Protein Thermal Shift technology

Optimizing buffer conditions and high-throughput screening of ligand-protein binding

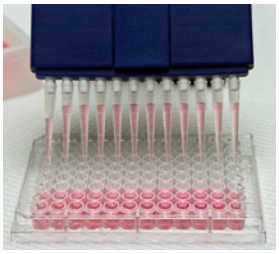
The Applied Biosystems™ Protein Thermal Shift™ assay measures protein thermal stability using a fluorescent protein–binding dye. Protein Thermal Shift experiments are conducted utilizing the melt capability of Applied Biosystems real-time PCR systems. This technology can be used for fast, economical screening of small-molecule and ligand libraries for drug discovery and other research applications. It is also ideal for screening buffer conditions to maximize protein stability for improved purification, storage, crystallization, and characterization. The Protein Thermal Shift assay uses <1 µg of protein per assay and can be run in a high-throughput fashion. This application note describes the Protein Thermal Shift assay and its uses, and introduces the Applied Biosystems Protein Thermal Shift Software v1.3 and reagent kits for performing the assay and analyzing the data.

How does the Protein Thermal Shift technology work?

The Protein Thermal Shift Software and reagents enable a protein melt assay that is an efficient screening tool for measuring protein thermal stability. Thermal stability of proteins is typically measured for specific applications, including identifying suitable buffer conditions and measuring protein–ligand interactions. The assay is simple, and time-to-results typically is from 0.5–2.0 hours, depending on the real-time PCR system and run conditions used for the assay (Figure 1).

The complete Protein Thermal Shift solution workflow typically takes 0.5–2 hours

1
Prepare assays

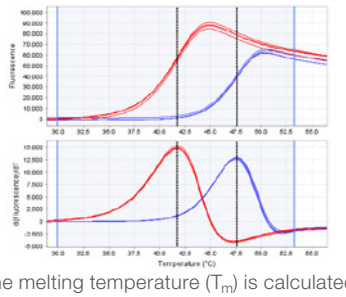


Mix protein, buffer, ligand (if applicable), and Protein Thermal Shift dye.

2
Run melt assay on real-time PCR system (QuantStudio™ 3, 5, 6, 7, 12K Flex systems)

The protein unfolds with increasing temperature, and the Protein Thermal Shift dye binds to exposed hydrophobic regions and fluoresces.

3
Analyze data with Protein Thermal Shift Software



The melting temperature (T_m) is calculated from the melt curve; changes in T_m are correlated to changes in protein stability or ligand binding.

Figure 1. The Protein Thermal Shift assay workflow.

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Protein Thermal Shift applications

Protein stability screening:

- Improving protein preps (buffer pH, salt, excipients, additives)
- Profiling crystallization conditions
- Protein formulation and storage buffer optimization
- Effect of mutations or modifications on protein stability
- Protein prep QC (presence of contaminants or aggregated protein)

High-throughput ligand screening:

- Small-molecule and fragment library screens
- Antibody–target specificity
- Protein–protein interaction
- Inhibitor binding

A real-time melt experiment using the Protein Thermal Shift dye kit and any real-time PCR system will yield a fluorescence profile specific to a protein and its environment (buffer or ligands). The Protein Thermal Shift dye does not fluoresce in aqueous solutions but fluoresces in nonpolar environments. The protein is mixed with the dye and heated; as it unfolds or melts, hydrophobic parts of the protein are exposed and bind to the dye, resulting in a significant increase in fluorescence emission detected by the PCR system.

The basic steps for a typical Protein Thermal Shift assay include:

1. Mix protein, Protein Thermal Shift dye, Protein Thermal Shift buffer, and ligand (if applicable).

2. Run a melt curve on a real-time PCR instrument.

3. Transfer the *.eds file to Protein Thermal Shift Software for analysis.

4. Calculate the melting temperature (T_m) from the melt curve.

5. Correlate changes in protein stability or ligand binding to changes in T_m.

The Protein Thermal Shift Software calculates the T_m from each fluorescence profile using the Boltzmann method (from a plot of fluorescence intensity vs. temperature) and the derivative method (from a plot of d(fluorescence)/dT vs. temperature). The derivative T_m values are taken from the top of the peak in the derivative plot, while the Boltzmann T_m values are taken from the inflection point of the fluorescence plot.

