Chapter 15

Thermofluor-Based Analysis of Protein Integrity and Ligand Interactions

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Abstract

Thermofluor is a fluorescence-based thermal shift assay, which measures temperature-induced protein unfolding and thereby yields valuable information about the integrity of a purified recombinant protein. Analysis of ligand binding to a protein is another popular application of this assay. Thermofluor requires neither protein labeling nor highly specialized equipment, and can be performed in a regular real-time PCR instrument. Thus, for a typical molecular biology laboratory, Thermofluor is a convenient method for the routine assessment of protein quality. Here, we provide Thermofluor protocols using the example of Cdc123. This ATP-grasp protein is an essential assembly chaperone of the eukaryotic translation initiation factor eIF2. We also report on a destabilized mutant protein version and on the ATP-mediated thermal stabilization of wild-type Cdc123 illustrating protein integrity assessment and ligand binding analysis as two major applications of the Thermofluor assay.

Key words Thermofluor, Thermal shift assay, Differential scanning fluorimetry, SYPRO Orange, Protein stability, Ligand binding, ATP, Cdc123, eIF2

1 Introduction

Recombinant proteins are widely used for biochemical analyses, and frequently the question comes up whether the purified or stored protein is still stable. Methods suitable to give an answer, such as CD spectroscopy or differential scanning calorimetry, typically require large protein amounts and expensive specialized instruments, which are not available in many laboratories. In contrast, Thermofluor is a low-cost and straightforward technique well suited as a routine protein quality control in most molecular biology laboratories. Such quality control is a must for batch-to-batch comparisons and to improve experimental reproducibility.

Thermofluor, a thermal shift assay also known as differential scanning fluorimetry (DSF), has become a versatile technique for the measurement of protein stability. Thermofluor makes use of an environmentally sensitive fluorescent dye, mostly SYPRO Orange

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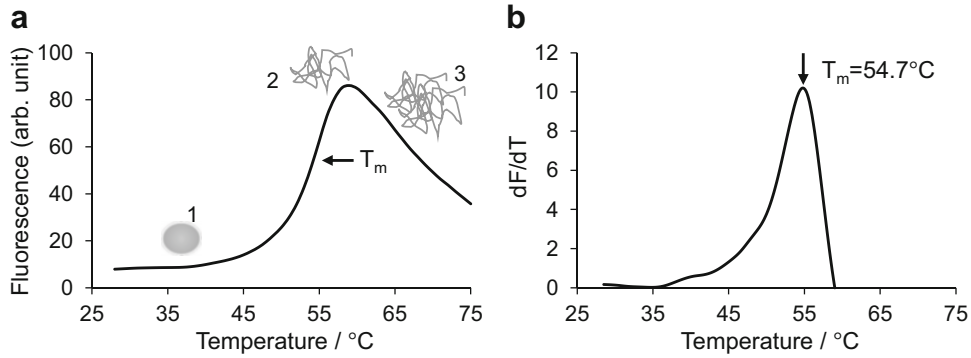

Fig. 1 A typical Thermofluor profile. Data shown were obtained with SpCdc123-his6 in the presence of 5× SYPRO Orange. (**a**) At low temperatures the protein is well-folded (gray sphere). The fluorescent dye SYPRO Orange is quenched in the aqueous environment. Thus, only basal SYPRO Orange fluorescence emission is measured at 555 nm upon excitation at 470 nm (1). As the protein gradually unfolds (gray ravel), SYPRO Orange binds to exposed hydrophobic regions. This leads to a strong increase in fluorescence emission (2). The protein's melting temperature (T_m, arrow) is given by the inflection point where 50% of the protein is unfolded. Following the peak of fluorescence intensity (protein is completely unfolded), a decrease of intensity is observed (3). This is probably due to protein aggregation, which removes protein from the solution and prevents SYPRO Orange from interacting with hydrophobic patches. (**b**) First derivative of the fluorescence as a function of temperature. The protein's melting temperature (T_m, arrow) is easily identified as the peak of the curve (*see* **Note 13**)

[1, 2], to monitor the thermal unfolding of proteins. The dye is quenched in an aqueous environment, but undergoes a pronounced increase in fluorescent quantum yield upon binding to exposed hydrophobic regions of the protein as the protein unfolds (Fig. 1). The gradual increase of temperature as well as the concomitant detection of the fluorescent signal can be performed by standard real-time PCR instruments. Data fitting using the real-time PCR instrument's accompanying software quickly provides the melting temperature (T_m) of the protein under various conditions. The T_m serves as a measure of protein stability [1, 3] and the shape of the curve as an indicator for protein integrity ([2, 4] and Fig. 2).

