecule additives. It has been demonstrated a number of times that increased thermal stability is correlated with increased structural order and decreased protein flexibility. This is often accompanied by increased conformational homogeneity of the protein sample (Ericsson *et al.*, 2006; Vedadi *et al.*, 2006; Nettleship *et al.*, 2008). Consequently, the identification of an optimized buffer cocktail or additive may have beneficial effects for protein-production procedures (Mezzasalma *et al.*, 2007) and may also increase the chances of the protein crystallizing.

The Thermofluor assay makes use of an environmentally sensitive dye, *e.g.* a hydrophobic fluorophore, which allows one to distinguish between folded and unfolded states of a protein. In the ideal case, no fluorescence is observed at low temperature because the protein is completely and correctly folded and no hydrophobic areas are exposed. Upon an increase in temperature the protein starts to unfold and hydrophobic areas become exposed. The fluorophore can now

Table 1
Composition of the 48 buffer screen solutions.

The concentrations given are the final concentrations used during the experiment.

Well	Buffer	Salt
A1	Water	
A2	50 mM sodium citrate pH 4.0	
A3	50 mM sodium acetate pH 4.5	
A4	50 mM sodium citrate pH 5.0	
A5	50 mM sodium citrate pH 5.5	
A6	50 mM bis-tris pH 6.0	
A7	50 mM sodium phosphate pH 6.0	
A8	50 mM MES pH 6.2	
A9	50 mM bis-tris propane pH 6.5	
A10	50 mM ADA pH 6.5	
A11	50 mM MES pH 6.7	
A12	50 mM PIPES pH 6.7	
B1	50 mM sodium phosphate pH 7.0	
B2	50 mM MOPS pH 7.0	
B3	50 mM HEPES pH 7.0	
B4	50 mM HEPES pH 7.5	
B5	50 mM Tris–HCl pH 7.5	
B6	50 mM HEPES pH 8.0	
B7	50 mM Tris–HCl pH 8.0	
B8	50 mM Bicine pH 8.0	
B9	50 mM Tris–HCl pH 8.5	
B10	50 mM CHES pH 9.0	
B11	50 mM CHES pH 9.5	
B12	50 mM CHES pH 10.0	
C1		250 mM NaCl
C2	50 mM sodium citrate pH 4.0	250 mM NaCl
C3	50 mM sodium acetate pH 4.5	250 mM NaCl
C4	50 mM sodium citrate pH 5.0	250 mM NaCl
C5	50 mM sodium citrate pH 5.5	250 mM NaCl
C6	50 mM bis-tris pH 6.0	250 mM NaCl
C7	50 mM sodium phosphate pH 6.0	250 mM NaCl
C8	50 mM MES pH 6.2	250 mM NaCl
C9	50 mM bis-tris propane pH 6.5	250 mM NaCl
C10	50 mM ADA pH 6.5	250 mM NaCl
C11	50 mM MES pH 6.7	250 mM NaCl
C12	50 mM PIPES pH 6.7	250 mM NaCl
D1	50 mM sodium phosphate pH 7.0	250 mM NaCl
D2	50 mM MOPS pH 7.0	250 mM NaCl
D3	50 mM HEPES pH 7.0	250 mM NaCl
D4	50 mM HEPES pH 7.5	250 mM NaCl
D5	50 mM Tris–HCl pH 7.5	250 mM NaCl
D6	50 mM HEPES pH 8.0	250 mM NaCl
D7	50 mM Tris–HCl pH 8.0	250 mM NaCl
D8	50 mM Bicine pH 8.0	250 mM NaCl
D9	50 mM Tris–HCl pH 8.5	250 mM NaCl
D10	50 mM CHES pH 9.0	250 mM NaCl
D11	50 mM CHES pH 9.5	250 mM NaCl
D12	50 mM CHES pH 10.0	250 mM NaCl

Table 2
Composition of the 48 additive screen solutions.