The Protein Thermal Shift technology is useful for many research applications, several of which are described below.

Optimizing buffer conditions for protein stability

Protein stability changes with buffer pH, salt content, and the presence of various cofactors in a protein's environment. The Protein Thermal Shift assay was used to screen buffers to identify the pH where the protein RecA was most stable (Figure 2). The buffer panel consisted of four buffers with different pH values (pH 5.5, 6.0, 7.0, and 7.5). The pH 7.0 buffer had the highest melting temperature indicating that the protein is most stable at this pH. The data also demonstrate the high level of reproducibility for each condition tested. Subsequent experiments could further refine the data by testing a pH range near the optimal pH (7.0) identified here. Optimal salt concentration for RecA stability was also tested (Figure 3). Identifying optimal buffer and salt conditions for a protein of interest will increase protein structure stability, lower the tendency for aggregation, and improve the success rate of crystallization.

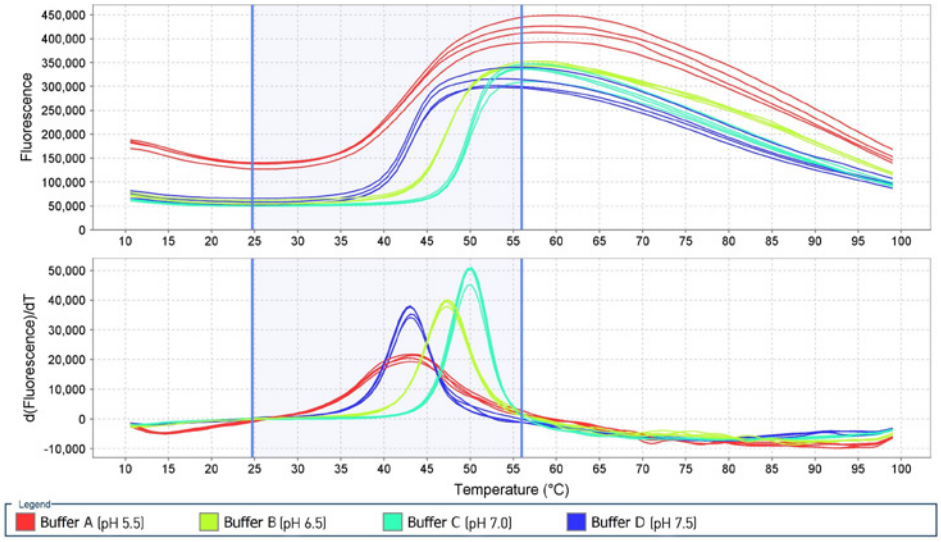

Figure 2. Effect of buffer pH on RecA thermal stability. The Protein Thermal Shift assay was used to screen for the effect of various buffer conditions on RecA thermal stability. Data from Protein Thermal Shift Software show the Boltzmann (upper panel) and derivative (lower panel) melt profiles of buffer A (pH 5.5), B (pH 6.0), C (pH 7.0), and D (pH 7.5). Data were collected at 1°C intervals from 25°C through 99°C on the Applied Biosystems StepOnePlus™ Real-Time PCR System and analyzed using the Protein Thermal Shift Software.

Studying protein–ligand interactions

The Protein Thermal Shift assay is ideal for screening small molecules or other types of compounds that can enhance protein thermal stability. In addition, this assay enables identification of ligands that can lead to successful co-crystallization. The Protein Thermal Shift assay can be used to study binding interactions between proteins and antibodies, drugs, and other proteins. When a small-molecule ligand preferentially binds to the native form of a protein, the ligand stabilizes the protein and the T_m of the complex is higher than that of the unbound protein. Proteins with stabilizing ligands exhibit positively shifted melt curves in comparison to proteins without bound ligands.

