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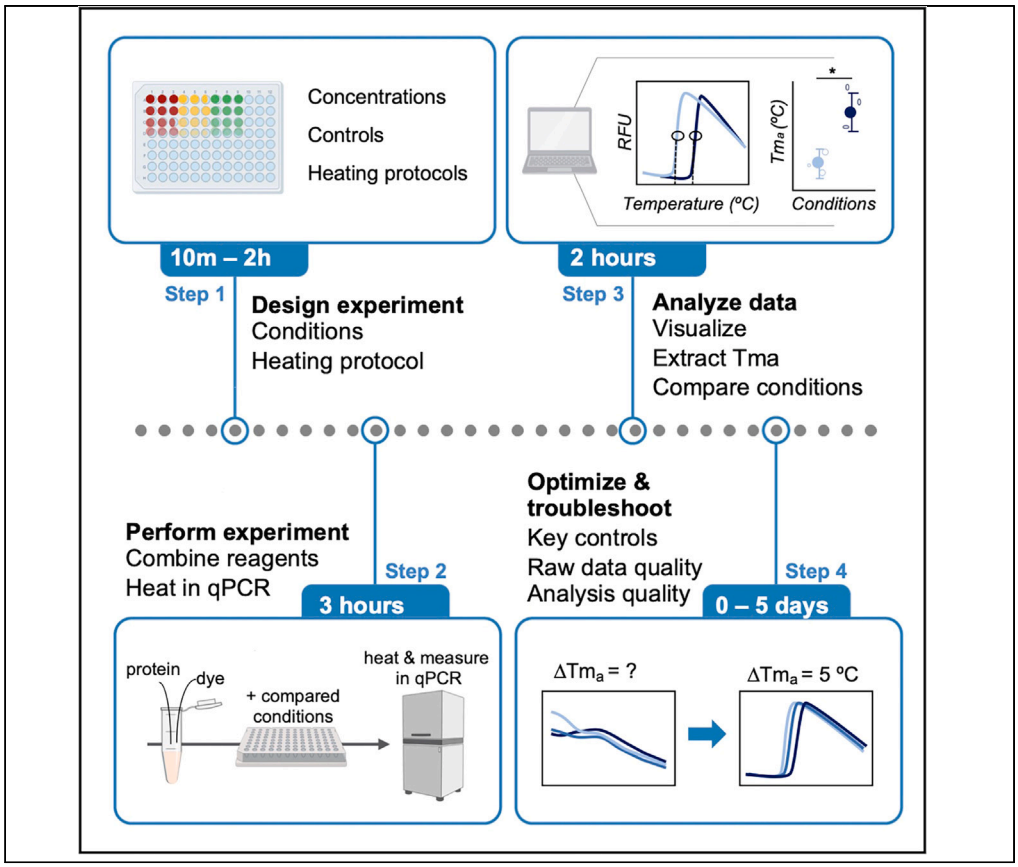
Protocol for performing and optimizing differential scanning fluorimetry experiments



Differential scanning fluorimetry (DSF) is a widely used technique for determining the apparent melting temperature (Tma) of a purified protein. Here, we present a protocol for performing and optimizing DSF experiments. We describe steps for designing and performing the experiment, analyzing data, and optimization. We provide benchmarks for typical Tmas and ΔTmas, standard assay conditions, and upper and lower limits of commonly altered experimental variables. We also detail common pitfalls of DSF and ways to avoid, identify, and overcome them.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

This protocol describes how to design and conduct a typical DSF experiment

The approaches to robust DSF data analysis are described

Common technical problems and methods for troubleshooting are included

Additional resources for planning and interpreting DSF experiments are listed

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Protocol for performing and optimizing differential scanning fluorimetry experiments

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SUMMARY

Differential scanning fluorimetry (DSF) is a widely used technique for determining the apparent melting temperature (Tma) of a purified protein. Here, we present a protocol for performing and optimizing DSF experiments. We describe steps for designing and performing the experiment, analyzing data, and optimization. We provide benchmarks for typical Tmas and ΔTmas, standard assay conditions, and upper and lower limits of commonly altered experimental variables. We also detail common pitfalls of DSF and ways to avoid, identify, and overcome them.

BEFORE YOU BEGIN

Differential Scanning Fluorimetry (DSF) is an *in vitro* technique that allows the apparent thermal stability of a protein to be measured using minimal protein and without specialized instrumentation. DSF is also referred to as the Thermal Shift Assay (TSA) or ThermoFluor. It is distinct from thermal stability experiments performed on cells or lysates, such as CETSA[1] or Cellular Thermal Shift Assays, because DSF is performed *in vitro* with purified protein. DSF is also distinct from nanoDSF[2] despite their similar names; nanoDSF uses a specialized instrument to measure unfolding via tryptophan fluorescence, while DSF uses a standard qPCR instrument to measure unfolding via selective dye fluorescence. DSF was miniaturized and adapted into a standard benchtop assay in the early 2000s.[3] As a result, DSF has become commonly used at scales from single samples to industrial high-throughput screens.[4–6]

Setting up a DSF experiment is easiest when the following preparations are made before beginning the experiment: (i) the concentration of the protein stock solution is at least 5 μM, (ii) any compounds, peptides, or other assay components to be added are in concentrated solutions (i.e., any small molecules are prepared as 10 mM DMSO stock solutions), and (iii) the intended thermocycling protocol is established as a template on the qPCR instrument (see materials and equipment).

When testing more than 5 to 10 conditions, preparing a separate plate containing the experimental additives, such as ligands, reduces pipetting time and experimental errors because buffer, protein, and/or dye mixtures can be uniformly added to this plate. Finally, because some qPCR plates and tubes can induce misleading fluorescence, plasticware can optionally be pre-tested for DSF compatibility as described in troubleshooting 1: Artifactual dye fluorescence.

Table 1. Standard ranges and starting places for DSF experiments

Component	Starting point	Notes	Limits
DSF dye	SYPRO Orange	See Table 5 for alternative dyes	Incompatible with some proteins and reagents.[5,7,8] See Troubleshooting 1: Artifactual dye fluorescence.
DSF dye concentration	5× SYPRO Orange	"5×" corresponds to 10 μM[9]	Upper limit: dye insolubility Lower limit: loss of RFU signal (e.g., see Figure 3D)
Buffer	The preferred biochemical buffer for the protein	DSF is compatible with most biochemical buffers. Our default buffer is: 20 mM HEPES, pH 7.4, 100 mM NaCl	For SYPRO Orange: High background fluorescence in high viscosities (e.g., >10%) Quenched at pH < 5 or >10 High background fluorescence with micelles and membranes.
Protein concentration	5 μM	DSF dyes give stronger signals for some proteins than others, so optimal protein concentration can vary between proteins.	Upper limit: protein expense, or protein behavior Lower limit: loss of RFU signal (typically 100–500 nM)
Thermocycling protocol	Range: 25°C–95°C Heating rate: 1°C /min	See materials and equipment	See troubleshooting 2 and limitations
Additives	The preferred additives for the protein	DSF is compatible with many standard additives, such as reducing agents (DTT, TCEP), EDTA, 0.001% Triton X-100, peptides, compounds, or metal ions	Artifactual activation of the DSF dye. See troubleshooting 1: Artifactual dye fluorescence.
Additive concentrations	Four-point dose-response	Optimal additive concentrations are often higher in DSF than other assays.	Upper limit: compound solubility Lower limit: ΔTma sensitivity See limitations and troubleshooting 1: Artifactual dye fluorescence.

Design experiment and layout

Timing: 1 h

Important factors to consider while choosing conditions for DSF experiments are outlined below, alongside standard DSF conditions, for reference (Table 1).

Key steps in experimental design are outlined below.

1. Choose a protein concentration at which the sample is well behaved, and can reasonably undergo the studied process (e.g., relevant stoichiometries can be achieved between protein and other potential additives in the experiment). Standard: 1–5 μM.

   Note: Here, a "well-behaved" sample means that the protein does not aggregate or change its oligomerization state.

2. Choose buffer conditions that are typical for the protein.

   Note: DSF is compatible with most standard buffers. See Table 1 for limitations.

3. Choose a DSF dye that selectively detects the unfolded state of the protein to be tested. Standard: SYPRO Orange.

   Note: The DSF dyes appropriate for a given protein are determined empirically, and some sample DSF dyes are listed in Table 5 (see materials and equipment).

4. Choose a DSF dye concentration. Standard for SYPRO Orange is "5×" (10 μM).

   Note: Dye concentration should be high enough to produce raw fluorescence data with a signal to noise ratio that minimizes variability in Tma between technical replicates (see

Table 2. qPCR cycling conditions – continuous heating, also called "straight ramp"

Step	RFU read	Temperature	Time	ΔT	Cycles
1	Yes	25°C	50 s	1.0°C	70 cycles
2	No	25°C	1 min		

expected outcomes), but low enough that the dye does not aggregate or induce aggregation of the additives (see troubleshooting 1: Artifactual dye fluorescence.)

5. If testing additives (i.e., compounds, or peptides) choose the number and concentrations of additives to test, ideally with positive and negative controls.

   Note: For binding experiments, additives are typically tested in at least four concentrations. If an estimated K_D is already known for an additive, it can be best to choose concentrations such that this value falls in the middle of the tested range.

6. Choose control conditions to reveal experimental artifacts, such as aggregation.

   Note: Whenever possible, include a protein-free control for every tested condition to prevent the misinterpretation of artifactual, protein-independent fluorescence (see troubleshooting 1).

7. Choose additional control conditions to aid interpretation of the data, if possible. Standard: prevalidated ligands as positive controls.
8. Choose a thermocycling protocol that samples temperatures at which the protein is maximally folded to maximally unfolded. See Tables 1, 2 and 3 for standard protocols and examples.

   Note: The chosen thermocycling protocol should also yield reproducible and easily analyzed raw unfolding curves (see troubleshooting 2). Standard: 25°C–95°C at +1°C/min.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SYPRO Orange (5000×)	Thermo Fisher Scientific	Ref S6650
Other		
384-well qPCR plates	Axygen	PCR-284-LC480WNFBC
96-well qPCR plates	USA Scientific	1402-8590
Optically clear plate seals	Applied Biosystems	4311971
E100 ClipTip p125 matrix pipette	Thermo Fisher Scientific	#4671040BT
Software and algorithms		
DSFworld	Wu et al.[1]	https://gestwickilab.shinyapps.io/dsfworld/

MATERIALS AND EQUIPMENT

- Setting up qPCR protocols.

Any qPCR instrument can be used for DSF, as long as it can measure fluorescence at the wavelengths required by the DSF dye. See Table 4 for recommended and alternative fluorescence channels for SYPRO Orange. Thermocycling instructions for the two standard protocols, continuous and iterative heating, are found below.

Table 3. qPCR cycling conditions – iterative heating, also called "up-down mode"

Step	RFU read	Temperature (°C)	Time	ΔT	Cycles
1	Yes	25°C	10 s		70 cycles
2	No	25°C	30 s	1.0°C	

The "thermocycling protocol" is a general term that is used to refer to the method by which the protein is heated (Figure 1). "Continuous heating" (Table 2) is the default, standard thermocycling protocol used in DSF. The primary alternative, "Iterative heating" (Table 3), is slower and more prone to instrument-specific variations, but it is sometimes necessary to achieve reproducible unfolding curves. Please note that the unfolding processes monitored in continuous and iterative thermocycling protocols are theoretically distinct, although often used interchangeably. See troubleshooting 2.