Upon its first description in 2001 for high throughput drug discovery [5], dyes such as 1-anilino-8-naphthalenesulfonate (ANS) or dapoxyl sulfonic acid were used, but eventually the dye with the most favorable characteristics for Thermofluor turned out to be SYPRO Orange [1, 6]. It has a high increase in quantum yield, and its excitation and emission maxima of ~500 nm and ~600 nm, respectively [1, 6], are compatible with standard filter sets of most real-time PCR instruments [2].

The simplicity and economical protein requirement make Thermofluor very attractive for the routine quality control of purified recombinant proteins. Thermofluor allows to easily evaluate any adverse effects on the protein of choice that might occur during

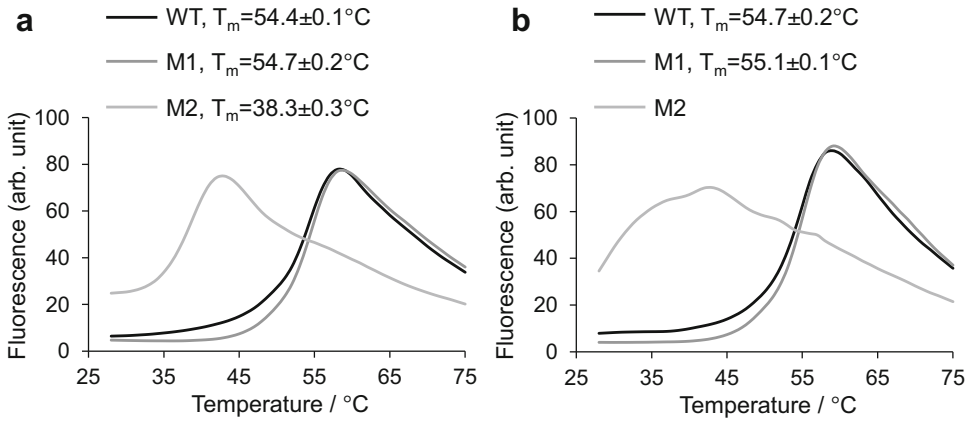

Fig. 2 Thermal destabilization of a mutant protein. Thermofluor was performed using 5x SYPRO Orange and 2 μg (2 μM) SpCdc123-his6 wild-type (WT, black line) or two SpCdc123-his6 mutants: mutant 1 (M1, dark gray line) and mutant 2 (M2, light gray line). In the thermal denaturation profiles shown, fluorescence emission is plotted versus temperature to monitor protein unfolding. (**a**) Proteins in imidazole-containing buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, approximately 160 mM imidazole) before dialysis. (**b**) Proteins in imidazole-free buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl) after dialysis (*see* **Note 3**). Shown is one representative curve per condition. The T_m (calculated from duplicate reactions) was derived as the peak of the first derivative of the fluorescence as a function of temperature, calculated by the melting curve analysis of the Rotor-Gene Q Software 2.3.4. Mutant 1 is similarly stable as wild-type SpCdc123-his6. Both proteins are essentially unaffected by imidazole. Mutant 2 is less stable than wild-type SpCdc123-his6. In imidazole-free buffer, it does no longer show a defined unfolding transition. Together the data indicate that amino acid replacements can affect protein stability and sensitize a protein toward buffer composition

affinity purification, protein concentration, buffer exchange (Fig. 2), freezing or prolonged storage. In particular for mutant proteins, Thermofluor quickly shows whether or not amino acid exchanges do influence protein stability (Fig. 2). Thermofluor analysis therefore provides valuable data for the interpretation of downstream assays.