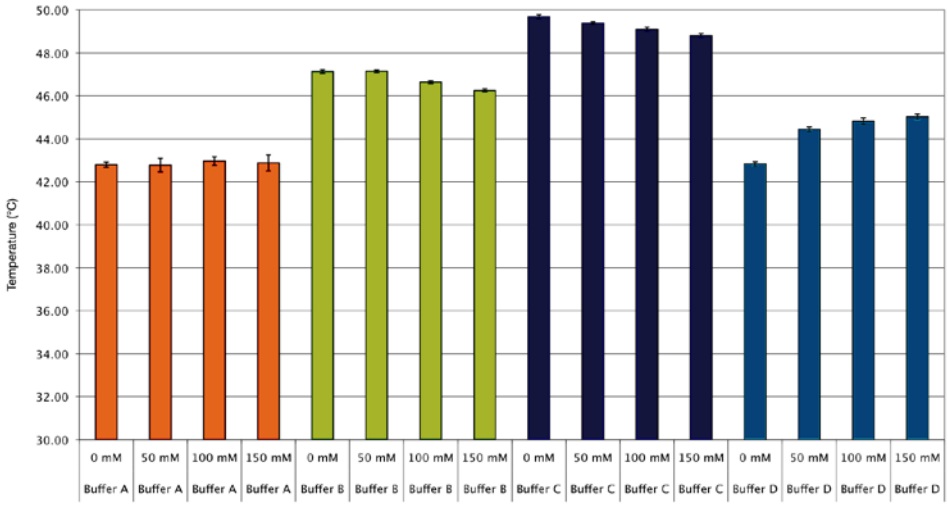

Figure 3. Effect of salt concentration on RecA thermal stability. Varying concentrations of salt were titrated for each pH condition tested in Figure 2 to further optimize conditions for RecA thermal stability. Increases in the salt concentration had no effect on RecA thermal stability in buffer A. Increased salt concentrations in buffers B and C decreased the thermal stability of RecA. However, in buffer D, increasing salt concentrations increased the thermal stability of RecA. Reaction conditions were the same as described in Figure 2.

Effect of ligand binding on protein thermal stability

An assay was used to demonstrate the thermal stability of RecA in the presence of ATP used as a ligand (Figure 4). Results showed that the thermal stability of RecA increased from 53.2°C to 57.7°C in the presence of 5 mM ATP based on median derivative T_m values.

In Figure 5, various concentrations of the Protein Thermal Shift control ligand were bound to the Protein Thermal Shift control protein and assayed for thermal stability. The results show that protein stability increased with increasing concentrations of the ligand.

