# High-Throughput Differential Scanning Fluorimetry of GFP-Tagged Proteins

Running title: High-Throughput DSF-GTP

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## i. Summary

Differential scanning fluorimetry is useful for a wide variety of applications including characterisation of protein function, structure-activity relationships, drug screening, and optimization of buffer conditions for protein purification, enzyme activity, and crystallization. A limitation of classic differential scanning fluorimetry is its reliance on highly purified protein samples. This limitation is overcome through differential scanning fluorimetry of GFP-tagged proteins (DSF-GTP). DSF-GTP specifically measures the unfolding and aggregation of a target protein fused to GFP through its proximal perturbation effects on GFP fluorescence. As a result of this unique principle, DSF-GTP can specifically measure the thermal stability of a target protein in the presence of other proteins. Additionally, the GFP provides a unique in-assay quality control measure. Here, we describe the workflow, steps and important considerations for executing a DSF-GTP experiment in a 96-well plate format.

## ii. Key Words

Fluorimetry, high-throughput screening, thermal shift assay, green fluorescent protein, ligand binding, enzyme inhibitors, selective protein unfolding, drug discovery

## 1. Introduction

Standard differential scanning fluorimetry techniques have been used to screen compounds for activity against target enzymes in drug discovery programs [1]. However, these techniques require solvatochromic dyes, such as 1,8-ANS and SYPRO Orange which can interfere with additives, ligands and the target protein. Differential scanning fluorimetry of GFP-tagged proteins (DSF-GTP) does not require any dye, making it ideal for studying protein-ligand interactions [2]. DSF-GTP is simple, fast, robust and insensitive to variations in reaction volumes, with a useful temperature and pH range of 25 – 75 °C and 5 – 11 respectively. The thermal stability of a target protein is obtained within 1 - 2 hr by simply subjecting a GFP-tagged protein in a given condition to a standard melt curve protocol in a real-time thermal cycler (Fig. 1) [2-6]. DSF-GTP has been applied for the high-throughput screening of optimal buffer conditions such as pH and salt, the identification of protein-specific substrates, cofactors and other complex ligands (e.g. DNA and RNA), the screening of small molecule libraries as well as structure-activity relationships and off-target effects, and the functional characterization of proteins [2-6]. DSF-GTP is the only DSF method that can selectively measure the transition midpoint ($T_m$) of a target protein in the presence of other proteins, enabling assessment of target engagement and off-target serum interaction studies [7]. Additionally, the C-terminal GFP tag provides a unique intra and inter-assay quality control reference GFP fluorescence $T_m$ peak that is unique to this method and allows detection of buffer effects and inconsistencies as well as non-specific ligand effects.

[Fig 1 near here]

## 2. Materials

### 2.1 Cloning of target protein in a GFP vector

1. pET-based GFP vector for cloning and expression of a target protein with N-terminal hexahistidine (6-His) tag, inter protein linker and C-terminal GFP (see **Note 1**, Fig. 1).

2. Single Step (KRX) Competent Cells (see **Note 2**). Alternatively, competent *E. coli* cloning strains such as DH12s and DH5$\alpha$ for vector amplification.

3. Appropriate antibiotics for selective growth media (e.g. ampicillin and chloramphenicol).

4. Luria-Bertani (LB) or SOC broth [8].

5. LB agar plates supplemented with (0.4% w/v) glucose and antibiotic.

6. Water bath (for transformation) and incubators.

### 2.2 Cell culture for protein expression

Protein expression requires the fresh transformation of a pET-based GFP vector containing the target coding sequence in KRX or an alternative T7 expression strain such as BL21(DE3) or derivative thereof [9,10].

1. Appropriate antibiotics for selective growth media (e.g. ampicillin and chloramphenicol).

2. Appropriate inducer (e.g. rhamnose, IPTG or lactose, see **Note 3**).

3. LB broth and LB agar plates supplemented with (0.4% w/v) glucose and antibiotic [8].

4. Terrific broth (TB) or auto-induction medium [11] supplemented with antibiotic.

5. A refrigerated centrifuge capable of processing 50 mL polypropylene tubes at >2,000 ×*g*.

6. A laboratory refrigerated incubator shaker suitable for 100 mL and 1 L conical flasks.

**2.3 Purification**

1. Refrigerated high speed centrifuge and rotors (40,000 ×*g*) for polypropylene/polycarbonate tubes up to 50 mL (depending on culture lysate volume).

2. Cell lysis system (e.g. French pressure cell press, see **Note 4**).

3. Gravity flow chromatography columns depending on cell lysate volume.

4. SDS-PAGE system and reagents to assess protein expression, solubility and purity.

5. Lysis buffer: 50 mM sodium phosphate (pH 7.8), 300 mM NaCl, 10 mM imidazole (optional), 2 mM β-mercaptoethanol (optional) and 10% glycerol v/v (optional).

6. Elution buffer: 50 mM sodium phosphate (pH 7.8), 300 mM NaCl, 200 mM imidazole, 2 mM β-mercaptoethanol (optional) and 10% glycerol v/v (optional).

7. Immobilized metal affinity chromatography (IMAC) nickel resin (e.g. BioRad Profinity IMAC nickel-charged resin).

8. Ammonium sulphate for protein precipitation (optional).

9. Appropriate resuspension buffer (dependent on target protein requirements for solubility and stability if known, or lysis buffer if requirements are unknown).