Note: The final 25°C step (Table 2, Step 2) is used to ensure that the qPCR block returns to approximately ambient temperature after the experiment concludes. This step prevents accidental pre-run protein melting if experiments are run back-to-back. Fluorescence is not read in step 2 because it is not a part of the DSF experiment.

Note: Many qPCR instruments give consistent, plate location-based variations in Tma (see troubleshooting 3). This effect is typically small, and can be ignored unless thermal shifts are small and require high precision (*e.g.*, $\pm$ 0.2°C). This effect has also been recently reported and characterized using DSF-GTP.[10]

Note: Minor variation in results collected on different instruments is rare but possible. Instrument-specific effects are more likely to be relevant in high throughput screening, where thermal shifts are small and require high precision (*e.g.*, $\pm$ 0.2°C), or when "up-down' thermocycling protocols are used. See troubleshooting 3 for more information on instrument-dependent effects.

Note: See https://www.aatbio.com/fluorescence-excitation-emission-spectrum-graph-viewer for alternative channel names and information.

- qPCR tubes or plates.

Not all qPCR plates or tubes are manufactured to be compatible with DSF, and, in our experience, this compatibility must be determined empirically. Because plate compatibility is fast and easy to test, but difficult to identify retroactively, it is recommended that each new lot of plates is tested for DSF compatibility prior to use, as described in troubleshooting 1.

- DSF dyes.

The standard DSF dye is SYPRO Orange (Table 4). However, SYPRO Orange is not able to selectively detect the unfolded states of some proteins[8] and in these cases an alternative dye must be

Table 4. Fluorescent channels for SYPRO Orange measurement

Channel	Comment	Excitation (nm)	Emission (nm)
Recommended channel			
FRET	SYPRO is brightest in this channel	490	600
Alternative channels			
JOE	Or other "green" channels	515	545
TAMRA	Or other "yellow" channels	535	580
ROX	Or other "orange-red" channels	565	605

SYPRO Orange can be measured in a variety of fluorescence channels (Figure S1).

Table 5. Alternative DSF dyes

Reagent or resource	λ (nm)	Working CONC.	Use	Source	Identifier
SYPRO Orange	490/600	"5×"	Standard DSF dye	Thermo Fisher	Ref S6650
Proteostat	480/600	"1×"	Aggregate detection	Enzo	ENZ-51027-K100
GloMelt	468/507	"1×"	Alternative DSF dye	Biotium	Ref. 33021-T
1,8-ANS	350/470	50 μM	Traditional DSF dye	Widely available[11]	CAS 82-76-8
bis-ANS	355/520	50 μM	Traditional DSF dye	Widely available[11]	CAS 65664-81-5
DCVJ	490/580	100 μM	mAb formulations	Menzen et al.[12]	CAS 58293-56-4
CPM	380/460	250 μM	Thiol-reactive dye	Alexandrov et al.[13]	CAS 76877-33-3
Red-Tris-NTA	618/660	200 nM	His-tagged proteins	Ronzetti et al.[14]	NanoTemper, #MO-L018
BODIPY FL-cystine	490/520	2 μM	Thiol-reactive dye	Hofmann et al.[15]	Invitrogen B20340
Aurora library	varies	1–50 μM	Protein-adaptive DSF	Wu et al.[8]	See Wu et al.[8]

identified. Alternative dyes (Table 5) can also sometimes overcome technical issues, including compound incompatibility[8] and fluorescent interference from additives. Historically, ANS and 1,8-ANS were the primary alternatives for DSF,[11] but these dyes absorb at shorter wavelengths than qPCR instruments typically measure, precluding their use in most modern DSF applications.

Dyes should be stored according to manufacturer's instructions. In general, most dyes can be stored for approximately one year as 5 mM DMSO stocks in the dark, either at ambient temperature (~25°C) or frozen (−30 to −80°C).

Note: The "5×" working concentration of SYPRO Orange corresponds to approximately 10 μM. See Figures S8 and S9.

STEP-BY-STEP METHOD DETAILS

The protocol below describes the specific steps needed to study the binding of 10 compounds to a protein of interest. Each compound is tested in triplicate at four concentrations (20, 10, 5, and 2.5 μM). We also use this general protocol to study interactions with additives other than compounds, such as metal co-factors or peptides. See Figure 2 for a simplified schematic of this protocol. See Figure S2 for a plate-view map of the full experiment and for plate layout.

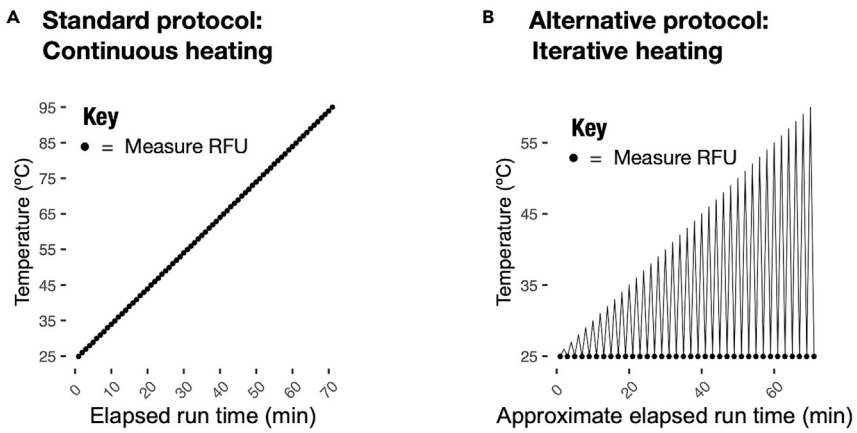

Figure 1. Schematic of continuous and interactive heating thermocycling protocols

(A) In continuous heating, temperature is increased incrementally over time, and RFU is measured at regular intervals throughout heating. A typical temperature range in continuous heating is 25°C–95°C.

(B) In iterative heating, in each step of the heating protocol, the sample is heated and then re-cooled, and RFU is measured following re-cooling. With each subsequent step of an iterative heating protocol, the high temperature to which the sample is heated increases incrementally. Like continuous heating, a typical temperature range in continuous heating is 25°C–95°C. For simplicity, the figure displays iterative heating only up to 55°C.

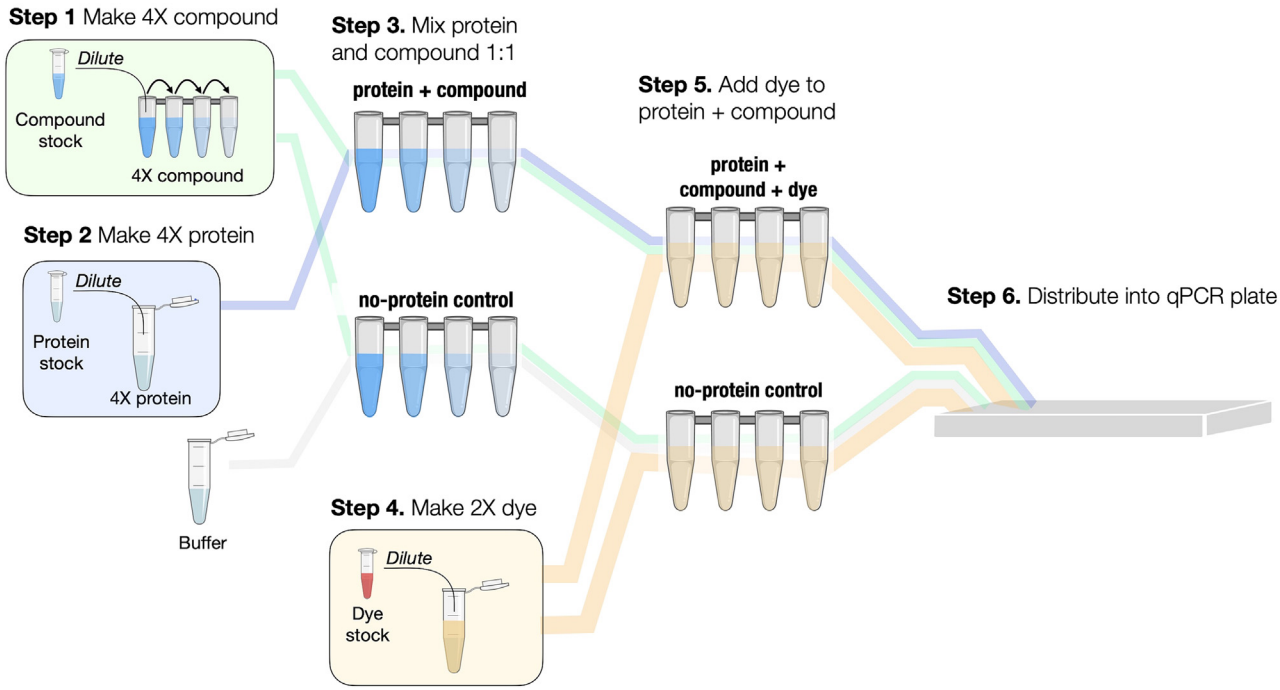

Figure 2. Schematic of step-by-step procedure for testing the binding of one small molecule by DSF

The step-by-step protocol describes the testing of ten compounds by DSF; for simplicity, only one compound is represented in this schematic. Steps 1–3: First, 4× solutions of compound and protein are prepared and combined in a 1:1 ratio. See "Combine protein and additives" in text. A complementary no-protein control, containing buffer and compound, is also prepared at this step. Step 4. Next, a 2× solution of DSF dye is prepared in buffer. See "make concentrated dye solution" in main text. Steps 5 and 6: The dye solution is then combined in a 1:1 ratio with the protein+compound samples and no protein controls. Step 6: These final samples are then distributed into the wells of a qPCR plate.

Combine protein and compounds

Timing: 30–60 min

We begin by creating 4× working solutions of both protein and compounds. These 4× solutions are then mixed in equal volumes, creating 2× solutions containing both protein and compound, and incubated. We prepare and incubate these solutions in PCR strip tubes.

Combining protein and additives as a first step allows this solution to incubate in the absence of dye. This dye-free incubation can reduce possible interference of the DSF dye with the folded protein and it minimizes competition between dye and compound for binding to the folded protein. This step also minimizes other artifacts, such as, dye-assisted binding of otherwise non-binding compounds,[16] and dye-induced compound aggregation (see troubleshooting 1).

Note: For experiments testing more than 5 to 10 conditions, we recommend completing all required calculations before beginning experimental set-up. This step is particularly helpful to identify how much excess volume should be added to the starting solutions to account for pipetting loss in consecutive dilution and transfer steps (*e.g.*, see steps 3d and 4a, 7d, and 9 below).

Note: In this sample experiment, each compound is tested at four concentrations: 20, 10, 5, and 2.5 μM, and each condition is tested in triplicate. The compound stock solution is 10 mM. The final protein concentration is 1 μM, and the stock protein concentration is 100 μM.