The concentrations given are the final concentrations used during the experiment.

Well	Additive 1	Additive 2
E1	50 mM sodium chloride	
E2	100 mM sodium chloride	
E3	150 mM sodium chloride	
E4	200 mM sodium chloride	
E5	400 mM sodium chloride	
E6	600 mM sodium chloride	
E7	800 mM sodium chloride	
E8	1000 mM sodium chloride	
E9	2.5%(v/v) glycerol	
E10	5%(v/v) glycerol	
E11	10%(v/v) glycerol	
E12	15%(v/v) glycerol	
F1	20%(v/v) glycerol	
F2	3% D-glucose	
F3	3% sucrose	
F4	10 mM magnesium chloride	
F5	10 mM calcium chloride	
F6	5 mM manganese chloride	
F7	5 mM nickel chloride	
F8	5 mM iron(III) chloride	
F9	5 mM zinc chloride	
F10	5 mM EDTA	
F11	100 mM potassium chloride	
F12	100 mM lithium chloride	
G1	50 mM sodium bromide	
G2	50 mM sodium iodide	
G3	100 mM sodium formate	
G4	100 mM sodium acetate	
G5	100 mM sodium malonate	
G6	100 mM sodium tartrate	
G7	100 mM sodium nitrate	
G8	100 mM sodium thiocyanate	
G9	100 mM sodium sulfate	
G10	100 mM ammonium sulfate	
G11	100 mM ammonium chloride	
G12	100 mM imidazole pH 7.6	
H1	500 mM imidazole pH 7.6	
H2	2 mM AMP	5 mM magnesium chloride
H3	2 mM ADP	5 mM magnesium chloride
H4	2 mM AMPPcP	5 mM magnesium chloride
H5	2 mM dCMP	5 mM magnesium chloride
H6	2 mM dGMP	5 mM magnesium chloride
H7	2 mM GDP	5 mM magnesium chloride
H8	2 mM TMP	5 mM magnesium chloride
H9	2 mM NAD	5 mM magnesium chloride
H10	30 mM L-arginine	30 mM L-glutamate
H11	5 mM dithiothreitol	
H12	5 mM β-mercaptoethanol	

bind to these areas and fluorescence occurs. For data analysis, the fluorescence intensity may be plotted as a function of temperature. The resulting curve is ideally sigmoidal and can be used to estimate the apparent melting temperature T_m of the protein of interest, which corresponds to the midpoint of the transition curve. Strictly speaking, the T_m value determined by Thermofluor corresponds to the temperature at which hydrophobic areas of the protein become exposed. This is generally accepted to be a good approximation of the actual melting temperature of the protein (Senisterra *et al.*, 2006), although methods such as differential scanning calorimetry (DSC) are specifically geared towards determination of the actual melting temperature.

Here, we present and discuss a novel 96-condition screen for Thermofluor experiments. The screen is based on a previously published screen (Nettleship *et al.*, 2008) and has undergone a number of rounds of development and improvement. Since the screen is suitable for a wide variety of protein samples, it may turn out to be useful as a starting screen for Thermofluor experiments with the goal of the identifying optimized buffer conditions for protein crystallization.

2. Development of the Thermofluor screen

The original screen (Nettleship *et al.*, 2008) was built up of two equally sized parts (each consisting of 48 conditions): a 'buffer screen' and an 'additive screen'. The buffer screen contained 12 buffers covering a pH range from 4.0 to 9.5. These buffers were combined with NaCl at concentrations of up to 500 mM. The additive screen contained nucleotide cofactors, metal salts, sugars, reducing agents and other chemicals.