Ligand interaction usually stabilizes the native protein [1, 7, 8], thus leading to an increase in melting temperature. This can be employed, on the one hand, for ligand screenings in drug design [5, 8–10], and on the other hand, to characterize the binding of natural ligands, such as nucleotides, to proteins [11, 12] (Fig. 3). Even an approximation of K_d values is possible [9, 13–15], which provides useful preinformation for biophysical methods like isothermal titration calorimetry (ITC) that consume larger quantities of protein. Thermodynamic parameters obtained from Thermofluor assays correlate well with those determined by other biophysical methods [4, 9, 16, 17]. Not only ligands but also solvents and additives affect the stability and thus the T_m of proteins. Accordingly, Thermofluor is a popular method for the determination of optimal buffer conditions for protein purification, storage and structural studies such as crystallization or NMR [2, 3, 16, 18–20].

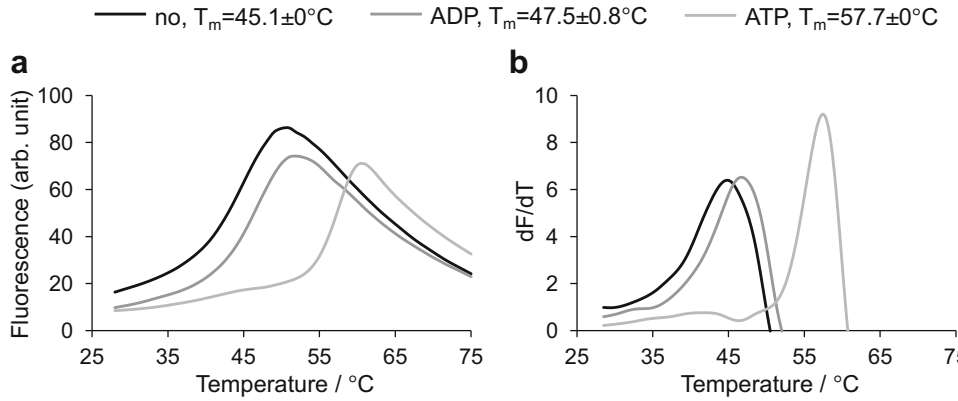

Fig. 3 Stabilization of hD123 through ATP. Thermofluor was performed using 2 μg hD123(1–290)-his6 (2.3 μM) and 5× SYPRO Orange without nucleotide (no, black line), or with 1 mM ADP (ADP, dark gray line) or 1 mM ATP (ATP, light gray line). One representative curve is shown. (**a**) In the thermal denaturation profiles shown, fluorescence emission is plotted *versus* temperature to monitor the unfolding of hD123 (1-290)-his6. (**b**) The first derivative of the fluorescence as a function of temperature was exported from the Rotor-Gene Q Software 2.3.4 and plotted using Microsoft Excel. The T_m is represented as the peak of the curve. The T_m values shown in the legend were calculated as an average of duplicates from three independent experiments. The data indicate that ATP-binding stabilizes hD123 by more than 10 degrees

Recombinant proteins are critical tools in biochemical studies such as the analysis of ribosome biogenesis and mRNA translation. Recently it has become clear that the well-studied eukaryotic translation initiation factor 2 (eIF2) requires a dedicated assembly factor [21]. This protein called Cdc123 is conserved among eukaryotic organisms and indispensable for the viability of yeast and human cells [21–24]. Protein structure analysis revealed that Cdc123 is related to ATP-grasp enzymes [25]. Here we use Cdc123 as an example to present Thermofluor protocols for the analysis of protein stability and nucleotide binding. We show data illustrating a mutant protein that became unstable after removal of imidazole by dialysis, while generating a proper unfolding curve before dialysis (Fig. 2). Furthermore, as an example of a nucleotide-binding assay, we show that Cdc123 is stabilized strongly by ATP, and to some extent also by ADP (Fig. 3). While several of the technical aspects discussed here are specific to the real-time PCR machine Rotor-Gene Q 2plex Platform (Qiagen), the method can be applied to any standard real-time PCR instrument [2, 3, 9, 10, 13, 15] typically available in molecular biology laboratories.