1. Prepare 10 mL of buffer containing 1% DMSO. This buffer is used in all subsequent steps of the protocol.

   Note: When working with small molecules, we include 1% DMSO in the buffer to improve the solubility of both compounds and DSF dye (see troubleshooting 1 for more information on additive and dye solubility in DSF experiments). In experiments where solubility is a major concern, such as high throughput screening or when an additive is known to be relatively insoluble, we also include 0.01% Triton X-100.[17,18]

2. Prepare 4× solutions of each compound at each concentration to be tested (Figure 2, Step 1) and set aside.
   a. Calculate the number of wells required for each compound at each concentration.

   1 concentration × 3 replicates = 3 wells per compound at each concentration

   Include a no − protein control for every tested condition :

   3 wells with protein + 3 wells without protein = 6 wells per compound at each concentration.

   b. Calculate the required volume of final (1×) compound at the highest tested concentration.

   6 wells per concentration × 10 µL per well = 60 µL

   c. Calculate the volume of 4× compound required to create all serial dilutions.
      i. To perform serial dilutions, begin by making double the required volume for the highest concentration.

      60 µL × 2 = 120 µL per compound at highest concentration

      ii. Calculate required volume of 4× compound at highest concentration.

      120 µL of 1 × solution ÷ 4 = 30 µL of 4 × solution

      Exact volume of 4 × solution at highest concentration = 30 µL of 4 × solution

      iii. Add excess volume of account for pipetting loss.

      30 µL of 4 × compound × 115% = 34.5 µL

      Round up to make the pipetting and calculations easier : 50 µL of 4 × solution.

      iv. Calculate the volumes of compound stock and buffer to combine.

      Final (1 ×) highest compound concentration = 20 µM

      4 × highest compound concentration = 80 µM

      Stock compound concentration = 10 mM (80 µM final concentration

      ÷ 10 mM stock concentration) × 50 µL final volume

      = 0.4 µL compound stock

      Round up to avoid pipetting inaccuracies below 1 µL : 1 µL compound stock.

Final recipe : 1 μL compound stock + 124 μL buffer

d. Prepare 4× solution of each compound at the highest tested concentration. To the first tube in a PCR strip tube, add 1 μL of 10 mM compound to 124 μL of buffer. Mix well by pipetting approximately half of the tube volume (~60 μL) up and down 20 times.

Note: if volumes at this step exceed 150 μL, we use 1.5 mL microcentrifuge tubes in place of PCR strip tubes.

Note: We perform DSF using 384 well plates with a 10 μL well volume by default, though larger well volumes (up to 30 μL) are also common. Larger well volumes (20–60 μL) may be required for 96 well plates.

e. Include a DMSO control by combining 1 μL of DMSO with 124 μL of buffer.
f. Create the serial dilutions of each compound and DMSO control.
   i. Add 60 μL of buffer to three tubes in each of the 11 PCR strips now containing the 4× solutions of each compound (10 compounds + 1 DMSO control)
   ii. For each compound, transfer 60 μL of the highest concentration solution into a tube containing 60 μL of buffer. Mix the new diluted solution thoroughly by pipetting up and down.

Note: For some compounds, changing pipette tips between each consecutive dilution noticeably increases the accuracy of concentrations across dilution series.

   iii. Create the remaining two serial dilutions by transferring 60 μL of the newly diluted solution into a new tube containing 60 μL of buffer and mixing in the same manner as before.

3. Prepare purified protein solution at 4× final concentration (Figure 2, Step 2).
   a. Calculate the number of protein-containing wells in the final experiment.

10 compounds + DMSO control × 4 concentrations per compound × 3 replicates = 132 wells

Final : Number of protein − containing wells : 132 wells

   b. Calculate required volume of 1× protein solution.

132 wells × 10 μL per well = 1,320 μL of 1 × protein solution

   c. Calculate exact required volume of 4× protein solution.

1,320 μL of 1 × solution ÷ 4 = 330 μL of 4 × protein solution

   d. Add excess volume to account for pipetting loss.

330 μL × 115% = 379 μL of 4 × protein solution

Increase excess volume to account for pipetting loss to the multiple transfers required to test

multiple compounds : 379 μL of 4 × protein solution + additional excess

= 420 μL of 4 × protein solution.

Note: Here, excess volume of protein solution is needed to account for pipetting loss (see step 4 below).

e. Calculate volumes of protein stock and buffer to combine.

$$\text{Final } (1\times) \text{ protein concentration} = 1\ \mu L$$

$$4\times \text{ protein concentration} = 4\ \mu L$$

$$\text{Stock protein concentration} = 100\ \mu M$$

$$(4\ \mu M \text{ final concentration} \div 100\ \mu M \text{ stock concentration}) \times 420\ \mu L \text{ final volume}$$

$$= 16.8\ \mu L \text{ protein stock}$$

$$\text{Final recipe} : 16.8\ \mu L \text{ protein stock} + 403.2\ \mu L \text{ buffer}$$

f. In a 1.5 mL microcentrifuge tube, add 16.8 μL of 100 μM protein stock to 403.2 μL buffer and mix well by pipetting approximately half of the total volume (~200 μL) up and down 20 times. Set aside.

Note: The protein sample can be filtered at this step to remove aggregates. We typically use Amicon Ultrafree-0.1 micron MC Centrifugal filters.

4. Create 2× solutions of protein with compound (Figure 2, Step 3).
   a. To the top four tubes of 11 strips of PCR strip tubes, pipette 9 μL of 4× protein solution.

   Note: After this step, there will be a maximum of 24 μL of 4× protein solution remaining, minus the volume lost to pipetting.

   b. Into each protein-containing tube, pipette 9 μL of 4× compound solution. Mix well by pipetting up and down.
   c. To the bottom four tubes of the same 11 PCR strip tubes, pipette 9 μL of buffer.
5. Create 2× solutions of protein-free controls with compound.
   a. Repeat the above step, using buffer in place of the 4× protein solution.
6. Incubate 2× solutions for 20 min at ambient temperature (approx. 25°C).

   Note: For systems which may require more time to equilibrate, increase the length of this incubation accordingly.

   Note: We perform steps 2–6 using a programmable electronic multichannel pipette (see key resources table).

Make concentrated dye solution

Timing: 5 min

This step allows thorough mixing of the dye solution before combining with the protein and additives, which decreases assay variability from uneven distribution of the dye across wells. This step also provides an opportunity to observe any SYPRO Orange aggregation (see troubleshooting 1: Use fresh SYPRO Orange) and remake any aggregated solutions with fresh dye before combining with more precious reagents such as protein or compounds (See troubleshooting 1).

7. Prepare a 2× solution of DSF dye (Figure 2, Step 4).
   a. Calculate the number of dye-containing wells in the final experiment.

   10 compounds + DMSO control × 4 concentrations per compound × 3 replicates = 132 wells

Include a no − protein control for every tested condition : 132 wells with protein
+ 132 wells without protein = 264 wells

b. Calculate the required volume of 1× dye.

$$264 \text{ wells} \times 10\ \mu\text{L per well} = 2,640\ \mu\text{L } 1\times \text{dye}$$

c. Calculate exact required volume of 2× dye solution.

$$2,640\ \mu\text{L } 1\times \text{dye} \div 2 = 1,320\ \mu\text{L } 2\times \text{dye}$$

d. Add excess volume to account for pipetting loss.

$$1,320\ \mu\text{L} \times 115\% = 1,518\ \mu\text{L } 2\times \text{dye}$$

Round up to 2,000 µL to simplify pipetting.

Note: Here, more excess volume of 2× dye is needed, to account for pipetting loss to each of the compounds tested (see step 9 below).

e. Calculate the volume of dye stock and buffer to combine.

$$\text{Final } (1\times) \text{ dye concentration} = \text{“}5\times\text{”} \text{ SYPRO Orange}$$

$$2\times \text{ dye concentration} = \text{“}10\times\text{”} \text{ SYPRO Orange}$$

$$\text{Stock dye concentration} = \text{“}5000\times\text{”} \text{ SYPRO Orange}$$

$$(\text{“}10\times\text{”} \text{ final concentration} \div \text{“}5000\times\text{”} \text{ stock concentration}) \times 2,000\ \mu\text{L final volume}$$

$$= 4\ \mu\text{L “}5000\times\text{”} \text{ SYPRO Orange}$$

$$\text{Final recipe : } 4\ \mu\text{L “}5000\times\text{”} \text{ SYPRO Orange} + 1,996\ \mu\text{L buffer}$$

8. In a 2 mL microcentrifuge tube, combine 4 µL of 5000× SYPRO Orange and 1,996 µL buffer and mix well by pipetting up and down.

Note: At this step, we watch carefully for any unusual coloration of the SYPRO Orange solution, as this can indicate that the dye stock has aggregated. See troubleshooting 1.

Combine protein and dye solutions

Timing: 15 min

Combine the 2× working solutions of dye and protein + additives in the PCR tubes. This step allows the protein, ligand and dye to mix prior to addition to the qPCR plate.

9. Pipette 18 µL of 2× dye into each of the PCR strip tubes prepared in steps 2–6 (Figure 2, Step 5).

Note: There will be approximately 400 µL of excess 2× dye solution remaining after this step, minus the amount lost to pipetting.

Note: Because the 2× solutions have already been thoroughly mixed, it is also acceptable to add the 2× dye solution directly to the qPCR plate at this step. The 2× protein + compound solutions can then be added to the 2× dye solutions in the qPCR plate. Wells do not require further mixing after addition to the qPCR plate. This step saves time and reduces experimental error from air bubbles being introduced during mixing.

10. Mix each solution well by pipetting up and down carefully 10–20 times and transfer 10 μL of each solution into a single well of the final qPCR plate that is to be used for heating and measurement (Figure 2, Step 6).

⚠ CRITICAL: Some makes, and even certain lots, of qPCR plates can be DSF-incompatible. See troubleshooting 1 for examples and a procedure for determining compatibility with DSF prior to experimental set-up.

Note: We use a programmable electronic multichannel pipette (see key resources table) to perform steps 9 and 10. For step 9, we repeatedly fit three matrix pipette tips into the 2 mL microcentrifuge tube, draw up the maximum allowable volume, and multi-dispense 18 μL of dye into the top edge of each PCR strip tube (Figure S3), carefully avoiding any cross-contamination between PCR strip tubes, and ensuring the full volume of dye combines with the protein and compound solution in each tube. For step 10, we program the pipette to first mix the solution by slowly pipetting 10 μL up and down 5 times, followed by a step to draw up 33 μL of solution, and then dispense 10 μL of solution three times.

Note: When pipetting small volumes into wells (*e.g.*, 2–5 μL), we pipette each component onto opposing vertical sides of each well, and then spin the plate down to settle and mix the components into the bottom of each well (Figure S3). This approach reduces the number of pipette-tip changes required to prevent cross-contamination.

Final DSF reaction mixture

Reagent	Final concentration	Amount
4× Purified protein (4 μM)	1 μM	2.5 μL per well
4× Compound (varies)	1×	2.5 μL per well
2× SYPRO Orange ("10×")	"5×" (~10 μM)	5 μL per well
Total	N/A	10 μL per well

11. Seal the plate with optically clear sealing film.
12. Spin the plate gently (e.g., 1 min at 1000 rcf) to settle any droplets to the bottoms of the wells.