Over the years the original screen was modified and extended, reflecting the accumulated experience with the screen in practical terms. The Thermofluor screen presented here is also composed of a buffer screen (Table 1) and an additive screen (Table 2). The buffer screen was extended to 23 different buffer solutions covering a pH range from 4.0 to 10.0. These are used as such or in combination with 250 mM NaCl. This not only allows the effect of pH to be investigated but also the effect that different buffer chemicals may have on the stability of the protein. For the additive screen, different groups of chemicals have been defined. During the initial phase of the development of the additive screen all additive solutions were prepared in

a basal buffer consisting of 50 mM Tris–HCl pH 7.5, 200 mM NaCl. However, since a variety of proteins have been found to be rather unstable in this buffer cocktail, the current additive screen is left unbuffered.

The roles of the different groups of chemicals in the additive screen are as follows. Conditions E1–E8 (Table 2) are included to analyze protein stability in the presence of NaCl in the concentration range 50–1000 mM. This allows investigation of whether low- or high-salt concentrations in the purification procedure are tolerated by the protein. Conditions E9–F3 are used to analyze the effect of glycerol, D-glucose and sucrose, which are frequently encountered as protein-stabilizing agents. In conditions F4–G11 different metal salts are tested. EDTA was also included in this group to assess the effect of the absence of divalent metal ions on protein stability. Imidazole is commonly used for the elution of proteins from immobilized metal-affinity chromatography (IMAC) columns. Two concentrations of imidazole (conditions G12 and H1) are included to assess protein stability at high imidazole concentrations. If the stability is found to be significantly reduced at high imidazole concentrations, care should be taken to remove the imidazole as soon as possible after elution of the protein, *e.g.* by dilution, dialysis or size-exclusion chromatography. Conditions H2–H9 contain various nucleotide cofactors supplemented with 5 mM magnesium chloride, which often assists in nucleotide-cofactor binding. Finally, the two amino acids Glu and Arg (Golovanov *et al.*, 2004) as well as two different reducing agents constitute the last group of the screen (conditions H10–H12).

The whole screen may be prepared in a 96 deep-well master block and stored at 253 K. However, multiple rounds of freezing and thawing may have adverse effects on some of the substances in the screen. It is therefore recommended to reformat the freshly prepared screen into 96-well thin-wall PCR plates, which are later directly used for the Thermofluor experiment, to seal them with low-cost sealing foil and to store the ready-to-use plate at 253 K for one-time use.

3. The Thermofluor experiment

For the Thermofluor experiment, no prior knowledge of the protein properties is required. Every standard real-time PCR machine (*e.g.* a MyiQ^{TM} Thermocycler equipped with a Single Color Real-Time PCR Detection System from Bio-Rad) is suitable for this experiment. The assay is performed using a 96-well thin-wall PCR plate (*e.g.* from Bio-Rad or Roche). The total reaction volume is 25 µl and the plate is set up on ice or in a cold room. Either a ready-to-use plate, which was previously prepared and stored at 253 K, is thawed for the experiment or the plate is freshly prepared. For the buffer screen, 20 µl of the screening solution is pipetted into the wells of the plate, ideally using a multi-channel pipette. Accordingly, for the additive screen the wells are filled with 15 µl of the screening solution and 5 µl water or 5× buffer (for instance, the most stabilizing buffer identified from the buffer screen or an alternative buffer system such as that used during the protein-purification procedure). A 5000× SYPRO Orange stock solution in DMSO (Invitrogen) is diluted 1:100 in water to yield a tenfold working solution, which can be stored in the dark at 277 K for several hours. The protein concentration in the purified protein solution should be in the range 20–100 µM. However, the concentration may be lower for higher molecular-weight samples. 2.5 µl protein solution and 2.5 µl of the tenfold SYPRO Orange working solution are added to the 20 µl well solution. The plates are sealed with Optical Quality Sealing Tape (*e.g.* from Bio-Rad) and centrifuged at 4000 rev min^{-1} for 2 min. For the Thermofluor experiment the plates are then heated from 277 to 368 K in increments of 0.5–1 K

in a thermocycler equipped with a single-colour real-time PCR detection system (scan rate of 1 K min^{-1}). The lid of the thermocycler is heated to 378 K to avoid condensation effects during the experiment. Fluorescence changes in the wells of the plate are monitored simultaneously using excitation and emission wavelengths of 492 and 516 nm, respectively. The whole experiment only takes about 1–2 h. It is also possible to start the Thermofluor experiment at a higher temperature than 277 K, *e.g.* 293 K; however, a lower starting temperature is recommended in order to ensure that proteins with a low melting temperature also exhibit clear baseline fluorescence before protein unfolding is initiated.