2 Materials

1. SYPRO Orange Protein Gel Stain, 5000× concentrate in DMSO (e.g., Thermo Scientific) (*see* **Note 1**), diluted 1:5 in DMSO to yield a 1000× working dilution in DMSO (*see* **Note 2**). The 1000× SYPRO Orange stock is stored at 4 °C.

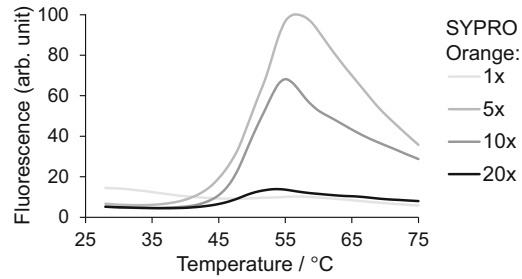

Fig. 4 Fluorescence dye optimization. Melting curves are shown using different SYPRO Orange concentrations as described in Subheading 3.1. Thermofluor was performed using 2 μg his6-SpCdc123 (2 μM) together with 1× (light gray line), 5× (gray line), 10× (dark gray line), or 20× (black line) SYPRO Orange. The gain was set to 9.33. In the thermal denaturation profiles shown, fluorescence intensity is plotted versus temperature to monitor the unfolding of his6-SpCdc123. The melting curve with the best dynamic rage was obtained with 5× SYPRO Orange

2. Purified recombinant proteins dialyzed against 2× Thermofluor (TF) buffer (*see* **Note 3**).

   We used Cdc123 from *Schizosaccharomyces pombe* (Sp), carrying a six histidine affinity-tag (his6) at the N- or C-terminus, as indicated: his6-SpCdc123 (39.1 kDa) (Fig. 4) and SpCdc123-his6 (37.9 kDa) (wild-type (wt), mutant 1 (M1) with 2 amino acid exchanges, and mutant 2 (M2) with an additional third exchange) (Figs. 1 and 2). In addition, a C-terminally truncated version of the human homolog hD123 (hD123(1-290)-his6; 34.8 kDa) (Fig. 3) was used. All proteins were expressed in *E. coli* BL21-CodonPlus and purified on an ÄKTA system using Ni-NTA columns (GE Healthcare). An imidazole gradient (20–350 mM) was used for elution of the proteins from the column.

3. 2× Thermofluor (TF) buffer (*see* **Note 4**): 50 mM Tris/HCl pH 7.5, 400 mM NaCl.
4. 500 mM magnesium acetate.
5. Ultrapure water.
6. 100 mM ATP (pH adjusted with NaOH, e.g., Thermo Fisher Scientific) stored at −20 °C in small aliquots to avoid refreezing.
7. Real-time PCR instrument such as Rotor-Gene Q 2plex Platform (Qiagen) with Rotor-Gene Q Software 2.3.4; 0.1 ml 4-strip PCR-tubes and caps, or plates, as suitable for the real-time PCR instrument.

3 Methods

Unless indicated otherwise, all steps are performed on ice.

3.1 Optimization of Protein Amount and SYPRO Orange Concentration (See Note 5 and Fig. 4)

1. Each reaction is prepared in duplicate. The final volume of each reaction is 25 µl.
2. Clear the protein sample from aggregates and precipitates (*see* **Note 6**) by filtration or centrifugation (4 °C, 20 min, 16,000 rcf [max speed]).
3. Protein master mixes for 2 µg and 5 µg protein per reaction are prepared, each for 9 reactions, according to Table 1.
4. 1000× SYPRO Orange in DMSO is diluted in H_2O to 5×, 25×, 50× and 100× SYPRO Orange (for 1×, 5×, 10×, and 20× SYPRO Orange in the final reaction). Prepare dilutions in H_2O fresh each time.
5. Distribute 20 µl of each protein mix from **step 3** to 8 × 0.1 ml PCR tubes (*see* **Note 7**).
6. For duplicate reactions, add 5 µl of each SYPRO Orange dilution from **step 4** to 2 aliquots of each protein mix from **step 5**. Mix by pipetting up and down three times.
7. Close the tubes, place in a 72-well rotor of a real-time PCR instrument (Rotor-Gene Q 2plex Platform, Qiagen) and shake the liquid to the bottom of the tubes (*see* **Note 8**).
8. Run the melt program with the 470 nm source and 555 nm detector filter (*see* **Note 9**) using different gain settings (*see* **Note 10**). Ramp from 28 °C to 75 °C (*see* **Note 11**) rising by 1 °C each step. Wait for 90 s of premelt conditioning on first step. Wait for 15 s for each step afterward.
9. Melting curves as shown in Fig. 4 are obtained. Choose the condition with the lowest protein amount that gives a good signal to noise ratio and sharp unfolding transition (*see* **Notes 5** and **12**).