Note: Prior to placing the plate in the qPCR machine, visually inspect the wells and make note of any uneven appearance, such as changes in solution color (See troubleshooting 1).

Heat and measure in qPCR instrument

Timing: ~1 h

This step produces the fluorescence data required to calculate Tma and ΔTma.

13. Place the prepared qPCR plate in a qPCR instrument, and heat and monitor fluorescence.

Note: See "Materials set up" for how to program thermocycling protocols on a qPCR machine. See troubleshooting 2 for key ways that a qPCR thermocycling protocol can be used to optimize and troubleshoot DSF experiments.

Table 6. Example of raw data measured on a qPCR instrument

Temperature	A1	A2	A3
25	1185.30594	3596.51177	807.581347
26	1245.61295	3933.54899	823.417192
27	1259.20655	4281.19015	842.943395
…	…	…	…
94	510.250524	1668.08898	869.663463

14. Following completion of the heating protocol, export the raw fluorescence data (Table 6) from the qPCR machine and proceed to data analysis and interpretation.

⚠ CRITICAL: Always ensure that the exported result file contains raw fluorescence data. Many qPCR instruments export semi-processed results by default, such as amplification data. These pre-processed results are not appropriate for DSF analysis.

EXPECTED OUTCOMES

The expected results for DSF experiments are summarized below. Expectations are broken down into three categories: expectations for raw fluorescence data, reproducibility, and sensitivity to common experimental variations. In addition, a visual guide to the components of a DSF curve is included (Figure 3), which includes examples of raw DSF curves which meet (Figure 3C) or violate

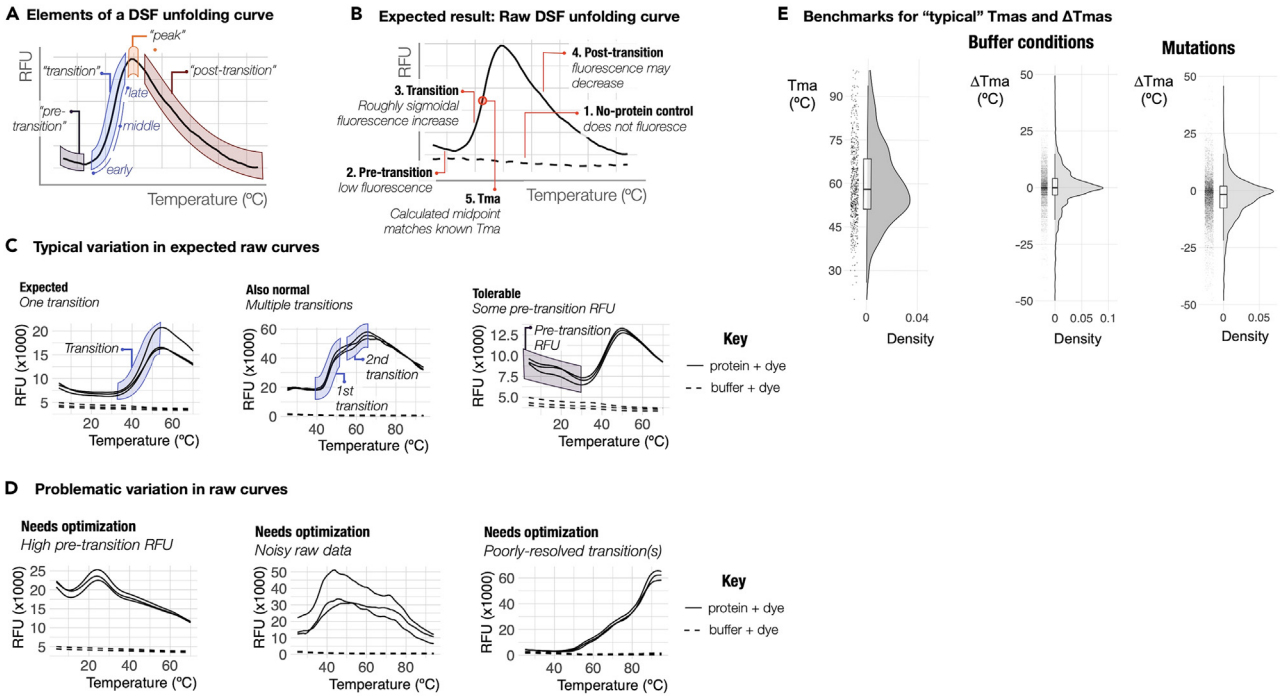

Figure 3. Summary of expected results for DSF experiments

(A) A raw DSF unfolding curve. Key parts of the curve are highlighted and labeled.

(B) Description of expected results for DSF curves. Solid line: protein + dye. Dashed line: buffer + dye (no protein control).

(C) Expected variation in DSF curves between proteins, and technical replicates. Each plot contains experiments performed in technical triplicate. Each individual line represents a single replicate.

(D) Common problematic feature in DSF curves. Each plot contains experiments performed in technical triplicate. Each individual line represents a single replicate.

(E) left: *In vitro* Tm_as for unique proteins, aggregated from the ProThermDB dataset.[19] 59.3% of proteins have Tm_as between 45°C–70°C. Data is colored by the organism of protein origin, revealing that this distribution is true of many organisms. middle: *In vitro* ΔTm_a s resulting from point mutations, measured in the same buffer conditions and colored by organism. right: *In vitro* ΔTm_a s resulting from changes to buffer, buffer concentration, and/or pH, measured in the same buffer conditions and colored by organism.

Table 7. Example of raw data measured on a qPCR instrument

Temperature	Well	RFU
25	A1	1185.305941
26	A1	1245.612947
27	A1	1259.206548
…	A1	…
95	A1	510.2505239
25	A2	3596.511772
26	A2	3933.54899
27	A2	4281.190151
…	A2	…
95	A2	1668.088983
25	A3	807.5813474
26	A3	823.4171917
27	A3	842.9433951
…	A3	…
95	A3	869.6634628

(Figure 3D) expectations for successful DSF experiments, alongside a summary of common Tmas and ΔTmas (Figure 3E) from the ProThermDB database.[19]

QUANTIFICATION AND STATISTICAL ANALYSIS

DSF data analysis has three primary steps.

1. Visualize raw RFU data (Tables 6 and 7). Critical inspection of raw RFU data plays an important role in both artifact identification and informing the approach taken to extracting Tma values (See troubleshooting 1). We use a standard procedure to inspect raw data for all experiments (below).
2. Calculate Tma. The Tma is defined as the mid-point of the unfolding transition. The best approach to Tma calculation is often protein- and application-specific. We provide an overview of current methods for Tma extraction in Table 8.
3. Calculate thermal shifts (Δ Tma) (Tables 9, 10, and 11). The impact of a given perturbation (e.g., ligand binding) is expressed as a shift in the Tma (ΔTma). ΔTma is calculated by subtracting the Tma of a control condition from the Tma of a tested condition. Statistical significance of ΔTmas is determined using standard statistical procedures and is therefore not discussed.

⚠ CRITICAL: As stated in the protocol above, always ensure that the exported DSF results contain raw fluorescence data. Many qPCRs export semi-processed data by default, such as amplification data. These values can visually resemble raw fluorescence data, but are not appropriate for DSF experiments.

Table 8. DSF data analysis softwares

Software	Source
DSFworld	Wu et al.[9]
GraphPad Prism	
Meltdown	Rosa et al.[20]
MeltTraceur	Lee et al.[21]
HTSDSF Explorer	Martin-Malpartida et al.[22]
TSACRAFT	Lee et al.[23]
SimpleDSFviewer	Sun et al.[24]
DMAN	Wang et al.[25]
ThermoQ	Phillips et al.[26]

Table 9. Expected outcomes for raw fluorescence data

Category	Expectation	See figure
Protein-dependent fluorescence	Samples that do not contain protein (negative controls) show minimal changes in fluorescence across the measured temperature range	Figures 3B and 3C
Unfolding transition(s)	Samples that contain protein show a clear unfolding transition, visible as an increase in fluorescence within the measured temperature range	Figures 3A–3C
Unfolding transition(s)	Unfolding transitions is roughly sigmoidal in shape	Figures 3A–3C
Unfolding transition(s)	Unfolding transitions may contain multiple, overlapping transitions	Figures 3A–3C
Post-transition	Unfolding transition fluorescence may steadily decrease after reaching peak intensity	Figures 3A–3C

Visualize raw RFU data

For all DSF experiments, we begin the analysis step by plotting the raw RFU data. An example of typical exported raw data for a DSF experiment are presented below, in Tables 6 and 7.

4. Create a plot that overlays the raw RFU data for both "with-protein" samples and "no-protein" controls for each tested condition (for examples, see Figures 3B–3D, 5B, and 7).
   a. Examine this plot to confirm that significant temperature-dependent fluorescence appears only in the protein-containing sample. If temperature-dependent fluorescence occurs in the no-protein samples, we do not proceed with analysis. Instead, we optimize the DSF conditions to reduce protein-independent fluorescence (i.e., see troubleshooting 1).
   b. If the no-protein control does not reveal artifactual fluorescence, qualitatively assess the shape of the raw data curves. Qualitative assessments include how many transitions are visually apparent and their approximate steepness, and the presence of undesirable fluorescent signal at pre-unfolding temperatures (Table 9). See expected outcomes.
   c. If no unexpected changes in curve shape are observed, note features of the curve to help inform the approach taken to Tma quantification. For example, if two distinct transitions are visually apparent in the raw data, it may be important to use a DSF data analysis program capable of handling multi-transition data. See expected outcomes and quantification and statistical analysis.

Calculate Tma

Tma is calculated from raw DSF curves using either of two general approaches: first derivative or sigmoid fitting. These methods don't represent conflicting interpretations of DSF data; rather, they are different mathematical approaches to extracting the mid-point of a sigmoidal transition.

A common approach is to calculate Tmas is by fitting raw DSF curves to a Boltzmann sigmoid using general software packages, such as GraphPad Prism. This approach is widely used, simple, and requires no specialized tools. However, it typically requires truncating the raw DSF data at its maximum value to remove the post-peak decrease (Figures 3A and 3B).

Table 10. Expected outcomes for reproducibility of DSF results

Category	Expectation	See figure
Raw curve	total fluorescence intensity may vary 10%–50% between replicated conditions	Figures 3C and 3D
Raw curve	the shape of the transition is very similar between replicated conditions, such that the normalized raw data should visually overlay very closely	Figures 3C and 3D
Tma	Tmas vary widely between proteins; ~60% of proteins have Tmas between 45°C–70ºC	Figure 3E
Tma	Day-to-day variation for replicated conditions does not exceed ~1°C	Figures 3B and 3C
Tma	Lab-to-lab variation for replicated conditions does not exceed ~1°C–3°C	Figures 3A–3C
ΔTma	The maximum ΔTma achieved with saturated ligand binding is different between proteins. e.g., while maximum ΔTmas of many protein-ligand interactions is ~10°C, the ΔTma of saturated binding of biotin to streptavidin is 37°C.[27]	Figure 3C

Table 11. Expected outcomes for sensitivity of DSF experiments

Category	Expectation	See figure
Tma variation	Tmas vary widely between proteins; ~60% of proteins have Tmas between 45°C–70ºC	Figure 3E
ΔTma	ΔTmas for buffer conditions are often >10°C	Figure 3E
ΔTma	ΔTmas for mutations vary widely, and are often destabilizing.	Figure 3E
ΔTma	For validated binding, ΔTma increases as ligand concentration increases	
ΔTma	Dose-response relationships between ligand concentration and ΔTma are not always sigmoidal	
ΔTma	Ligands included at or around their K_D often yield ΔTmas of 1°C–12°C. See Limitations regarding the use of DSF for K_D determination. NOTE: Statistically significant ΔTmas below 1°C have been reported for fragments[18] NOTE: ΔTmas exceeding 12°C do occur, and may be more common for covalent ligands[28]	
ΔTma	Though ligand binding is typically expected to increase Tma, negative ΔTmas have also been reported for validated binding interactions.[29,30]	

- Data analysis softwares.