4. Use of the screen

The Thermofluor screen presented in this work may be used in multiple ways. Firstly, all 96 conditions can be tested simultaneously. However, it needs to be emphasized again that the additive screen lacks a buffer system; this might result in unstable protein, leading to low-quality fluorescence curves. Therefore, it is recommended that the additive screen is used in conjunction with a buffer system of choice (see §3). Another option would be to split the screen into the two halves. The buffer screen is first investigated in order to identify the most promising buffer composition; then, in a second experiment the most stabilizing buffer may be used in the additive screen.

It is also clear that with a coarse screen such as that described here it is not possible to identify 'the best' buffer composition in one step. Further protein-specific fine-tuning may be required to achieve this. Furthermore, for some proteins it may initially be necessary to also screen against the nature and the concentration of the dye used in order to yield optimal results.

5. Data evaluation

5.1. Data analysis

Data analysis can be performed using a Boltzmann model (Ericsson *et al.*, 2006). However, in everyday laboratory work the approach, which is frequently used, is just a simple qualitative test. Its evaluation is simply based on visual inspection of superposed
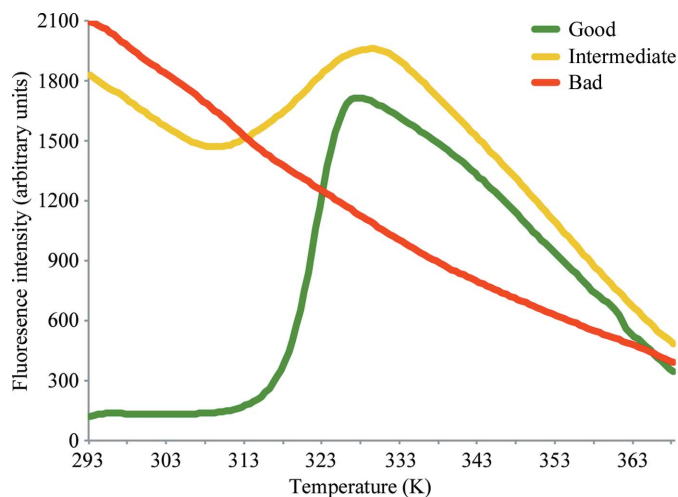
Figure 1
Different shapes of protein-unfolding curves in a Thermofluor experiment. Examples of unfolding curves of good (green), intermediate (yellow) and bad (red) quality are presented. The unfolding curve of good quality can be used for fitting procedures using the Boltzmann model (Ericsson *et al.*, 2006), resulting in this case in an apparent T_m value of 321.8 K.