Table 1
Protein mix for optimization of SYPRO Orange and protein concentration

In final reaction (25 µl)	Stock	1×	9×
2 µg or 5 µg protein in 2× Thermofluor buffer	*x* µg/µl	*y* µl	
1× Thermofluor buffer	2×	12.5–*y* µl	
20 mM magnesium acetate	500 mM	1 µl	9 µl
H_2O to 20 µl		6.75 µl	60.8 µl

Table 2
Buffer mix for protein stability assay

In final reaction (25 µl)	Stock	1×	9×
20 mM magnesium acetate	500 mM	1 µl	9 µl
5× SYPRO Orange (conc. as optimized)	1000×	0.125 µl	1.13 µl
H_2O to 12.5 µl		11.38 µl	102.4 µl

3.2 Protein Stability Assay

1. Each reaction is prepared in duplicate. The final volume of each reaction is 25 µl. This example is calculated for the analysis of three different proteins (wild-type SpCdc123-his6 and two mutants).
2. Clear the protein sample from aggregates and precipitates (*see* **Note 6**) by filtration or centrifugation (4 °C, 20 min, 16,000 rcf [max speed]).
3. Per reaction, complete 2 µg protein to 12.5 µl with 2× TF buffer. A master mix for 3 reactions is prepared for each protein.
4. The buffer-mix is prepared as a master mix for 9 reactions as described in Table 2.
5. 12.5 µl protein-mix from **step 3** or 2× TF buffer is distributed in duplicate to 0.1 ml PCR tubes (*see* **Note 7**). 2× TF buffer serves as a negative control to measure background fluorescence.
6. 12.5 µl buffer-mix from **step 4** is added to the proteins and buffer control and mixed by pipetting three times up and down. The final reaction condition is 2 µg protein (= 2 µM SpCdc123-his6), 25 mM Tris pH 7.5, 200 mM NaCl, 20 mM magnesium acetate, 5× SYPRO Orange.
7. Continue as described in Subheading 3.1 **steps 7** and **8**.
8. Analyze the data using the instrument's accompanying software (Rotor-Gene Q Software 2.3.4) to obtain the T_m. The first derivative of the fluorescence is plotted as a function of temperature (dF/dT). The maximum peak of the curve represents the T_m (*see* **Note 13**).
9. The result for wild-type SpCdc123-his6 and two mutants is shown in Fig. 2. The data indicate that mutant M2 is thermally destabilized and sensitive toward buffer exchange.

3.3 Nucleotide Binding Assay

1. Proceed as described in Subheading 3.2, except that in addition to Subheading 3.2 **step 4** two more buffer mixes are prepared containing 1 mM ATP or ADP (Table 3). Accordingly, a protein master mix for 7 reactions is prepared as described in Subheading 3.2 **step 3**.

Table 3
Buffer mix for nucleotide binding assay

In final reaction (25 µl)	Stock	1×	9×
20 mM magnesium acetate	500 mM	1 µl	9 µl
5× SYPRO Orange (or conc. as optimized)	1000×	0.125 µl	1.13 µl
1 mM ATP or ADP	100 mM	0.25 µl	2.25 µl
H_2O to 12.5 µl		11.13 µl	100.1 µl

2. The melting curves of wild-type hD123 (hD123(1-290)-his6) in the absence of nucleotide and in the presence of ADP and ATP are shown in Fig. 3. The data indicate that ATP stabilizes hD123 by more than 10 degrees.