10. Spectrophotometer and protein quantification assay (e.g. Bradford, BCA).

**2.4 Transition midpoint ($T_m$) peak determination**

1. Real-time thermal cycler capable of performing melt curve analyses (e.g. Bio-Rad CFX96 or equivalent).

2. Clear 96-well PCR plates, tubes or strips.

3. PCR plate sealing films, caps or cap strips.

4. Soluble GFP-tagged target protein (≥1 μM in resuspension buffer).

5. Assay buffer at 2x concentration (dependent on target protein requirements for activity if known, or lysis buffer if unknown, see **Note 5**).

6. Test reagents (see **Note 5**).

7. Compound screening: hard-shell clear 96-well skirted PCR plates and seals for high-throughput screening (see **Note 6**).

8. Compounds (5 mM in DMSO).

## 3. Methods

### 3.1 Cloning of target protein coding sequence in a GFP fusion vector

The pET-uvGFP vector (pIM013) [12] enables the directional cloning of a target protein coding sequence without a stop codon flanked with 5′ EcoRV and 3′ NheI sites and its expression as a C-terminal GFP fusion protein. The pIM013 vector is available on request.

1. Obtain target protein coding sequence that is codon optimized for *E. coli* (optional, see **Note 7**).

2. Clone target protein coding sequence into a GFP expression vector such as pIM013 [12].

3. Transform the recombinant vector in KRX cells and select transformants on LB agar supplemented with glucose and ampicillin (see **Note 8**).

4. It is good practice to confirm presence and integrity of target sequence before storage and use (optional see **Note 9**).

### 3.2 Small scale cultures for evaluation of protein expression in KRX cells

We advise to first assess a few clones for GFP fusion protein expression in soluble and fluorescent form using a small scale auto-induction system culture to identify the highest yielding colonies.

1. Always freshly transform competent KRX cells with recombinant expression vector.

2. Select transformants (i.e. single colonies) on LB glucose + ampicillin plates (See **Note 10**). Important: re-plate the selected colonies on a fresh master LB glucose + ampicillin plate for large scale protein expression and temporary storage.

3. Inoculate a small-scale test culture with a single colony (10 mL TB media + ampicillin in a 100 mL flask) and incubate at 37°C with shaking at 200 RPM until $OD_{600}$ reaches ~ 1.

4. Take a pre-induction sample, centrifuge and combine with SDS-PAGE sample buffer for subsequent target protein production assessment.

5. To the remaining culture, add rhamnose to 0.1% (w/v) final concentration and reduce temperature of shaker to 16°C (see **Note 11**) with shaking at 200 RPM for 2 days.

6. Take a post-induction sample as per Step 4 (see **Note 12**).

7. Harvest cells by centrifugation (see **Note 13**) and discard supernatant (optional: freeze the pellet for small-scale purification if desired).

**3.3 Large scale protein expression**

Identification of the best producing clone is described in 3.2. The colony is selected from a fresh LB glucose + ampicillin replica plate to inoculate the culture. A 100 mL culture yields sufficient proteins for at least 1,000 DSF-GTP reactions.

1. Inoculate 100 mL TB media + ampicillin in a 1 L flask with a full inoculation loop of bacteria that was scrapped off an overnight master LB glucose + ampicillin plate and incubate at 37°C with shaking at 200 RPM until $OD_{600}$ reaches 1-1.5.

2. Add rhamnose to 0.1% final concentration (or perform auto-induction) and reduce temperature of shaker to 16°C with shaking at 200 RPM for 2 days (see **Note 11**).

3. Harvest cells by centrifugation and discard supernatant (see **Note 14**).

**3.4 Protein purification**

All purification steps are performed on ice or at 4°C.

1. Resuspend cell pellet with 7 mL of ice cold lysis buffer per gram of wet cell paste.

2. Lyse cells by passing the cell suspension twice through an ice cold French pressure cell with the French press set at 10,000-12,000 psi. For optimal cell lysis, we slowly open the valve of the pressure cell to allow the cell suspension to drip out at a rate of 1-2 drop per second (see **Note 4**). Set aside a small aliquot.

3. Clarify lysate by centrifugation at 40,000 g for 20-40 min at 4°C. Keep the supernatant and set aside a small aliquot.

4. Load the clarified lysate into a gravity column packed with 1-2 mL of IMAC nickel resin or a similar resin. Follow the manufacturer's guidelines for purification (see **Note 15**). Using this set-up, we generally recover and combine about 4-8 mL of GFP fusion protein containing fractions and keep a small aliquot for each fraction to assess protein purity and integrity by SDS-PAGE (see **Note 16**).

5. Fractions containing GFP fusion proteins can be precipitated with 0.5 g/mL ammonium sulphate on ice with gentle mixing for 1 hr (optional).

6. Protein precipitate is recovered by centrifugation at 40,000 g for 20-40 min at 4°C and supernatant is checked for GFP fluorescence before discarding it (optional: only necessary if step 5 is performed).

7. Resuspend protein pellet in lysis buffer or alternative buffer suitable for the stability of the target protein at a final concentration of 50-100 μM (optional: only necessary if steps 5 and 6 are performed) (see **Note 17**).