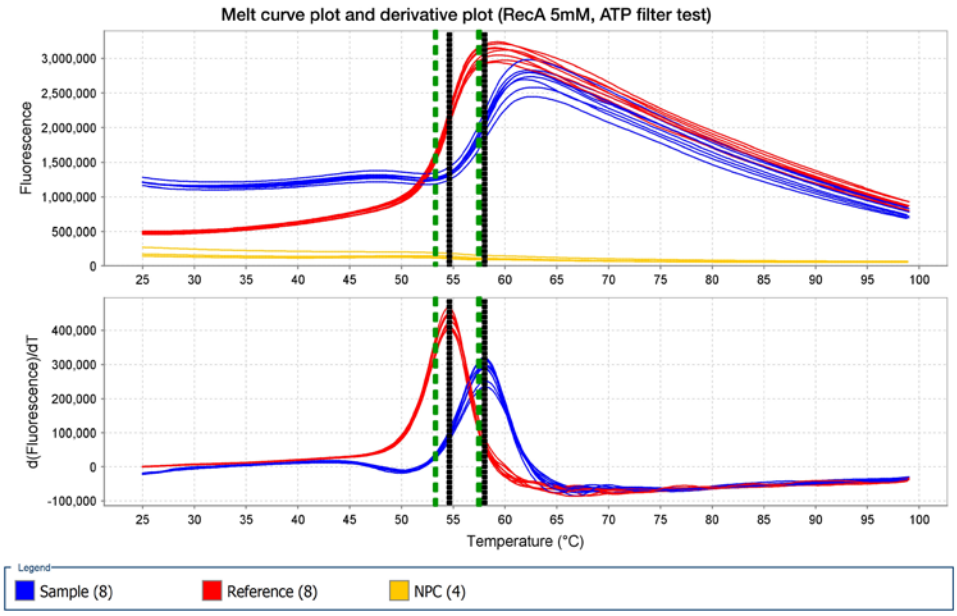

Figure 4. Thermal stability of RecA with binding of ATP. RecA at a final concentration of 0.2 mg/mL was mixed with a ligand (5 mM ATP) and the Protein Thermal Shift dye. Eight replicate reactions with and without ligand were run on an Applied Biosystems QuantStudio™ 5 Real-Time PCR System using melt curve filter setting at x1-m4, continuous data collection, and a ramp rate of 0.05°C/sec from 25°C through 99°C. Data were analyzed using Protein Thermal Shift Software v 1.3. **Top:** Fluorescence vs. temperature. **Bottom:** Derivative plot of the change in fluorescence vs. temperature. The median Boltzmann T_m and median derivative T_m values are shown as green dashed and black dotted vertical lines, respectively.

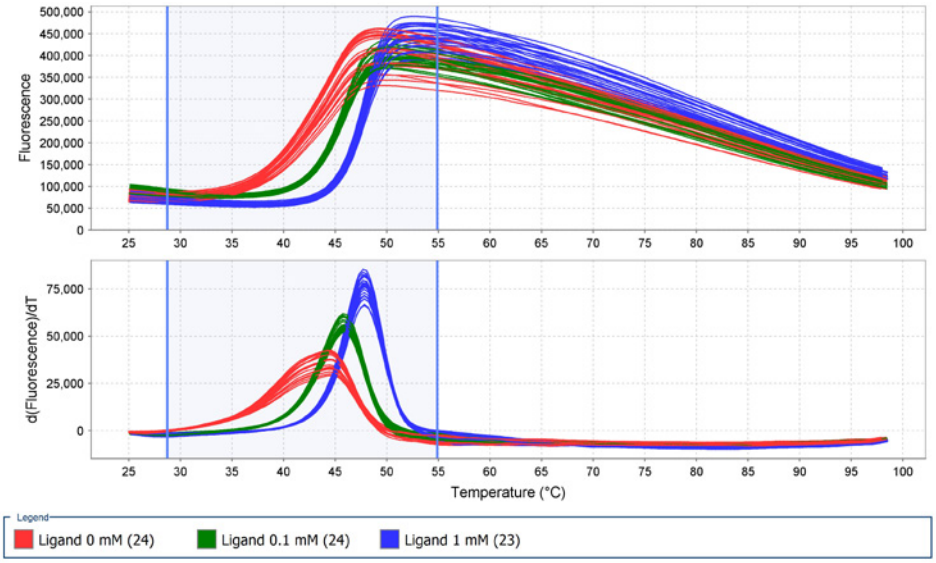

Figure 5. Effect of ligand concentration on thermal stability. Different concentrations (0 mM, 0.1 mM, and 1 mM) of the Protein Thermal Shift Control Ligand were bound to the Protein Thermal Shift Control Protein and analyzed using the Protein Thermal Shift assay. The data show the slope of the melt curve increasing with the concentration of the ligand, thus indicating more stable binding of the protein at higher concentrations of ligand. Data were collected at 1°C intervals from 25°C through 99°C on the StepOne Real-Time PCR System and analyzed using the Protein Thermal Shift Software.

Screening against a panel of ligand mixes (high-density screening)

Pooled ligands are used as an initial step to assign protein binding to a specific ligand group. If a significant thermal shift is detected with a ligand pool (hit pool), the protein is then screened against all individual ligands in the pool to determine the specific binding ligand(s). Figures 6 and 7 show the results of a study in which 96 ligand mixes were screened against the protein YraM to assess improvement in thermal stability. Mixes D10 and D11 demonstrated a ΔT_m of 1.5°C, significantly different from the control and other mixes tested against YraM. A positive ΔT_m indicates improved thermal stability with the ligand mix tested.