curves, which allows identification of the condition with the highest apparent melting temperature.
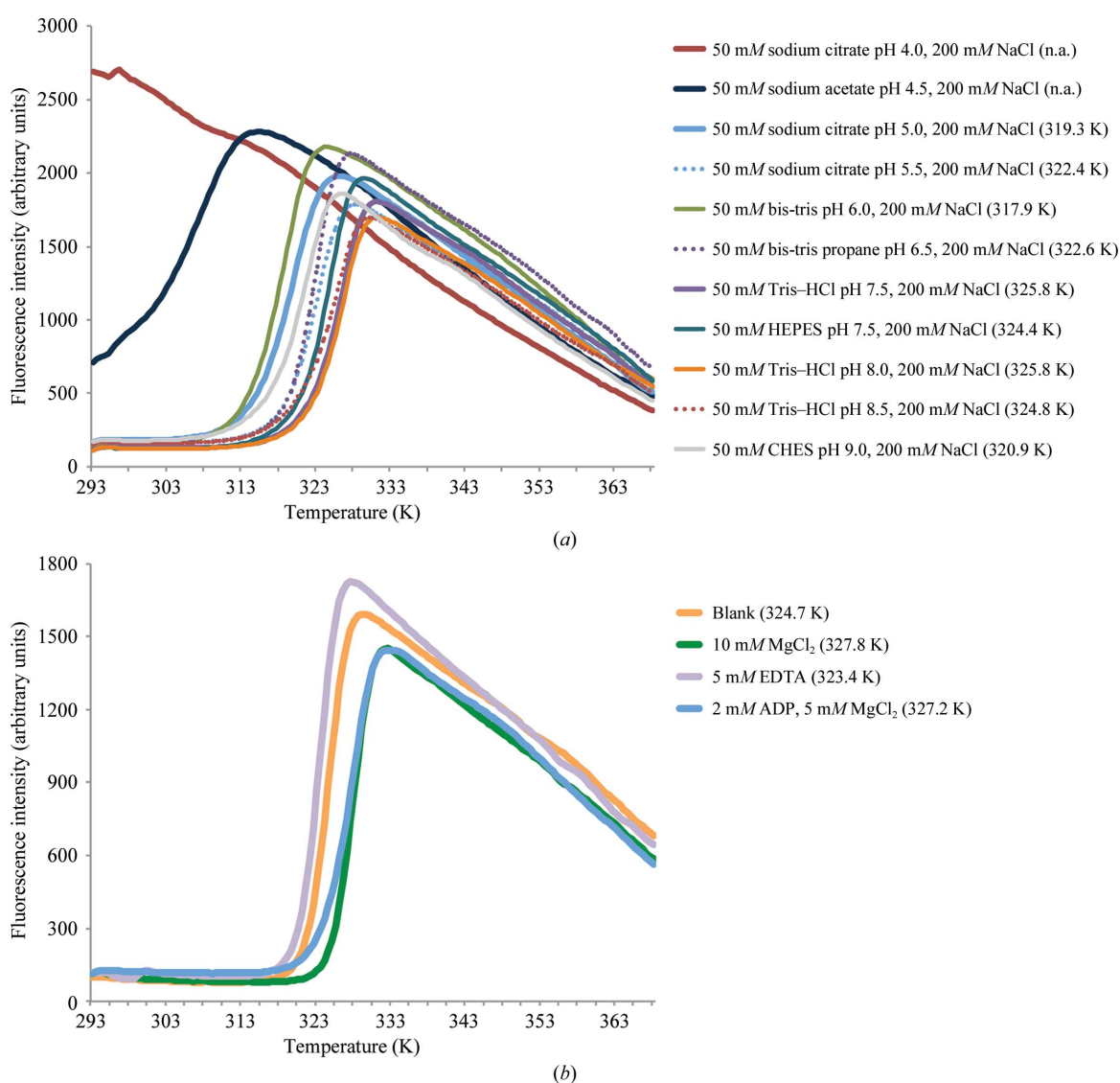
5.2. Non-ideal curve shape

In practice, the unfolding curves obtained from a Thermofluor experiment will mostly not exhibit an ideal sigmoidal shape. Fig. 1 depicts three possible outcomes. A good unfolding curve has no or only low fluorescence intensity before unfolding is initiated, followed by a sharp transition (green curve in Fig. 1). After reaching the maximum fluorescence a decay in fluorescence intensity is observed, which might be explained by thermal motion of the fluorescent dye and/or protein aggregation.