8. Further purification and dialysis can be performed if desired (see **Note 18**).

9. Determine protein concentration (see **Note 19**).

### 3.5 Validation of Target-GFP fusion protein by DSF-GTP

Preliminary screens can be run with any fraction containing the Target-GFP protein, i.e. straight after elution from the column. Initial $T_m$ determination can be performed with as little as 20-50 μL of a 1-3 μM Target-GFP protein solution corresponding to an initial RFU value of 4,000-8,000 (see **Note 19**). Further buffer optimization can then be performed to identify ideal buffer conditions. If no $T_m$ is apparent, the protein may be in an unstable or non-homogenous oligomeric form and may require the addition of cofactors (see **Note 20**) to produce a homogenous complex capable of producing a $T_m$ peak [2]. Thus, for proteins with known or suspected ligands and/or cofactors, reactions should be supplemented with specific ligands and/or cofactors at concentrations ranging from 1-10 mM. Alternatively, the protein may have a $T_m$

greater than that of GFP which might require the addition of destabilizing molecules to lower the $T_m$ of the target protein.

1. Reactions containing 1-3 μM Target-GFP protein equivalent to initial RFUs ~4,000-8,000 in elution buffer (20-50 μL final volume) are set up in 96-well plates and sealed with PCR plate sealing film (see **Note 21**). At this stage, the effect of a cofactor or ligand can be tested.

2. Set up a melt curve program ranging from 25 to 90°C with 0.5°C increments for 30 sec in a suitable real-time PCR thermal cycler (see **Note 22** and Fig. 2).

[Fig 2 near here]

3. Run the melt curve program with the FAM channel selected (see **Note 23**).

4. $T_m$ curves are obtained and $T_m$ values are recorded for each reaction (see **Note 24**, Fig. 3). All data and graphs can be exported and processed further (see Fig. 4).

[Fig 3 near here]

[Fig 4 near here]

**3.6 High-throughput screening by DSF-GTP**

DSF-GTP can be used to screen for compounds that interact directly with a target protein in 96-well plate format (see **Note 6**). The $T_m$ value of GFP provides a unique

in-assay quality control measure which is useful to detect interferences or non-specific interactions that can occur with a given compound or test condition. Applications such as hit or ligand identification and structure activity relationship investigation are within the scope of DSF-GTP. Of special note, DSF-GTP has been shown to be the only DSF method suited for the detection of off-target effects such as non-specific binding or neutralization of a compound in a complex biological sample such as serum or cell lysate [7]. Below, we provide an example of high-throughput experimental setup that was applied to BirA-GFP [7].

1. Target-GFP fusion protein is diluted to 1 μM in a suitable diluent buffer. For a 96-well plate, 5 mL of protein solution at 1 μM will be required.

2. Compounds are distributed in the 96-well PCR plate in identical solvent and concentration (e.g. 1 μL of a 5 mM solution of compound in DMSO).

3. A volume of 49 μL diluted protein is aliquoted and mixed in each well containing 1 μL of a 5 mM solution of compound in DMSO. Two control wells contain 1 μL of pure DMSO (see **Note 25**).

4. Seal and equilibrate the plate at room temperature for 10 min.

5. Run a melt curve and export data as described in Section 3.5.

6. Determine $\Delta T_m$ by subtracting the $T_m$ of the fusion protein alone from $T_m$ of the fusion protein with compound in identical buffer conditions.

7. Determine an appropriate $\pm\Delta T_m$ cut-off value and select hits for further investigation (see **Note 26**).

### 3.7 Salt and pH dependence

Ionic strength and pH of buffers affect the stability of proteins and protein:ligand complexes. DSF-GTP can be applied to evaluate the effect of ionic strength and pH on a particular protein or protein:ligand complex. Reactions can be run with the fusion protein in the presence or absence of a ligand or cofactor of interest at different pH or salt concentrations (see **Note 27**). Buffers with pH ranging from 5-11 have been successfully used in DSF-GTP (see **Note 28**). An example of experimental setup to determine the effect of NaCl is provided below:

1. Dilute protein sample to 2 μM in buffer at twice the intended buffer concentration.
2. Dilute NaCl in water at twice the intended final concentration (see **Note 29**). Pure water is combined with the protein solution in the control reaction.
3. Combine equal volumes of protein and NaCl solutions in a well (50 μL reaction volume).
4. Seal and equilibrate the plate at RT for 10 min.
5. Run a melt curve and export data as described in Section 3.5.

### 3.8 Cofactors and coenzymes

The binding of a cofactor or coenzyme to a protein can significantly affect its thermal stability. Examples of cofactors that can easily be assessed with DSF-GTP include divalent metal ions, coenzymes, nucleotides, and vitamins. Care must be taken with transition metals as they can significantly affect GFP at low mM concentrations (**Note 30**).

1. Dilute protein sample to 2 μM in assay buffer (see **Note 17**) at twice the intended final concentration.

2. Dilute cofactor in water at twice the intended final concentration.

3. Combine equal volumes of protein and cofactor solutions in a well (50 μL reaction volume). Pure water is combined with the protein solution in the control reaction (see **Note 31**).