Common DSF data analysis softwares are presented in Table 8. Certain softwares are particularly suited to certain applications, and these specifications are noted. DSFworld is listed in the key resources table (doi: https://doi.org/10.5281/zenodo.8432908), as it was used to analyze the results in this protocol. Note that most qPCR instrument manufacturers do not provide data analysis software that is suitable for DSF.

LIMITATIONS

For a system to be studied using DSF, a non-membrane protein must unfold at a temperature accessible by a qPCR instrument, *e.g.*, 15°C–85°C. Most proteins meet this requirement (Figure 3E), with notable exceptions including ultra-stable proteins from thermophilic species, or certain structural families such as hexameric ATPases.[31,32] Other limitations of DSF are often application-specific, and related to whether or not the perturbation of interest (e.g., ligand binding) can be clearly and reliably detected. These limitations, while different for each case, often have common features:

Dye incompatibilities. For some proteins, the available DSF dyes may not be able to selectively detect unfolding[8] (see Table 5 and troubleshooting 2). If a GFP-tagged version of the protein is available, DSF with GFP-tagged proteins (DSF-GTP) may be performed instead.[10,33,34] Otherwise, Circular Dichroism[35] (CD) and Differential Scanning Calorimetry[36] (DSC) remain the gold-standard techniques to determine protein Tmas.[37]

Unreliable source of thermodynamic parameters. For many proteins, thermal unfolding can be complex. As a result, thermodynamic quantities like ΔG, ΔH, and ΔS cannot be reliably extracted from DSF data. Accordingly, the calculation of ligand K_Ds from DSF data, though demonstrated in the literature,[38] may be less reliable than other methods, such as isothermal titration calorimetry (ITC), time-resolved FRET (TR-FRET), or fluorescence polarization (FP). A practical manifestation of this idea is that ligands often appear to bind at higher concentrations than expected (e.g., the concentration of ADP ribose required to observe binding to nsp3 macrodomain 1 was reported to be higher for DSF than in ITC or homogenous time-resolve fluorescence (HTRF) experiments[18]).

Membrane proteins. Most current examples of DSF involving integral membrane proteins involve the use of cysteine-reactive dyes, largely CPM.[13] Though some membrane proteins contain the required, buried cysteines, DSF applications remain less common with membrane proteins than for soluble ones and are anecdotally regarded as less reliable. Further, interpretation of DSF results

for membrane proteins can be particularly challenging, because theories of thermal denaturation for membrane proteins are less well developed.[39]

TROUBLESHOOTING

When issues with DSF experiments do occur, they typically fall into two general categories: (1) artifactual fluorescence and (2) complex or inaccurate unfolding curves (Table 9). These categories and potential solutions are discussed in individual sections below.

Problem 1: Artifactual dye fluorescence

In our experience, artifactual dye fluorescence is the most common cause of failed or misinterpreted DSF results. By artifactual dye fluorescence we mean the appearance of fluorescence by something other than the tested protein. For example, SYPRO Orange can fluoresce aberrantly in the presence of glycerol, detergents, lipids and EDTA.[7] Artifactual dye fluorescence is typically revealed during visualization of raw RFU data, by the presence of strong fluorescent signal in the protein-free control (see quantification and statistical analysis). However, it also arises from issues in experimental design or set-up (see Design experimental and layout).

The raw DSF curve alone does not provide enough information to discern legitimate fluorescence from artifacts. Instead, artifactual fluorescence is best revealed by the appearance of fluorescent signal in no-protein controls, which differ only from tested conditions in the absence of protein. When the inclusion of no-protein controls is impractical, such as in high-throughput screening, we strongly recommend re-introducing them at the earliest possible validation step. See the 'potential solutions' below for some common, and often pernicious, sources of protein-independent fluorescence in DSF.

In addition to the solutions described below, protein-independent fluorescence can also be reduced using the following standard practices.

- Minimize high-viscosity buffer components such as glycerol, as many DSF dyes are more fluorescent in higher viscosity environments.
- Use aggregation-prone reagents such as detergents or small molecules at concentrations below their Critical Aggregation Concentration[40] (CAC) when possible. When compounds and SYPRO Orange are mixed, aggregation has been observed at lower concentrations than is expected for either alone (see troubleshooting 1), as the combined CAC may be lower than expected. To reduce compound-induced dye fluorescence, we often include 0.001% Triton X-100 in DSF buffers.
- Try using fresh filtered stocks of any buffer additives, such as detergents or EDTA.
- If no example of successful DSF for the tested protein is available for comparison, it can be helpful to include a positive control protein, such 10 μM hen egg white lysozyme and 10 μM ("5×") SYPRO Orange.

Note: Most qPCR instruments can reach temperatures from 4°C to 100°C. Because robust Tma calculation requires data points at both the lower- and upper-plateaus of the unfolding curve, the compatible Tma range for a given qPCR includes temperatures from ~10°C above the minimum measurable temperature to ~10°C below the maximum measurable temperature.

Note: Though best characterized for SYPRO Orange, all DSF dyes likely have the capacity for aberrant activation.

Note: For a discussion of dye fluorescence by folded proteins, see troubleshooting 2.

Note: SYPRO Orange activation or quenching at ambient temperature (approximately 25°C) is sometimes accompanied by a change in visual pigmentation during experimental set-up (Figure S4).

Note: See troubleshooting 3 for a possible approach to identify artifactual fluorescence in primary compound screens, where performing no-protein controls is not feasible.

Potential solution: Ensure plate and tube compatibility with dye

A common, and often-overlooked, DSF artifact is the induction of dye fluorescence by qPCR plasticware (protocol step 13), such as PCR tubes or microwell plates. Unlike more familiar forms of background, this fluorescent signal seems to result from the interaction of the plasticware with the DSF dye. To ensure tube or plate compatibility, it is recommended to pre-test new lots of plasticware prior to use (for a sample procedure in 384-well plates, see Figure 4A). Plastic-based dye activation looks different for different plate and tube types, and can even resemble a bright, seemingly normal protein unfolding curve (Figure 4C). One way to rapidly identify this artifact is to examine the raw data of a protein-free control.

⚠ CRITICAL: Plastic compatibility can vary between different lots of the same plate type, meaning that plate-induced fluorescence can occur after opening a new box of a previously compatible plate or tube type, even from the same manufacturer. We typically test one plate per lot, because, in our experience, its compatibility is representative of the lot. The lot number is typically printed on the plate packaging.

Potential solution: Use fresh SYPRO orange

SYPRO Orange can aggregate, and these aggregated dye stocks can produce low-quality DSF curves (Figure 5). This problem is often apparent during the visualization of raw RFU data (see quantification and statistical analysis), but may also be observed visually during dye dilution in the experimental set up (protocol step 8). Aggregated stocks of SYPRO Orange appear cloudy, and more lightly colored, than fresh dye (Figure 5A). In our hands, this aggregation can occur regardless of storage conditions (i.e., anywhere between 25°C and −80°C) and is often aggravated by freeze-thaw cycles. If the SYPRO Orange stock appears off-color or cloudy at any point and produces poor quality results (Figure 5B), we recommend re-starting with fresh dye. More broadly, one should visually inspect SYPRO Orange solutions when a new buffer, additive or small molecule is introduced (Figure 5C). Changes in color can portend unreliable results. For example, the color intensity of SYPRO Orange solutions decrease below ~ pH 5. Conversely, an increase in visual pigmentation of SYPRO Orange solutions can occur due to aberrant interactions with small molecules (Figure S10, Methods S1).

Potential solution: Check if additive is activating the DSF dye

Some small molecules induce DSF dye fluorescence, particularly at higher concentrations. This type of artifactual dye activation is often revealed during the visualization of raw RFU data (see quantification and statistical analysis), but may also be observed by a change in solution color (protocol step 10). In these cases, the small molecule does not need to be intrinsically fluorescent; rather, the combination of the small molecule and the DSF dye often produces the protein-independent fluorescence. Examples are provided (Figure 6) and extended examples are also shown (Table S1; Figures S5–S7). This compound-induced, protein-independent fluorescence can obscure melting transitions, producing inaccurate Tmas and/or ΔTmas (Figure 6B). The best way to avoid this artifact is to minimize compound aggregation: (i) Compound aggregation can be sensitive to storage conditions and the age of the stock solution,[40] such that aggregation can sometimes be eliminated by using fresh, filtered stocks, (ii) Because SYPRO Orange is reported to be detergent incompatible, detergents are typically not included in DSF assays. However, this incompatibility may be application-specific, because we regularly include 0.001% Triton X-100 to reduce compound aggregation, and this addition can reduce

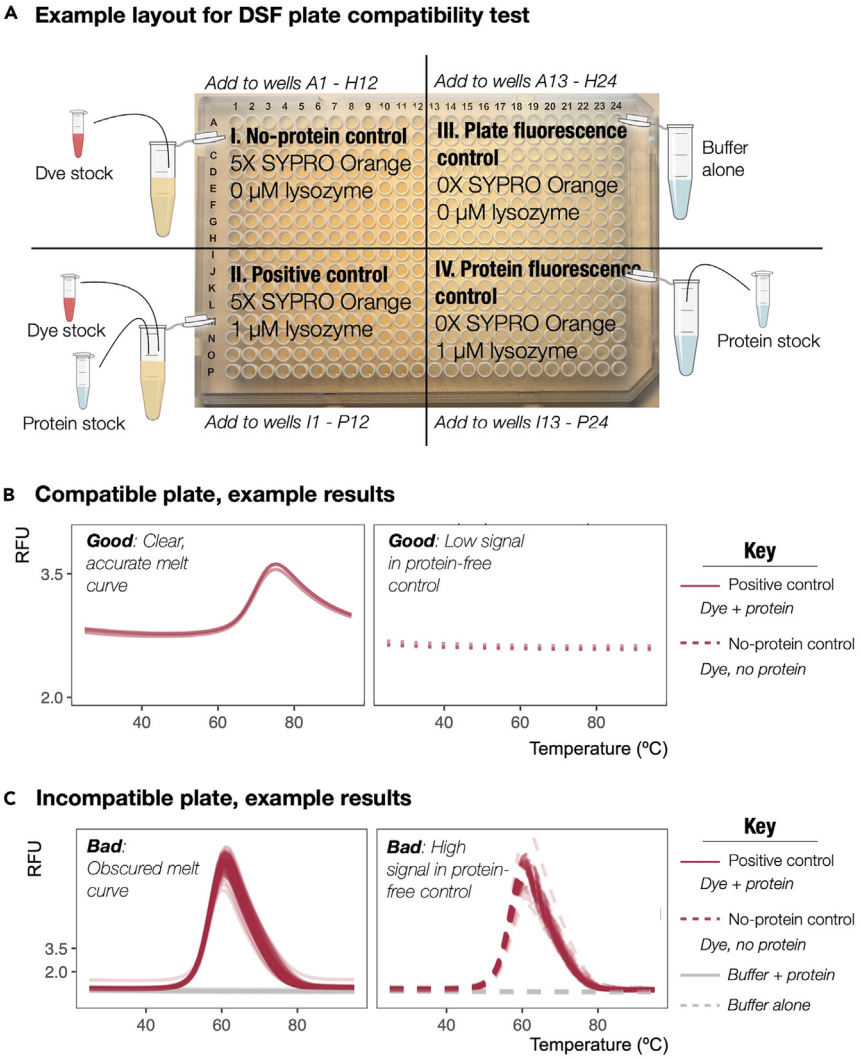

Figure 4. Every new lot of plates or tubes should be tested for DSF compatibility

(A) A general experimental layout to test new plate lots of DSF compatibility. If a specific buffer will be used in downstream experiments, it is best to use that buffer for the plate compatibility test. Otherwise, any standard, simple buffer can be used, such as 10 mM HEPES, 200 mM NaCl, pH 7.20.