The fluorescent dye SYPRO Orange may exhibit high background fluorescence below the transition temperature, which is often

accompanied by a broadened transition to a non-interpretable curve shape (yellow curve in Fig. 1). This happens if the protein is already partially unfolded, *e.g.* owing to a sub-optimal initial buffer composition or protein:SYPRO Orange ratio or if the protein exhibits hydrophobic patches on its surface, *e.g.* owing to a missing binding partner. Such curves are usually not useful for fitting procedures using the Boltzmann model. However, by careful visual inspection of broadened curves a stabilizing buffer can often still be identified which might result in better melting curves in subsequent Thermofluor experiments (Crowther *et al.*, 2009).

For some proteins, no useful unfolding transition at all can be obtained (red curve in Fig. 1) because the protein is already unfolded at the beginning of the experiment and hence no further transition takes place. This observation is made for some proteins in the extreme regions of the pH buffer screen (*e.g.* pH 4.0 and 10.0).

(a)

- 50 mM sodium citrate pH 4.0, 200 mM NaCl (n.a.)
- 50 mM sodium acetate pH 4.5, 200 mM NaCl (n.a.)
- 50 mM sodium citrate pH 5.0, 200 mM NaCl (319.3 K)
- 50 mM sodium citrate pH 5.5, 200 mM NaCl (322.4 K)
- 50 mM bis-tris pH 6.0, 200 mM NaCl (317.9 K)
- 50 mM bis-tris propane pH 6.5, 200 mM NaCl (322.6 K)
- 50 mM Tris–HCl pH 7.5, 200 mM NaCl (325.8 K)
- 50 mM HEPES pH 7.5, 200 mM NaCl (324.4 K)
- 50 mM Tris–HCl pH 8.0, 200 mM NaCl (325.8 K)
- 50 mM Tris–HCl pH 8.5, 200 mM NaCl (324.8 K)
- 50 mM CHES pH 9.0, 200 mM NaCl (320.9 K)

(b)

- Blank (324.7 K)
- 10 mM MgCl$_2$ (327.8 K)
- 5 mM EDTA (323.4 K)
- 2 mM ADP, 5 mM MgCl$_2$ (327.2 K)

Figure 2

Thermofluor-based protein-unfolding curves of *Mtb*-DapD. The values in parentheses are T_m values as determined using the Boltzmann model (Ericsson *et al.*, 2006). A selection of protein-unfolding curves obtained in the buffer screen (*a*) and the additive screen (*b*) is shown. The buffer system used for the additive screen was 50 mM Tris–HCl pH 7.5, 200 mM NaCl. The Thermofluor experiment was prepared as described in §3 using 50 µM protein. The experiment was performed from 293 to 368 K in increments of 0.5 K. Please note that in a deviation from the screen described in this manuscript, the NaCl concentration of the screen was 200 mM instead of 250 mM. (*a*) The most stable protein sample was obtained in 50 mM Tris–HCl pH 7.5 or 8.0 supplemented with 200 mM NaCl. In 50 mM sodium citrate pH 4.0, 200 mM NaCl, DapD is unfolded at the beginning, resulting in a non-interpretable curve. (*b*) The additive screen showed that the T_m value for DapD was increased by about 3 K in the presence of MgCl$_2$, whereas the removal of divalent metal ions using EDTA resulted in destabilization. Adenosine-based nucleotides also result in a slight stabilization of DapD, probably because DapD uses succinyl-CoA as a cofactor, which exhibits an ADP moiety, and/or because of the presence of MgCl$_2$ in the nucleotide conditions.