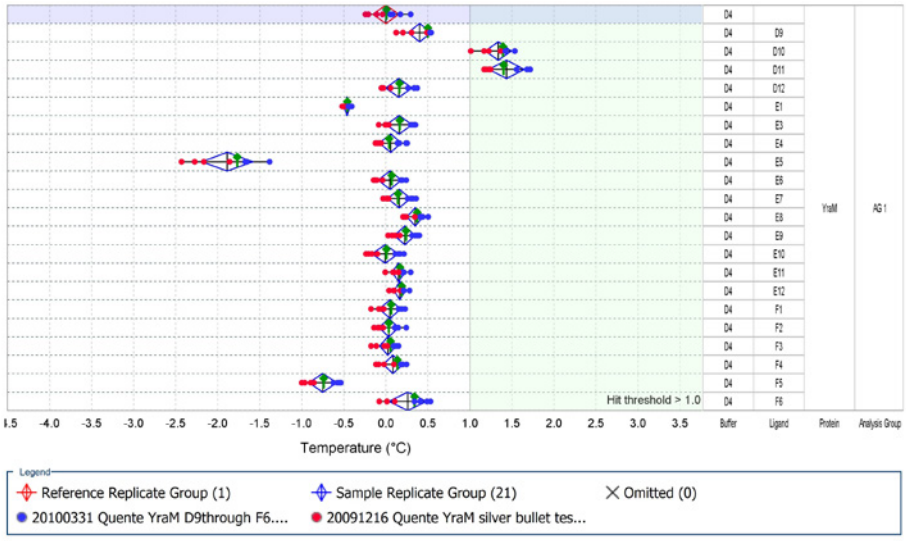

Figure 6. Screening YraM against a panel of ligand mixes. Several different ligand mixes were tested for YraM binding using the Protein Thermal Shift assay. Mixes D10 and D11 showed the highest stability, as indicated by the increased T_m. The D10 and D11 mixes included $CaCl_2$, $MgCl_2$, $CdCl_2$, $CoCl_2$, $ZnCl_2$, $CuCl_2$, $MnCl_2$, and $NiCl_2$ along with other components. T_m data were generated using the Boltzmann method. ΔT_m was calculated by comparing the T_m values for YraM without ligand to those of YraM with ligand. Data were collected at 1°C intervals from 25°C through 99°C on the StepOne Plus Real-Time PCR System and are shown in the replicate result plot view of the Protein Thermal Shift Software.