(B) Spaghetti plot of DSF data from a DSF-compatible microwell plate lot, demonstrating a clear melting curve with low inter-well variation, and no protein-independent fluorescence. Each curve represents a single replicate.

(C) Spaghetti plot of DSF data from a DSF-incompatible microwell plate lot, demonstrating a bright fluorescent response which mimics a protein melting curve, when dye and buffer are heated in the absence of protein. Each curve represents a single replicate.

compound-induced fluorescence dramatically, and (iii) The simplest approach is to use compounds at lower concentrations, provided the Tma is sensitive enough to produce statistically significant ΔTmas.

Note: Colloidal aggregation may occur at sub-CMC concentration when small molecules and SYPRO Orange are combined (Figure S5).

Note: See Figures S8 and S9 for the chemical characterization of SYPRO Orange. A standard "5×" working concentration corresponds to ~10 µM.

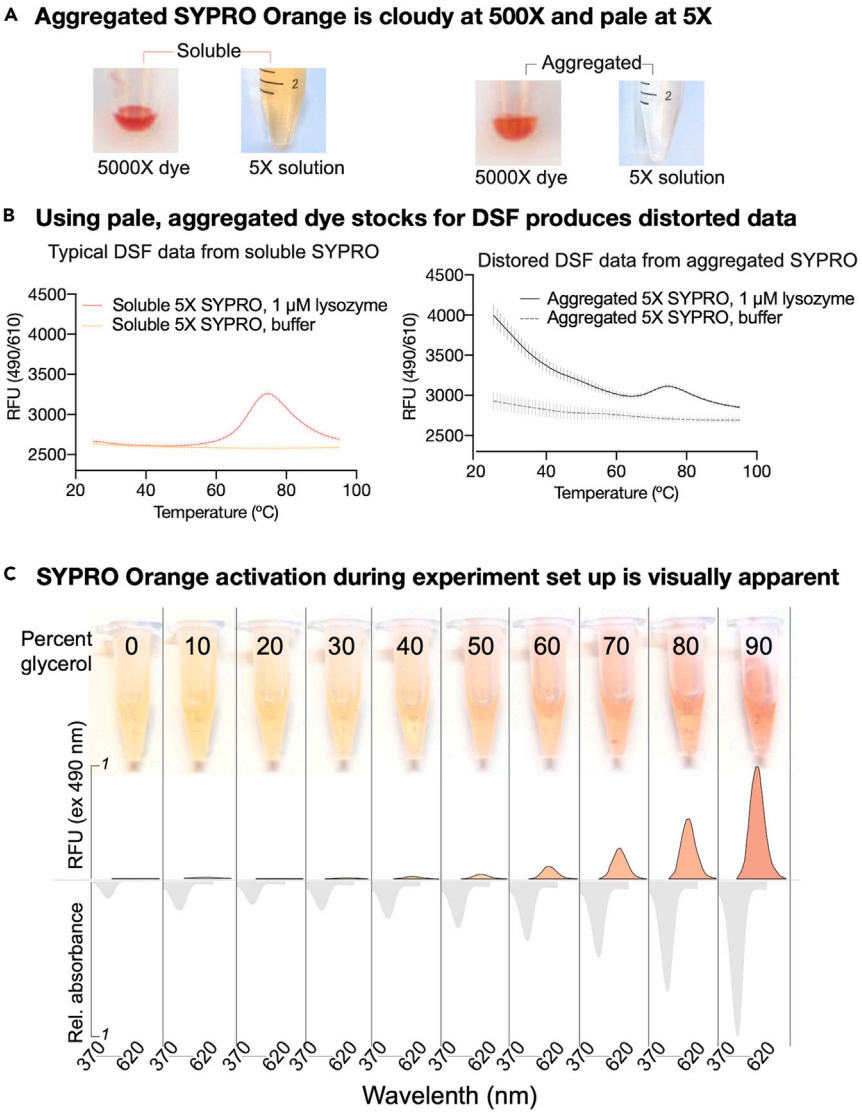

Figure 5. Atypical color suggests non-optimal conditions

(A) Typical, soluble 5000× SYPRO Orange stock appears dark and clear, and produces orange 5× working solutions (left), while spontaneously aggregated freshly-thawed 5000× SYPRO Orange appears cloudy and light, and produces markedly pale 5× working solutions.

(B) DSF data collected for the model protein lysozyme using soluble 5× SYPRO Orange shows a clear, accurate melting curve and no protein-independent fluorescence (left). The same experiment performed using the pale, aggregated stock produces high room-temperature fluorescence and an obscured melting curve and increased protein-independent fluorescence (right). Results are shown as the average of experiments performed in triplicate and error bars are standard deviation (SD).

(C) Room-temperature activation of SYPRO Orange, in this figure by increasing concentrations of glycerol, often produces pink-pigmented solutions during experimental set-up.

Problem 2: Unfolding curve is difficult to interpret

Even in the absence of artifactual, protein-independent fluorescence (troubleshooting 1), the DSF unfolding curve for a protein can be difficult to analyze. This difficulty can sometimes be resolved by adjusting either the experimental design (See design experiment and layout) or analysis method. Difficulty interpreting unfolding curves arises during data visualization or analysis (see quantification and statistical analysis) and, if not corrected, they can appear as poor fit quality or unusually high variability in calculated Tmas (see expected outcomes).

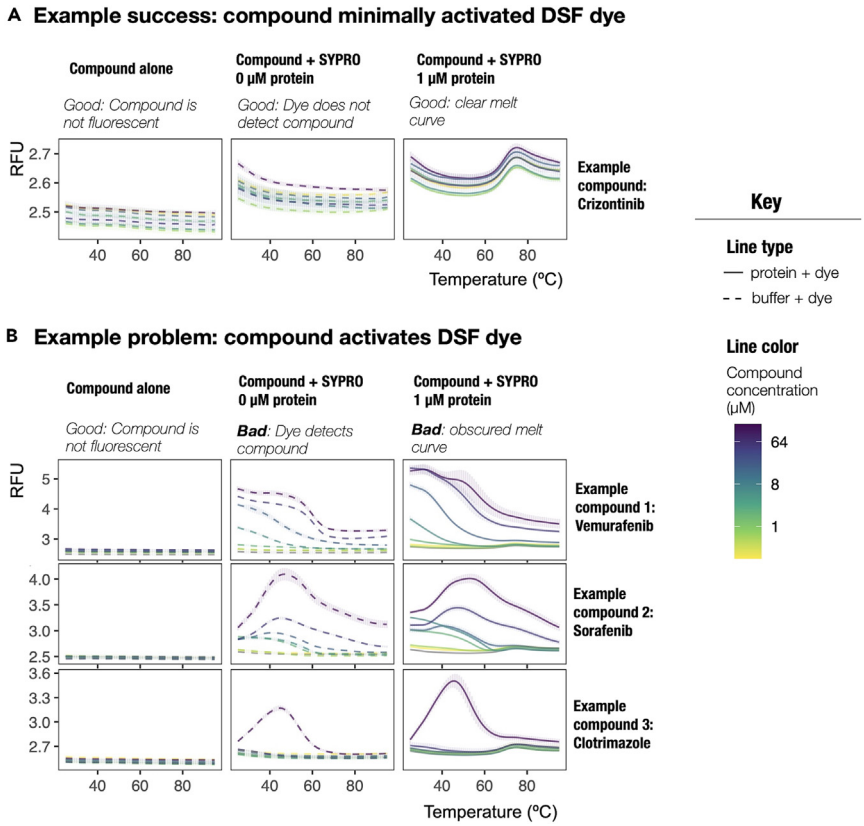

Figure 6. Compounds can induce artifactual DSF dye fluorescence

DSF was performed with four compounds at eight concentrations (0, 0.2, 0.6, 1.9, 5.6, 16.7, 50, and 150 µM). Each compound was tested in buffer alone (negative control for compound fluorescence), with 5× SYPRO Orange (negative control for compound-induced SYPRO Orange fluorescence), and with 5× SYPRO + 1 µM lysozyme. All experiments performed in technical triplicate. Plotted lines = mean. Error bars = standard deviation. A. Example compound which does not induce SYPRO Orange fluorescence, for reference. B. Three compounds which do induce SYPRO Orange fluorescence. Compound-induced fluorescence obscured the melting transition of lysozyme. All results are the average of triplicates and the error is SD.

Some atypical unfolding curves arise because the starting protein sample is either impure or aggregated. If a DSF unfolding curve is difficult to interpret, the first step is to ensure that the protein sample is pure (>90%), and not misfolded or unfolded. We often begin by filtering the protein solution prior to remove potential protein aggregates. However, even pure, well folded proteins can have complex unfolding trajectories, resulting in similarly complex DSF results (see Figure 3B). Robustly extracting Tmas from complex curves can require DSF-specific softwares (see Table 5). When selecting conditions to yield interpretable DSF curves, we use the following criteria.

- DSF experiments should reproduce the Tma determined by a gold-standard approach (e.g., CD) under the same buffer conditions.
- When choosing conditions used for DSF (e.g., buffer, concentration), select those similar to conditions used in other, well-established biochemical assays.
- Ensure that the dye is not interacting with the folded protein. For example, if pre-transition fluorescence is high (Figures 3A and 3D), it is possible that the DSF dye is binding to the folded protein, and potentially interfering with its normal behavior.