(a)   (b)
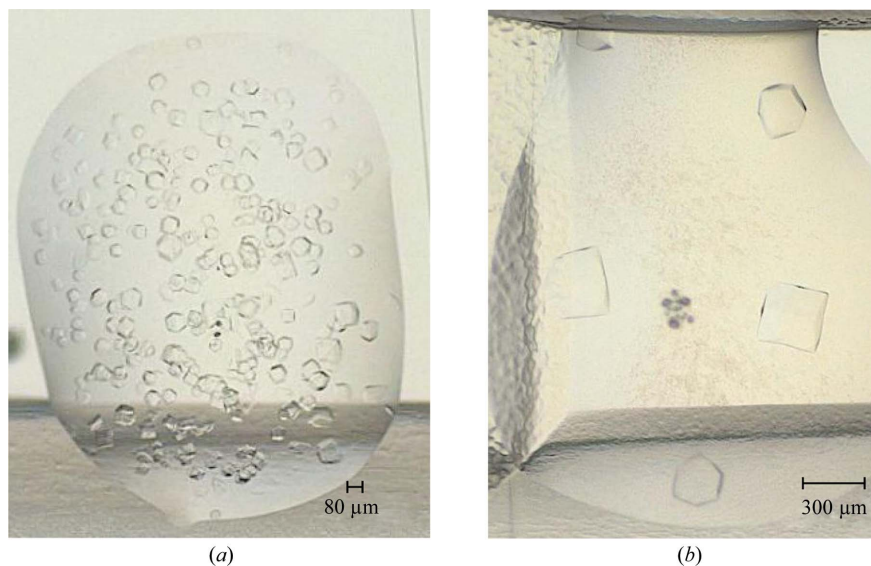
Figure 3
Crystals of *Mtb*-DapD obtained in the absence (*a*) and presence (*b*) of 10 m*M* MgCl$_2$. The crystals grew in the presence of a high concentration of sodium acetate pH 7.0 (Reinhard *et al.*, unpublished work). The protein buffer was 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 3 m*M* DTT. As shown previously, the enzymatic activity of *Mtb*-DapD is enhanced in the presence of MgCl$_2$ (Schuldt *et al.*, 2009).

However, if the buffer used for protein purification is so unfavourable, it can be envisaged that the whole Thermofluor screen will not yield any useful results. In such cases different initial buffer systems should be tried, although it may not be apparent from the Thermofluor assay which one to choose. Moreover, no unfolding transition is also observed when the analyzed protein does not have a hydrophobic core. This is, for instance, the case for some DNA-binding proteins.

5.3. When is a shift significant?

The effect of different compounds on the shift of the melting temperature cannot be predicted and the shift can range from as little as only a few degrees to several tens of degrees. The accuracy of the measurement is correlated with the shape of the unfolding curve (Crowther *et al.*, 2009) and hence can be considered as a protein-dependent factor. If desired, the precision of the measured value can be determined by performing multiple independent Thermofluor experiments. All changes larger than the determined error can be considered as being significant.

5.4. Negative shift in melting temperature

Obviously, for most proteins the goal of the experiment is the identification of an increased melting temperature in order to obtain a protein sample with higher stability and hopefully homogeneity. However, it is also feasible to look for the opposite: considering the very high melting temperatures of more than 363 K observed for some proteins from thermophilic microorganisms, it may also be worth identifying a buffer system which is destabilizing. In such a buffer system, protein solubility would be decreased and consequently the chance of crystallization may be enhanced.

Moreover, information about a negative shift can be used during protein-production procedures, *e.g.* destabilization of the protein in high imidazole or extremely high- or low-salt concentrations.