4. Seal and equilibrate the plate at RT for 10 min.

5. Run a melt curve and export data as described in Section 3.5.

### 3.9 Effect of ligand binding affinity and concentration on target protein $T_m$

In this section we illustrate how to set up a concentration dependence experiment to inform about ligand binding affinity with a target protein. The compound concentrations should range between 1-1000 μM. We recommend a twofold serial dilution.

1. Dilute protein sample to 2 μM in buffer at twice the intended buffer concentration.

2. Prepare a twofold serial dilution of the compound (see **Note 29**). For this, dilute the compound in water at twice the intended final concentration.

3. Combine equal volumes of protein and compound solutions in a well (50 μL reaction volume). Pure water is combined with the protein solution in the control reaction.

4. Seal and equilibrate the plate at RT for 10 min.

5. Run a melt curve and export data as described above.

6. Determine the net change in $T_m$ ($\Delta T_m$) between the $T_m$ values obtained for a control reaction (with water) and a test condition (with compound) for each concentration (see **Note 32** and Fig. 5).

7. Plot $\Delta T_m$ values *vs* $\log_{10}$ of compound concentrations (Fig. 5B) using a curve fitting software such as GraphPad.

8. Perform linear regression analysis of the linear portion of the curve and determine the compound concentration value at $\Delta T_m = 0$ (See **Note 33** and Fig. 5B).

[Fig 5 near here]

### 3.10 Special considerations for nucleic acids interactions

Proteins binding to single and/or double stranded RNA or DNA can be assessed by DSF-GTP. Nucleic acids binding to a protein usually stabilizes the protein [2-4,24]. An important point to consider when examining these complex interactions by DSF-GTP is that in the case of RNA or DNA their secondary structures can melt during the melt curve run whether they are single or double stranded. It is nevertheless possible to perform such reactions as long as these specific limitations are considered [3]. It is always advisable to confirm RNA or DNA binding to the Target-GFP using EMSA or GFP-EMSA [5]. Ensure the buffer used does not interfere with binding [13].

## 4 Notes

**1.** We routinely use the services of a gene synthesis provider as it is economical, fast and DNA sequencing of the target sequence is included in the service. Some gene synthesis providers will directly clone the codon optimized target protein coding sequence in a GFP expression vector if provided. It may be possible to perform DSF-GTP with GFP fusion proteins using different GFP expression vectors. However, to date it has only been performed with Cycle 3 GFP [12,14]. When DSF-GTP was tested with mCherry we did not observe a $T_m$ peak, mainly due to a progressive photobleaching of the fluorophore during the run.

**2.** KRX competent cells are useful as they can be used for both cloning and over-expression [15]. The tight expression control is also useful when the target protein is likely to be toxic (or has unknown toxicity).

**3.** Rhamnose and lactose are used for auto-induction of KRX and BL21(DE3) expression strains respectively [16,11]. Typical induction with IPTG can be achieved with BL21(DE3) strains, but requires monitoring of optical density for optimal induction of expression.

**4.** Alternative cell lysis methods can be used such as lysozyme digestion and/or osmotic shock. Sonication is not recommended.

**5.** Assay buffers (e.g. Tris and HEPES), and reagents such as salts (e.g. NaCl and KCl) divalent metals (e.g. $MgCl_2$ and $ZnCl_2$), co-factors, co-enzymes and ligands (e.g. nucleotides, NADH and biotin), and small molecules obtained from compound libraries (stocks at 5 mM in DMSO) can be rapidly screened in high throughput using a 96-well plate format.

**6.** HT-DSF-GTP for screening compound interactions with target proteins has only been performed in hard-shell skirted PCR plates to date. This is due to the requirements of the dispensing equipment of the compound supplier we use. If the validation of compound application can be performed using other liquid handling systems such as the QIAsymphony, it would be possible to perform HT-DSF-GTP using a Rotor-Gene Q. While we have not trialed HT-DSF-GTP in 384-well PCR plates to date, DSF-GTP has been performed successfully in 25 μL aliquots indicating upscaling of the high-throughput capacity is possible.

**7.** Having the coding sequence for the target protein synthesised enables codon optimization, which maximizes protein expression in the host expression system. An additional benefit is the ability to eliminate potentially problematic restriction sites if further cloning is desired while ensuring the protein sequence is not affected.

**8.** Glucose repression of the rhamnose promoter prevents leaky expression. Alternatively, standard cloning strains can be used for initial transformation. Transformants positive for the target protein sequence can be identified by colony PCR. This can be performed via conventional PCR using primers for the T7 promoter and GFP coding sequence.

**9.** Confirm target protein coding sequence insertion by restriction digest and sequencing if the target protein sequence has been amplified by PCR.

**10.** We typically select 4 transformants for assessment, with re-plating on a master plate divided into quadrants. This provides sufficient bacterial biomass for large scale expression and short-term storage of desired clones.

**11.** Auto-induction of protein expression can be performed if desired [16]. In this case glucose and rhamnose are added to 0.1% (w/v) final concentrations and pre-induction samples are taken prior to temperature reduction. We find that performing protein expression at lower temperature reduces the rate of expression, enabling improved protein production (correct folding, increased solubility and overall yield).

**12.** Pre and post-induction samples can be assessed for relative yield and correct fusion protein size by SDS-PAGE.

**13.** Pellets can be checked for fluorescence by exciting with long wave UV or blue light using a transilluminator or flashlight to provide a rapid assessment of fusion protein production in soluble form [17].