Even if the conditions are optimized, some DSF curves are challenging to interpret. In these cases, we suggest three approaches that are discussed in more below: (1) optimize detection of the

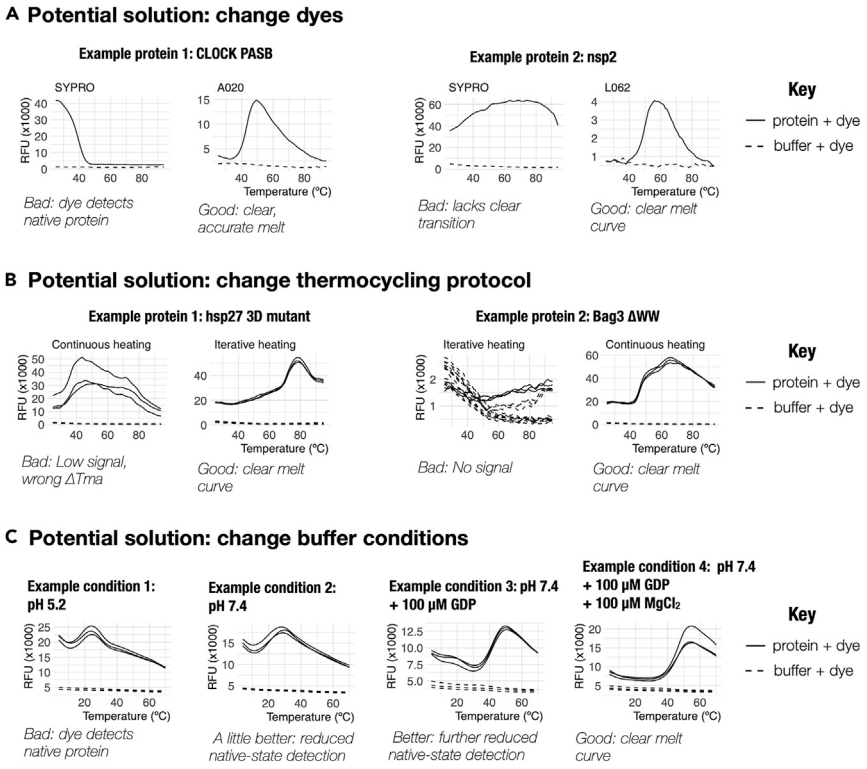

Figure 7. Potential solutions to uninterpretable DSF curves

See annotations below each plot for brief descriptions of issues and solutions; see Figure 3 for description of expected DSF curve shapes.

(A) Change DSF dyes. Results shown for two proteins. Left: use of dye A020 for CLOCK PASB. Right: use of dye L082 for SARS-CoV-2 non-structural protein 2 (nsp2).

(B) Change thermocycling protocols. Results shown for two proteins. Left: use of iterative heating to generate reproducible melts at the known Tma for hsp27 3D mutant. Right: use of continuous heating for Bag3 ΔWW domain, which shows no unfolding signal in iterative heating. Each plot contains experiments performed in technical triplicate. Each individual line represents a single replicate.

(C) Change buffer conditions. Results shown for one protein, LRRK2, in four different conditions. Each plot contains experiments performed in technical triplicate. Each individual line represents a single replicate.

unfolded state by changing DSF dyes, (2) optimize how the unfolded state is generated by changing thermocycling protocols, and (3) optimize the unfolding process itself by changing the environment (e.g., buffers) or the folded state itself.

Potential solution: Change DSF dyes

Sometimes atypical DSF curves arise because the dye used for the DSF experiments (protocol step 8) fails to selectively detect the unfolded states[8] (see design experiment and layout). In these cases, the most effective solution is to switch DSF dye (Figure 7A). Candidate dyes must be ordered and tested empirically for compatibility with the protein of interest (see Table 5).

> *Note:* Though nearly impossible to prove in all cases, it is generally thought that DSF dyes increase in fluorescence when bound to unfolded protein. Thus, in general, inaccurate reporting of unfolding indicates that there is a mismatch between the environments generated upon protein unfolding (*e.g.*, exposure of hydrophobic surfaces), and the environments in which the dye will become fluorescent (*e.g.*, selective binding to those hydrophobic surfaces). Thus, different dyes might detect different unfolded states, potentially resolving incompatibility issues. When switching dyes, it is best to re-optimize the buffer (see Potential

Solution: Optimize buffer conditions), or how the unfolded states are generated (see Potential Solution: Optimize thermocycling protocol).

Potential solution: Optimize thermocycling protocol

For many proteins, DSF results are sensitive to the thermocycling protocol—that is, the rate at which the sample is heated, and whether or not it is re-cooled between heating steps (protocol step 13; Table S2; Figures S10 and S11). While continuous heating is both faster and more common, some proteins require iterative heating to generate clear, reproducible unfolding curves (Figure 7B, left). In contrast, for other proteins, an unfolding curve is only measurable in continuous heating (Figure 7B, right). It is currently difficult to predict which thermocycling protocol will yield the best results.

Note: DSF results are sensitive to the thermocycling protocol because protein thermal denaturation is influenced by both thermodynamics and kinetics (Table S2; Figures S10 and S11). In general, proteins subject to up-down mode can sample the irreversibly unfolded states at the lower temperatures, while continuous-ramp protocols sample a mix of reversible and irreversible states. The standard, continuous ramp DSF heating protocol of 1°C per minute was first established in the 1970s and 1980s, because this rate was thought to be sufficiently slow that samples would reach pseudo-equilibrium.[41] However, this is only true for some systems.

Note: It is possible that, for some studies, bespoke thermocycling protocols (*e.g.*, with longer incubation or cooling steps) could be optimal. This possibility has not been widely explored in the literature.

Potential solution: Optimize buffer conditions

DSF curve shape can also be optimized by changing the conditions of the folded protein (e.g., by optimizing buffers, or adding stabilizing ligands). In one example, we used the GTPase domain of Leucine-rich repeat kinase 2 (LRRK2), a large kinase implicated in the development of Parkinson's disease. The LRRK2 GTPase domain binds both GDP and divalent metal cofactors, preferably Mg^{2+} or Mn^{2+}. Addition of GDP with either Mg^{2+} or Mn^{2+} reduces initial dye fluorescence (Figure 7C), making the raw DSF curve more interpretable. Other examples of using buffer conditions to simplify DSF curve shape are reported.[42]

Potential solution: Estimating artifactual fluorescence without no-protein controls

The fluorescent spectra of SYPRO Orange may help discern between compound- and protein-induced dye fluorescence. For example, compared to protein-dependent fluorescence, compound-induced SYPRO Orange fluorescence has a higher FAM:TAMRA ratio. Thus, measuring in both of these channels is a straightforward way to allow detection of artifacts.

Problem 3: Variation in results between different qPCR instruments

Instrument-specific variations are typically minor and can often be safely ignored. However, in some cases, being aware of these variations may be helpful (e.g., in high-throughput automated workflows, or if an application is intolerant to minor variations between Tma). Two instrument-specific parameters that can impact DSF experiments are (i) the rate at which a sample is heated during a temperature increment, and (ii) the time required to take a fluorescence reading.

- *Rate at which a sample is heating during a temperature increment.* Using the instrument default for heating rate is standard for DSF experiments. While some instruments allow heating rates to be set as an experimental parameter (e.g., in 30 s, increment +1°C at a rate of X °C/s), it is unclear if changes in this parameter meaningfully impact results. However, in "up-down mode" protocols, which require more heating and cooling, heating rates can have a large impact on the overall run time.

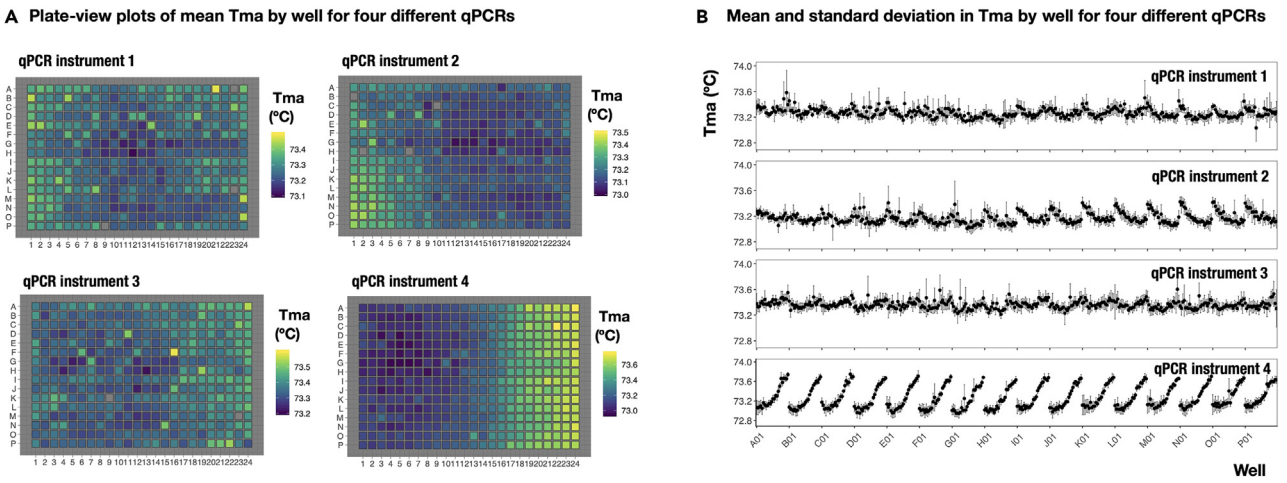

Figure 8. Instrument-specific, plate-location effects in Tma

In each of four different qPCR instruments, DSF was performed on identical 384-well plates containing the same DSF conditions in every well (1 μM hen egg white lysozyme, 5× SYPRO Orange, 10 mM HEPES pH 7.20, 200 mM NaCl). Four plates were run in each instrument, followed by Tma calculation. If no plate-location based variation was observed, Tma would be identical across all wells.

(A) Plate-view plots of the mean Tma for each instrument, demonstrating instrument-specific variation in plate location-based effects on Tma.

(B) Scatter plot displaying mean Tma and standard deviation in each instrument, demonstrating reproducibility in location-based variation in Tma.

- *Time required to take a fluorescence reading.* The time required to measure fluorescence is typically not accounted for in the instrument's projected run-time. Moreover, it is not a variable that can easily be changed. The time required to take a fluorescence reading is often best determined by calculating the difference between the projected and actual elapsed time for a run. For example, a continuous heating protocol that includes 70 cycles of a 1-min step would be projected to complete in 70 min. If this protocol actually completes in 82 min, then this equates to ~10 additional seconds per cycle. In this example, to achieve an actual heating rate of 1°C/min, the instrument's cycle duration must be reduced from 1 min to 50 s.

Problem 4: Systematic variation in Tma by plate location

Many qPCR instruments have some reproducible variation in Tma by well location. This variation is most easily observed when Tma is plotted as a plate-view heat map (Figure 8A). For many instruments, this variation is small enough to be safely ignored. However, for some qPCRs, such as qPCR instrument 4 in the example (Figure 8A), the variation is large enough to complicate interpretations.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jason Gestwicki (Jason.gestwicki@ucsf.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Aa DSF data was analyzed in DSFworld (doi: https://doi.org/10.5281/zenodo.8432908), which is available as an open source website at https://gestwickilab.shinyapps.io/dsfworld/, and open-source code at https://github.com/gestwicki-lab.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102688.