6. Results and discussion

In recent years the screen presented in this work, or earlier versions of it, have been used for buffer optimization of more than a dozen proteins, of which more than 50% were successfully crystallized. One example is the enzyme *Mtb*-DapD, a tetrahydrodipicolinate-*N*-succinyltransferase from *M. tuberculosis*, the successful crystallization and X-ray structure of which have been described previously (Schuldt *et al.*, 2008, 2009). Looking at results from a Thermofluor assay performed using DapD, it seems obvious that the Thermofluor assay is potentially very useful in order to identify conditions in which the protein is stable and therefore crystallizable (Figs. 2 and 3). For instance, DapD is significantly less stable in HEPES buffer than in Tris–HCl although both have the same pH value (Fig. 2*a*). Moreover, MgCl$_2$ was identified as a stabilizing agent (Fig. 2*b*). Subsequently, DapD was successfully crystallized from two different crystallization conditions. The first condition was based on a precipitant solution which contained 200 m*M* MgCl$_2$ (Schuldt *et al.*, 2008). In the second condition, the precipitant solution contained a high concentration of sodium acetate pH 7.0. Here, addition of Mg^{2+} significantly increased the crystal size from about 80 to 300 µm (Fig. 3; Reinhard *et al.*, unpublished work). In retrospect, this behaviour is nicely explained by the crystal structure of DapD obtained from the MgCl$_2$ crystallization condition (Schuldt *et al.*, 2009), which exhibits two tightly coordinated magnesium ions along the threefold axis of the DapD homotrimer.

7. Summary and outlook

The Thermofluor assay provides a quick and easy approach for identifying an optimized buffer composition for a protein of interest. In day-to-day laboratory work many cases were encountered in which optimization of the protein buffer had a positive effect on the quality, quantity and crystallizability of a protein sample. Consequently, it is strongly recommended that a Thermofluor experiment should be performed for every new protein target that is being worked on. However, just like every other experimental method, the Thermofluor method does not guarantee success and has its limitations.

laboratory communications

The Thermofluor screen presented in this work provides a general starting point for the identification of optimized buffer conditions and is suitable for a very wide variety of protein samples. Modifications of the screen are easy to perform in order to assess, for instance, more protein-specific properties such as the binding of certain ligands and inhibitors. Considering how easy it is to perform the experiment and also the small amount of protein sample that is required to perform a Thermofluor screen, it may be anticipated that this approach will play an even more important role in future structural biology projects.

References

Crowther, G. J. *et al.* (2009). *J. Biomol. Screen.* **14**, 700–707.
Ericsson, U. B., Hallberg, B. M., DeTitta, G. T., Dekker, N. & Nordlund, P. (2006). *Anal. Biochem.* **357**, 289–298.
Geerlof, A. *et al.* (2006). *Acta Cryst.* D**62**, 1125–1136.
Golovanov, A. P., Hautbergue, G. M., Wilson, S. A. & Lian, L.-Y. (2004). *J. Am. Chem. Soc.* **126**, 8933–8939.
Mezzasalma, T. M., Kranz, J. K., Chan, W., Struble, G. T., Schalk-Hihi, C., Deckman, I. C., Springer, B. A. & Todd, M. J. (2007). *J. Biomol. Screen.* **12**, 418–428.
Nettleship, J. E., Brown, J., Groves, M. R. & Geerlof, A. (2008). *Methods Mol. Biol.* **426**, 299–318.
Niesen, F. H., Berglund, H. & Vedadi, M. (2007). *Nature Protoc.* **2**, 2212–2221.
Pantoliano, M. W., Petrella, E. C., Kwasnoski, J. D., Lobanov, V. S., Myslik, J., Graf, E., Carver, T., Asel, E., Springer, B. A., Lane, P. & Salemme, F. R. (2001). *J. Biomol. Screen.* **6**, 429–440.
Rupp, B. (2009). *Biomolecular Crystallography: Principles, Practice, and Application to Structural Biology*. New York: Garland Science.
Schuldt, L., Weyand, S., Kefala, G. & Weiss, M. S. (2008). *Acta Cryst.* F**64**, 863–866.
Schuldt, L., Weyand, S., Kefala, G. & Weiss, M. S. (2009). *J. Mol. Biol.* **389**, 863–879.
Senisterra, G. A., Markin, E., Yamazaki, K., Hui, R., Vedadi, M. & Awrey, D. E. (2006). *J. Biomol. Screen.* **11**, 940–948.
Vedadi, M. *et al.* (2006). *Proc. Natl Acad. Sci. USA*, **103**, 15835–15840.