**14.** We recommend to centrifuge 50 mL polypropylene tubes at 4,000 ×*g* for 5-10 minutes. Weigh the tube before and after the centrifugation step to determine the wet cell pellet biomass. Optional step: freeze the cell pellet in liquid nitrogen and store at -80°C for later purification if desired.

**15.** Fractions containing the fusion protein are easily identifiable due to the GFP tag. Most fusion proteins we have produced have been eluted at a sufficient concentration to be visible to the naked eye due to GFP fluorescence.

**16.** For SDS-PAGE analysis we typically keep small aliquots of pre and post clarification steps (protein solubility), column flow-through (protein integrity and retention), initial and final wash steps as well as each eluted fractions (protein purity and integrity).

**17.** It is important to remove as much supernatant as possible from the ammonium sulphate precipitation. Re-centrifugation is recommended to ensure maximum buffer removal. Buffers can interfere with downstream testing. For example, assessment of divalent metal specificity can be impaired in phosphate buffer due to the formation of insoluble metal phosphate salts [18]. Protein pellets should be resuspended in a buffer that is compatible with subsequent assay conditions [13]. The addition of glycerol generally increases protein stability [19]. Resuspending the precipitated protein from 4-8 mL of eluted fractions in 1-2 mL of buffer will generally result in 50-100 μM target protein concentration (can be more or less depending on expression yield).

**18.** If needed, proteins can be further purified, desalted and dialysed. However, this is not essential for DSF-GTP, which can be performed with crude samples (e.g. If protein yields are low).

**19.** Protein concentration can be determined by Bradford assay or other standard colorimetric assays. Protein concentration can also be determined using a real-time PCR thermal cycler set on the FAM channel to record initial GFP fluorescence data (i.e. background corrected relative fluorescence value of a well). A short isothermal run at 25°C for 2-5 min is sufficient to produce a standard curve of a serial dilution of a known Target-GFP.

**20.** For example, an enzyme known to bind ATP and $Mg^{2+}$ ions can be tested in the presence or absence of ATP and $Mg^{2+}$ individually or in combination.

**21.** Non-skirted 96-well PCR plates and plate sealing film can be cut to size, reducing wastage. Hard-shell skirted PCR plates can be re-used, by cleaning with a reagent capable of removing protein aggregates, rinsing with RO water and drying thoroughly.

**22.** This protocol is based on the Bio-Rad CFX-96, but DSF-GTP has been performed with a Rotor-Gene Q, and can be performed in other real-time PCR thermal cyclers with melt curve capability.

**23.** Initial screens are performed from 25-90°C in order to record the $T_m$ values of the target protein and GFP in the chosen buffer system. Once the target protein $T_m$ has been determined the melt curve window can be reduced, so long as the GFP $T_m$ can still be determined. For example, the window can be reduced to 35-80°C, or 35-75°C depending on the buffer conditions (e.g. the GFP $T_m$ may drop in lower pH buffers). Theoretically, if a protein is capable of refolding following thermal denaturation, it should show an identical $T_m$ peak in the subsequent melt curve as long as the GFP fluorophore was not denatured in the first run (i.e. the first run is stopped before GFP unfolding starts). Most proteins are incapable of refolding following thermal denaturation and in our hands this has never occurred, but this should be tested as it provides an additional control to confirm the independent unfolding of a target protein which can then be further evaluated using GFP-Basta or similar aggregation based protein assays [12,20].

**24**. The $T_m$ obtained by DSF-GTP has been confirmed to correspond to the target protein unfolding and aggregation [2,12]. Real-time thermal cyclers, such as the Bio-

Rad CFX96 automatically determine, display and provide an option to export $T_m$ data (see Fig 3.).

**25.** Take care not to introduce bubbles in the wells as they will interfere with the light path of both the excitation LED and GFP emission spectra. If bubbles have formed they should be eliminated prior to plate sealing (easily broken with a needle or by centrifugation).

**26.** DSF-GTP can also be performed in diluted serum to determine non-specific binding of a hit to serum proteins and its concentration effects [7].

**27.** If the pH dependence of ligand and/or cofactor interactions is being assessed it is recommended to prepare these at twice the concentration of the final assay in order to combine the protein:ligand complex 1:1 with the buffer samples.

**28.** It is best to avoid buffering systems that have strong temperature dependence such as Tris. Phosphate is preferable as it has a lower temperature coefficient, and a gradient is easily prepared with different ratios of monobasic and dibasic sodium phosphate salts. However, this may not suit the desired pH range or divalent metal requirements of your target protein. Select a buffer with an appropriate pH range that has the lowest temperature coefficient possible. Use a buffer temperature coefficient table to determine the actual pH at the protein $T_m$ [21].

**29.** When testing a particular compound, it can be useful to prepare serial dilutions of the test compound that can then be combined with the protein solution.

**30.** Low concentrations of divalent $Mg^{2+}$ and $Mn^{2+}$ ions (i.e. up to 10 mM) can be tested in phosphate buffer systems containing NaCl. However, divalent metal ions

often form insoluble phosphate salts [18] and thus phosphate buffers should be avoided. Buffer systems should be assessed for suitability in terms of pH range and metal binding capacity [13]. Some transition metal ions ($Cu^{2+}$ and $Zn^{2+}$) can significantly affect the GFP fluorophore at relatively low concentrations (50-100 μM).