4 Notes

1. SYPRO Orange is light sensitive and should therefore be kept in the dark.
2. To avoid DMSO concentrations above 2% in the final reaction, the SYPRO Orange working stock solution should be as highly concentrated as possible. The SYPRO Orange dye (Invitrogen) is provided as 5000× solution in 100% DMSO. We found a 1000× SYPRO Orange in DMSO working stock dilution to be optimal. For 5× SYPRO Orange in the final reaction, DMSO is at 0.05%, while pipetting volumes are still acceptable.
3. The dialysis serves to remove the imidazole, which is in the elution buffer of our Ni-NTA–purified recombinant proteins. Imidazole influences the T_m and the imidazole concentrations differ in each gradient-eluted protein fraction. After dialysis against a defined buffer, T_m values are well reproducible. Furthermore, the dialysis against 2× TF buffer has the advantage, that even if high protein volumes have to be added to the reaction (in case of low initial protein concentrations) it is easy to keep the final buffer composition identical in all conditions. Half of the final reaction volume corresponds to protein volume plus 2×TF buffer.
4. The Thermofluor buffer composition can be adjusted to the protein's needs [3, 16, 18–20]. Tris is used here because of additional downstream assays.
5. We recommend the optimization of protein and SYPRO Orange concentration. The SYPRO Orange concentration strongly influences the increase in fluorescence upon protein denaturation (Fig. 4). Too much or too little SYPRO Orange

relative to protein leads to reduced quantum yield (*see* Fig. 4 20× and 1× SYPRO Orange). Choose the lowest protein amount that uses the full dynamic range of the photomultiplier, a good signal to noise ratio and sharp unfolding transition. Another approach, starting with a fixed 10× SYPRO Orange concentration and adjusting only protein amount, will in many cases consume more protein than necessary. We found that for most proteins optimal conditions are at around 2 μg protein (0.08 mg/ml) and 5× SYPRO Orange. However, in some cases protein concentrations might have to be varied from 0.01 to 0.2 mg/ml and SYPRO Orange from 1× to 20× to obtain suitable conditions.

6. Removing denatured protein from the sample is essential, since it might otherwise produce high background fluorescence.
7. Place the liquid approximately 3/4th down the tube to leave enough space above to add the SYPRO Orange–containing mix.
8. After placing the tubes in the 72-well rotor and attaching the locking ring, shake all liquid to the bottom of the tubes. Otherwise, no fluorescent signal will be collected at the beginning of the run, until the centrifugal force of the rotor forces the liquid to the bottom of the tube.
9. The emission and excitation peaks of protein-bound SYPRO Orange are at approximately 500 nm and 600 nm, respectively [1]. Using the standard 470 ± 10 nm excitation and 557 ± 5 nm emission filters provided with the Rotor-Gene Q 2plex Platform (Qiagen), we obtained substantial fluorescence increases: around 13-fold for lysozyme (3.8 μg) in 10× SYPRO Orange or around 22-fold for his6-SpCdc123 (2 μg) in 5× SYPRO Orange.
10. The gain determines the sensitivity of the photomultiplier that converts fluorescence photons to electronic signals. The gain setting is a means to modulate the signal amplification. If the gain is too high, the signal will be oversaturated. If the gain is too low, the signal will disappear in the background noise. To use the complete dynamic range of the photomultiplier, it is best to acquire the data on three to four channels with different gains in parallel. In the optimal gain setting, fluorescence values will approach the maximum threshold. We usually work with gains between 7.67 and 9.33.
11. For very stable proteins (e.g., lysozyme $T_m = 72$ °C) increase the temperature range of the melting curve.
12. If the protein fails to generate a melting curve, the protein (1) might be already denatured, which results in high fluorescence, (2) might contain intrinsically disordered regions, which

also results in high background fluorescence, or (3) might lack hydrophobic regions. The latter is often the case for small proteins, for example, ubiquitin (data not shown) and [2].

13. Here, the T_m is determined by plotting the first derivative of the fluorescence as a function of temperature, where the T_m is represented as the peak of the curve. Alternatively, the T_m, as midpoint of the unfolding transition, can be determined by nonlinear fitting to a Boltzmann equation [3, 17]. The data can be easily exported from the real-time PCR instrument's software as a text file and analyzed with a method and software of choice.

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