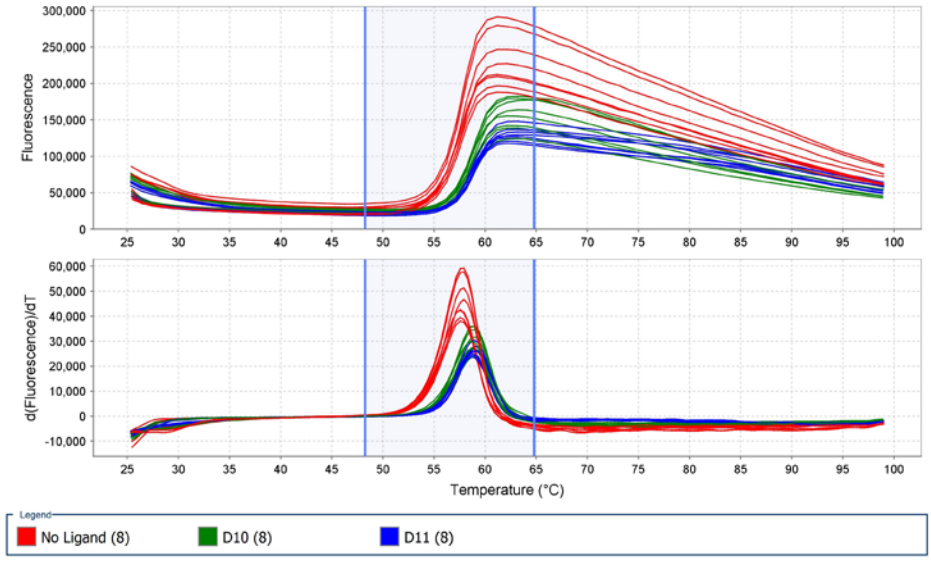

Figure 7. Screening YraM against D10 and D11 ligand mixes. The detailed Boltzmann and derivative plot views, in the Protein Thermal Shift Software, comparison of no ligand and the D10 and D11 ligand mixes tested with the YraM protein, as described in Figure 6.

Mutation or protein modification screening

The Protein Thermal Shift assay can also be used to easily monitor the effect of mutations on the thermal stability of a protein. Here, the Protein Thermal Shift assay was used to discriminate point mutation variants of Molony murine leukemia virus (M-MLV), Invitrogen™ SuperScript™ II, and SuperScript III reverse transcriptases (RTs) (Figure 8). The assay showed that SuperScript III RT has the highest thermal stability among the three proteins. This result demonstrates that increasing numbers of thermostabilizing mutations improves the thermostability of the reverse transcriptases and thus will improve reverse transcription efficiency.

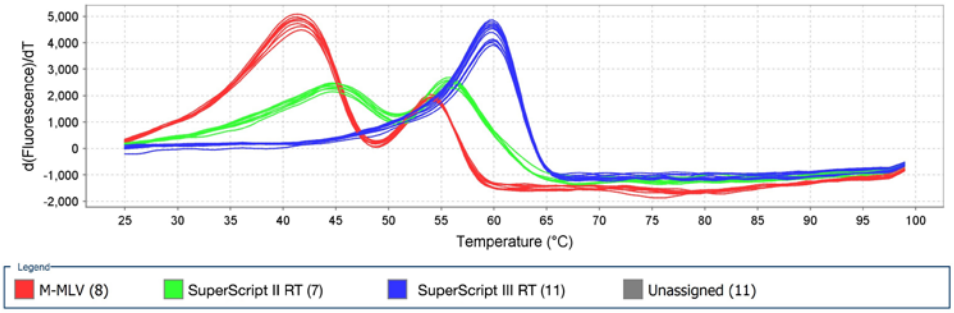

Figure 8. Effect of mutations on protein thermal stability. Each protein was analyzed at a concentration of 0.2 mg/mL using the Protein Thermal Shift assay. Data were generated on an Applied Biosystems ViiA™ 7 Real-Time PCR System and collected at a ramp rate of 0.05°C/sec from 25°C through 99°C and analyzed using the Protein Thermal Shift Software. The data show that SuperScript III RT has the highest thermal stability among the three reverse transcriptases.

Protein–protein interaction analysis

Protein–protein interactions are critical to many bimolecular processes. These interactions include enzyme-substrate binding, and protein-antibody or protein-receptor interactions. Understanding these interactions can provide insight into how the protein functions in a biological system. The Protein Thermal Shift assay can detect thermal stability changes induced by these interactions. To demonstrate this type of analysis, human decorin protein and a monoclonal antibody were used (Figure 9). Melt data for decorin alone, the decorin–antibody complex, and the antibody alone are shown.