**31.** Multiple compounds can be tested simultaneously, so long as the final buffer concentration is the same as in the control reaction.

**32.** We typically use a general spreadsheet software such as Excel for general calculations. Graphs can also be produced in Excel, however we prefer using GraphPad due to the more extensive curve fitting options available.

**33.** To determine the equilibrium dissociation constant ($K_D$), values of $T_m$ against $Log_{10}$ of ligand concentrations can be plotted and fitted to various mathematical models depending on the type of affinity and stoichiometry of complex formation [22,23]. In most cases, the concentration of the target protein is not negligible and the reaction is not performed in standard temperature conditions, hence the data cannot simply be fitted to a Langmuir binding isotherm equation. This becomes particularly obvious with high-affinity ligands. In most cases we have been able to derive a value for the affinity of a protein:ligand complex ($K_{obs}$) by simply determining the x-intercept value of the tangent (i.e. linear part) of the curve obtained by plotting $\Delta T_m$ values against $Log_{10}$ of ligand concentration. While not directly comparable, we found that $K_{obs}$ is proportional to $K_D$ and provides a good estimate of the stability of a protein:ligand complex. In general and for a variety of target proteins, $K_{obs}$ values are in good agreement with their reported $K_D$ [2-6]. Determination of $K_{obs}$ requires at

least four consecutive $T_m$ data over a concentration range of at least two orders of magnitude for adequate determination (see Fig. 5B).

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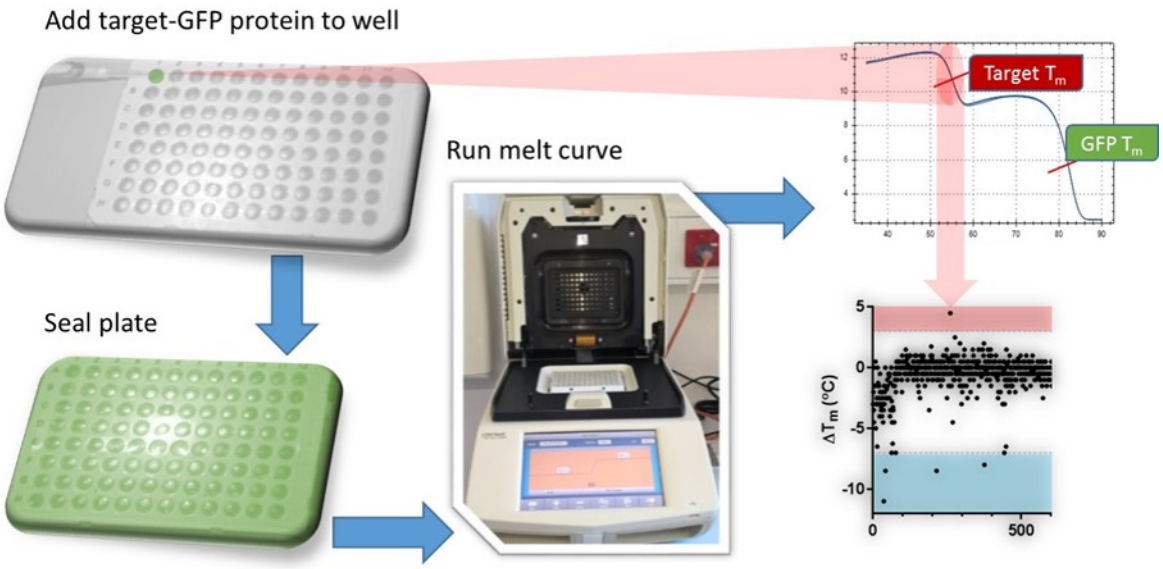


**Figure 1: Standard HT-DSF-GTP workflow**

The target protein sequence is cloned into a suitable GFP vector with a flexible 10 aa linker (example linker shown). Target-GFP protein is expressed and purified. Target-GFP protein is added to a well with or without a test compound. The plate is sealed and a melt curve program is run in a real time thermal cycler. The transition midpoints ($T_m$) for the target protein and GFP are determined. The $T_m$ of GFP provides an internal quality control. The change in $T_m$ ($\Delta T_m$) induced by the binding of a test compound is determined relative to the $T_m$ of free target protein. Compounds can stabilize or destabilize the target protein, resulting in a positive (highlighted in red) or negative (highlighted in blue) $\Delta T_m$.