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AUTHOR CONTRIBUTIONS

T.W. wrote the manuscript, designed and performed the experiments, and analyzed and interpreted data. M.H. performed the experiments and identified microtiter plates as a key source of protein-independent fluorescence signal. L.Z. and K.M.S. provided LRRK2 and associated reagents and designed the LRRK2 experiments. J.E.G. wrote the manuscript and designed and interpreted the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Tolvanen, T.A. (2022). Current advances in CETSA. Front. Mol. Biosci. *9*, 866764.

2. Real-Hohn, A., Groznica, M., Löffler, N., Blaas, D., and Kowalski, H. (2020). nanoDSF: In vitro Label-Free Method to Monitor Picornavirus Uncoating and Test Compounds Affecting Particle Stability. Front. Microbiol. *11*, 1442.

3. 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., and Salemme, F.R. (2001). High-density miniaturized thermal shift assays as a general strategy for drug discovery. J. Biomol. Screen *6*, 429–440.

4. Simeonov, A. (2013). Recent developments in the use of differential scanning fluorometry in protein and small molecule discovery and characterization. Expert Opin. Drug Discov. *8*, 1071–1082.

5. Gao, K., Oerlemans, R., and Groves, M.R. (2020). Theory and applications of differential scanning fluorimetry in early-stage drug discovery. Biophys. Rev. *12*, 85–104.

6. Ristic, M., Rosa, N., Seabrook, S.A., and Newman, J. (2015). Formulation screening by differential scanning fluorimetry: how often does it work? Acta Crystallogr. F Struct. Biol. Commun. *71*, 1359–1364.

7. Kroeger, T., Frieg, B., Zhang, T., Hansen, F.K., Marmann, A., Proksch, P., Nagel-Steger, L., Groth, G., Smits, S.H.J., and Gohlke, H. (2017). EDTA aggregates induce SYPRO orange-based fluorescence in thermal shift assay. PLoS One *12*, e0177024.

8. Wu, T., Yu, J.C., Suresh, A., Gale-Day, Z.J., Alteen, M.G., Woo, A.S., Millbern, Z., Johnson, O.T., Carroll, E.C., Partch, C.L., et al. (2023). Conformationally responsive dyes enable protein-adaptive differential scanning fluorimetry. Preprint at bioRxiv. https://doi.org/10.1101/2023.01.23.525251.

9. Wu, T., Yu, J., Gale-Day, Z., Woo, A., Suresh, A., Hornsby, M., and Gestwicki, J.E. (2020). Three Essential Resources to Improve Differential Scanning Fluorimetry (DSF) Experiments. Preprint at bioRxiv. https://doi.org/10.1101/2020.03.22.002543.

10. Sorenson, A.E., and Schaeffer, P.M. (2023). Real-Time Temperature Sensing Using a Ratiometric Dual Fluorescent Protein Biosensor. Biosensors *13*, 338. https://doi.org/10.3390/bios13030338.

11. Semisotnov, G.V., Rodionova, N.A., Razgulyaev, O.I., Uversky, V.N., Gripas', A.F., and Gilmanshin, R.I. (1991). Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. Biopolymers *31*, 119–128.

12. Menzen, T., and Friess, W. (2013). High-throughput melting-temperature analysis of a monoclonal antibody by differential scanning fluorimetry in the presence of surfactants. J. Pharm. Sci. *102*, 415–428.

13. Alexandrov, A.I., Mileni, M., Chien, E.Y.T., Hanson, M.A., and Stevens, R.C. (2008). Microscale fluorescent thermal stability assay for membrane proteins. Structure *16*, 351–359.

14. Ronzetti, M.H., Baljinnyam, B., Itkin, Z., Jain, S., Rai, G., Zakharov, A.V., Pal, U., and Simeonov, A. (2022). Application of temperature-responsive HIS-tag fluorophores to differential scanning fluorimetry screening of small molecule libraries. Front. Pharmacol. *13*, 1040039.

15. Hofmann, L., Gulati, S., Sears, A., Stewart, P.L., and Palczewski, K. (2016). An effective thiol-reactive probe for differential scanning fluorimetry with a standard real-time polymerase chain reaction device. Anal. Biochem. *499*, 63–65.

16. Beher, D., Wu, J., Cumine, S., Kim, K.W., Lu, S.-C., Atangan, L., and Wang, M. (2009). Resveratrol is not a direct activator of SIRT1 enzyme activity. Chem. Biol. Drug Des. *74*, 619–624.

17. Gahbauer, S., Correy, G.J., Schuller, M., Ferla, M.P., Doruk, Y.U., Rachman, M., Wu, T., Diolaiti, M., Wang, S., Neitz, R.J., et al. (2023). Iterative computational design and crystallographic screening identifies potent inhibitors targeting the Nsp3 macrodomain of SARS-CoV-2. Proc. Natl. Acad. Sci. USA *120*, e2212931120.

18. Schuller, M., Correy, G.J., Gahbauer, S., Fearon, D., Wu, T., Díaz, R.E., Young, I.D., Carvalho Martins, L., Smith, D.H., Schulze-Gahmen, U., et al. (2021). Fragment binding to the Nsp3 macrodomain of SARS-CoV-2 identified through crystallographic screening and computational docking. Sci. Adv. *7*, eabf8711. https://doi.org/10.1126/sciadv.abf8711.

19. Nikam, R., Kulandaisamy, A., Harini, K., Sharma, D., and Gromiha, M.M. (2021). ProThermDB: thermodynamic database for proteins and mutants revisited after 15 years. Nucleic Acids Res. *49*, D420–D424.

20. Rosa, N., Ristic, M., Seabrook, S.A., Lovell, D., Lucent, D., and Newman, J. (2015). Meltdown: A Tool to Help in the Interpretation of Thermal Melt Curves Acquired by Differential Scanning Fluorimetry. J. Biomol. Screen *20*, 898–905.

21. Lee, M.E., Dou, X., Zhu, Y., and Phillips, K.J. (2019). Refolding Proteins from Inclusion Bodies using Differential Scanning Fluorimetry Guided (DGR) Protein Refolding and MeltTraceur Web. Curr. Protoc. Mol. Biol. *125*, e78.

22. Martin-Malpartida, P., Hausvik, E., Underhaug, J., Torner, C., Martinez, A., and Macias, M.J. (2022). HTSDSF Explorer, A Novel Tool to Analyze High-throughput DSF Screenings. J. Mol. Biol. *434*, 167372.

23. Lee, P.-H., Huang, X.X., Teh, B.T., and Ng, L.-M. (2019). TSA-CRAFT: A Free Software for Automatic and Robust Thermal Shift Assay Data Analysis. SLAS Discov. *24*, 606–612.

24. Sun, C., Li, Y., Yates, E.A., and Fernig, D.G. (2020). SimpleDSFviewer: A tool to analyze and view differential scanning fluorimetry data for characterizing protein thermal stability and interactions. Protein Sci. *29*, 19–27.

25. Wang, C.K., Weeratunga, S.K., Pacheco, C.M., and Hofmann, A. (2012). DMAN: a Java tool for analysis of multi-well differential scanning fluorimetry experiments. Bioinformatics *28*, 439–440.

26. Phillips, K., and de la Peña, A.H. (2011). The Combined Use of the Thermofluor Assay and ThermoQ Analytical Software for the Determination of Protein Stability and Buffer Optimization as an Aid in Protein Crystallization. Curr. Protoc. Mol. Biol. *94*. https://doi.org/10.1002/0471142727.mb1028s94.

27. González, M., Bagatolli, L.A., Echabe, I., Arrondo, J.L., Argaraña, C.E., Cantor, C.R., and Fidelio, G.D. (1997). Interaction of biotin with streptavidin. J. Biol. Chem. *272*, 11288–11294.

28. Kopra, K., Valtonen, S., Mahran, R., Kapp, J.N., Hassan, N., Gillette, W., Dennis, B., Li, L., Westover, K.D., Plückthun, A., and Härmä, H. (2022). Thermal Shift Assay for Small GTPase Stability Screening: Evaluation and Suitability. Int. J. Mol. Sci. *23*, 7095. https://doi.org/10.3390/ijms23137095.

29. Cimmperman, P., Baranauskiene, L., Jachimoviciūte, S., Jachno, J., Torresan, J., Michailoviene, V., Matuliene, J., Sereikaite, J., Bumelis, V., and Matulis, D. (2008). A quantitative model of thermal stabilization and destabilization of proteins by ligands. Biophys. J. *95*, 3222–3231.

30. Askin, S., Bond, T.E.H., Sorenson, A.E., Moreau, M.J.J., Antony, H., Davis, R.A., and Schaeffer, P.M. (2018). Selective protein unfolding: a universal mechanism of action for the development of irreversible inhibitors. Chem. Commun. *54*, 1738–1741.

31. Blackwood, J.K., Rzechorzek, N.J., Abrams, A.S., Maman, J.D., Pellegrini, L., and Robinson, N.P. (2012). Structural and functional insights into DNA-end processing by the archaeal HerA helicase–NurA nuclease complex. Nucleic Acids Res. *40*, 3183–3196.

32. Burgess, J.L., Jones, H.B., Kumar, P., Toth, R.T., Middaugh, C.R., Antony, E., and Dickenson, N.E. (2016). Spa47 is an oligomerization-activated type three secretion system (T3SS) ATPase from Shigella flexneri. Protein Sci. *25*, 1037–1048.

33. Sorenson, A.E., and Schaeffer, P.M. (2020). High-Throughput Differential Scanning Fluorimetry of GFP-Tagged Proteins. Methods Mol. Biol. *2089*, 69–85.

34. Moreau, M.J.J., Morin, I., Askin, S.P., Cooper, A., Moreland, N.J., Vasudevan, S.G., and Schaeffer, P.M. (2012). Rapid determination of protein stability and ligand binding by differential scanning fluorimetry of GFP-tagged proteins. RSC Adv. *2*, 11892–11900.

35. Greenfield, N.J. (2006). Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. Nat. Protoc. *1*, 2527–2535.

36. Freire, E. (1995). Differential scanning calorimetry. Methods Mol. Biol. *40*, 191–218.

37. Atsavapranee, B., Stark, C.D., Sunden, F., Thompson, S., and Fordyce, P.M. (2021). Fundamentals to function: Quantitative and scalable approaches for measuring protein stability. Cell Syst. *12*, 547–560.

38. Bai, N., Roder, H., Dickson, A., and Karanicolas, J. (2019). Isothermal Analysis of ThermoFluor Data can readily provide Quantitative Binding Affinities. Sci. Rep. *9*, 2650.

39. González Flecha, F.L. (2017). Kinetic stability of membrane proteins. Biophys. Rev. *9*, 563–572.

40. Ganesh, A.N., Donders, E.N., Shoichet, B.K., and Shoichet, M.S. (2018). Colloidal aggregation: From screening nuisance to formulation nuance. Nano Today *19*, 188–200.

41. Rees, D.C., and Robertson, A.D. (2001). Some thermodynamic implications for the thermostability of proteins. Protein Sci. *10*, 1187–1194.

42. Chari, A., Haselbach, D., Kirves, J.-M., Ohmer, J., Paknia, E., Fischer, N., Ganichkin, O., Möller, V., Frye, J.J., Petzold, G., et al. (2015). ProteoPlex: stability optimization of macromolecular complexes by sparse-matrix screening of chemical space. Nat. Methods *12*, 859–865.