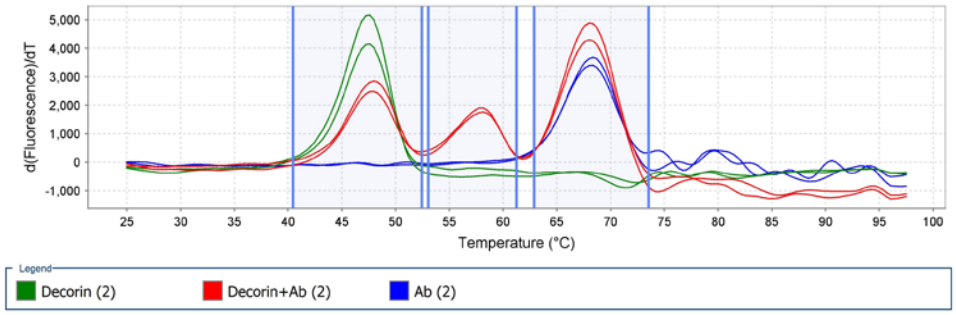

Figure 9. Human decorin protein- and antibody-binding analysis. Human decorin protein, a mixture of protein and antibody, and antibody alone were used in the Protein Thermal Shift assay. Data were generated on an Applied Biosystems 7500 Real-Time PCR System using continuous ramp mode at 1% ramp rate from 25°C through 99°C. Data were analyzed using the Protein Thermal Shift Software.

Summary: Protein Thermal Shift solutions for fast and efficient protein melt analysis

The Protein Thermal Shift Software and reagent kits enable easy Protein Thermal Shift analysis in a high-throughput manner, requiring <1 µg of sample per well, and at a cost that is significantly lower than those of other methods. The software and reagents can be used for high-throughput screening of proteins to identify ligands, mutations/modifications, or buffer conditions that increase melting temperature (T_m) and the relative stability of proteins. This application can also screen antibody-ligand binding as part of antibody development, or be used to assess protein preps for contaminants or aggregated protein.

The Applied Biosystems Protein Thermal Shift Dye Kit is available in a 2,000 reaction size and includes the Protein Thermal Shift buffer and dye. The Applied Biosystems Protein Thermal Shift Starter Kit includes the dye and the buffer, along with the Protein Thermal Shift control protein and control ligand. The starter kit is recommended for new users to become acquainted with the application and software.

The Protein Thermal Shift Software was developed specifically for analyzing protein melt fluorescence readings directly from real-time PCR instrument files. Different proteins will have different profiles, each with a unique melt-curve shape, slope, signal-to-noise ratio, and T_m range.

The Protein Thermal Shift Software generates one or multiple T_m values from these curves by the Boltzmann fit T_m and the derivative curve methods. The software allows quick comparison of T_m differences between assay conditions or ligands added to a sample, relative to a reference sample.

The Protein Thermal Shift assay can be run on any real-time PCR system, such as the Applied Biosystems QuantStudio 3, 5, 6 Flex, and 12K Flex Real-Time PCR Systems.

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Compatible instruments

System	System features	Cat. No.
QuantStudio 3 Real-Time PCR System	• Capacity: 96-well plates, 0.1 mL tubes or 0.2 mL tubes • Fast block: yes • Colors: 4	A28136 A28137
QuantStudio 5 Real-Time PCR System	• Capacity: 96-well plates, 0.1 mL tubes or 0.2 mL tubes, 384-well plates • Fast block: yes • Colors: 6	A28138 A28139 A28140
QuantStudio 6 Flex Real-Time PCR System	• Capacity: 96-well, 384-well • Fast block: yes • Colors: 5	4485697 4485694
QuantStudio 7 Flex Real-Time PCR System	• Capacity: 96-well, 384-well, TaqMan Array micro fluidic cards • Fast block: yes • Colors: 6 (21 filter combinations)	4485698 4485696
QuantStudio 12K Flex Real-Time PCR System	• Capacity: 96-well, 384-well, TaqMan Array micro fluidic cards, OpenArray plates • Fast block: yes • Colors: 6 (21 filter combinations)	4471050 4471134

Ordering information

Product	Size	Cat. No.
Protein Thermal Shift Dye Kit	2,000 rxns	4461146
Protein Thermal Shift Starter Kit	100 control rxns + (protein and ligand) + 2,000 rxns Protein Thermal Shift dye and buffer	4462263
Protein Thermal Shift Software	1 kit (10 licenses)	4466037
Protein Thermal Shift Software Additional license (separate software purchase required)	1 license	4466038
Protein Thermal Shift Software User Documentation Set	1 User Guide and 3 Quick Reference Cards	

For the latest product and application information, go to
thermofisher.com/proteinmelt

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