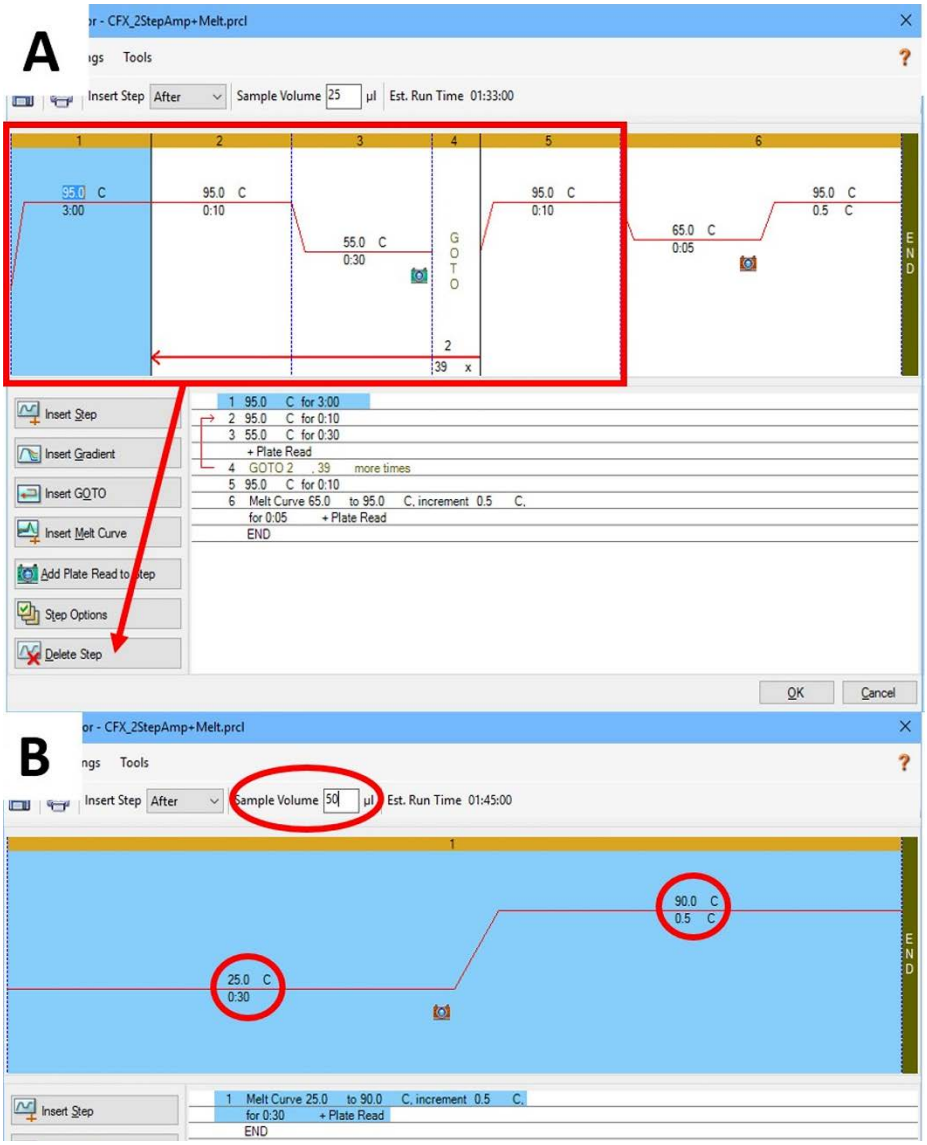


**Figure 2: Example of melt curve setup for DSF-GTP using Bio-Rad CFX-96**

(A) After selecting a generic qPCR protocol with melt curve, the preceding PCR steps are deleted (red box and arrow). (B) The melt curve can then be edited to suit the DSF-GTP run by changing the reaction volume, start and end temperature and dwell time (red circles). The FAM channel is selected with DSF-GTP and other channels can be de-selected. The plate template can be exported and then imported with the sample identification if required (most useful for drug screening where 94 compounds are tested per run).

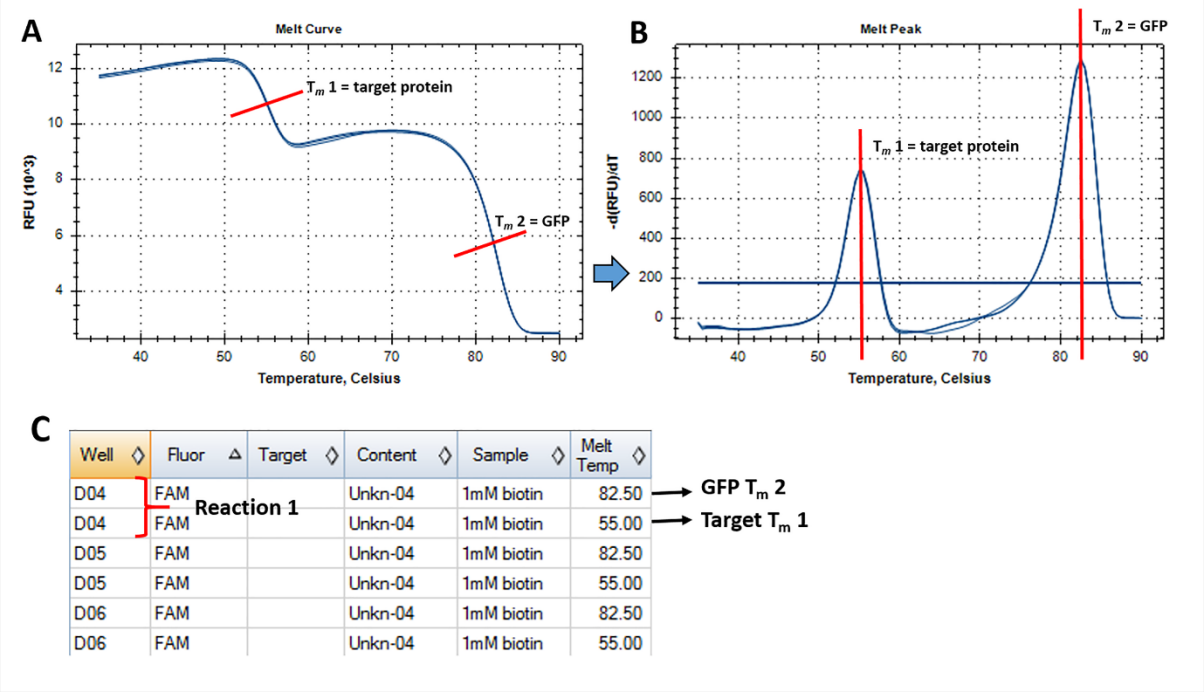


**Figure 3: Example of melt curve and $T_m$ data obtained with DSF-GTP using a Bio-Rad CFX96**

Reactions in this example were performed with 1 µM *E. coli* biotin protein ligase-GFP (Ec BirA-GFP) and 1 mM biotin in triplicate in PBS-T, pH 7.4. A) Raw data showing GFP fluorescence changes. At temperatures below the $T_m$ the target protein is in a folded state. The Target-GFP transitions to a lower fluorescent state when the target protein unfolds ($T_m1$). At higher temperature GFP unfolds leading to total loss of fluorescence ($T_m2$). B) Data is expressed as –d(RFU)/dT. C) The Bio-Rad CFX Maestro software automatically determines and tabulates $T_m1$ and $T_m2$ for each curve.

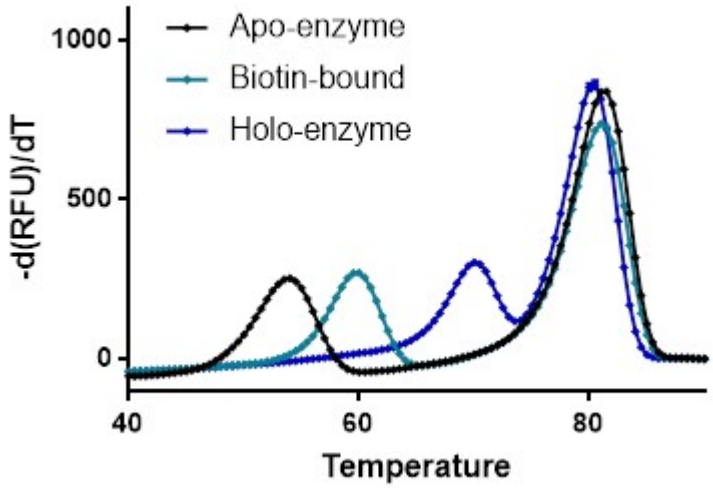


**Figure 4: Example of melt-curve data showing positive ligand effects on a target protein**

*M. tuberculosis* biotin protein ligase-GFP fusion protein (Mt BirA-GFP) with and without important ligands and cofactors. The melt curves show an increase in $T_m1$ of Mt BirA-GFP when combined with its ligand and cofactor. Reactions in this example were run in PBS-T with 1 μM Mt BirA-GFP. Data are presented using GraphPad Prism 7.

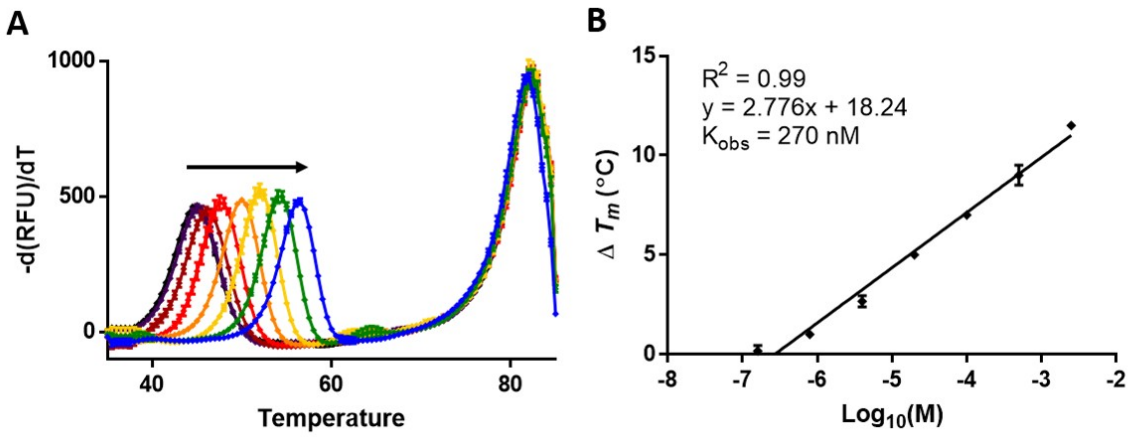


**Figure 5: Determination of $K_{obs}$ using DSF-GTP**

A) In this example, the increase in thermostability of Ec BirA-GFP (1 μM in PBS-T) upon binding to increasing concentrations of biotin (160 nM to 2.5 mM) can be seen with the rightward shift of $T_m$ peaks. B) The $K_{obs}$ for biotin binding to Ec BirA-GFP was derived from plotting $\Delta T_m$ against $Log_{10}$ of biotin concentrations and fitting to a linear regression model using GraphPad Prism 7 (see **Note 32**).