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Protein Thermal Shift™ Studies: Ligand Screening

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This quick reference is intended for advanced users who are familiar with the procedures in the *Protein Thermal Shift™ Studies User Guide* (PN 4461808).

Note: For safety and biohazard guidelines, refer to the "Safety" section in the *Protein Thermal Shift™ Studies User Guide* (PN 4461808). For every chemical, read the SDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Set up an experiment file for the instrument run

Note: You can modify the ramp speed, temperature range, and/or ramp rate according to your protein.

Setup	Setting
Experiment properties	• Experiment type: **Melt Curve** • Reagents: **Other** • Ramp speed: **Fast** or **Standard**
Target properties	• Reporter: **ROX** • Quencher: **None**
Plate layout	• Assign targets to all wells in use • Passive reference: **None**
Run method	• Reaction volume per well: **20 µL** • Ramp mode: **Continuous** • Thermal profile: Step 1: Temp: **25**°C, Time: **2 min** Step 2: Temp: **99**°C, Time: **2 min** • Ramp rate: – ViiA™ 7 System: Step 1: **1.6**°C**/s**, Step 2: **0.05**°C**/s** – StepOne™, StepOnePlus™, 7500, and 7500 Fast Systems: Step 1: **100%**, Step 2: **1%** • (ViiA™ 7 System only) Optical filters: – Excitation filter: **x4(580±10)** – Emission filter: **m4(623±14)**

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Prepare the protein melt reactions

We recommend that you prepare four replicates of each reaction.

Note: You can modify the protein:dye ratio according to your protein.

1. Prepare a fresh dilution of Protein Thermal Shift™ Dye (1000X) to 8X.
2. Place the reaction plate on ice, then add reaction components to the plate in the order listed:

Component	Volume
Protein Thermal Shift™ Buffer	5.0 µL
Water + protein + ligand	12.5 µL
Diluted Protein Thermal Shift™ Dye (8X)	2.5 µL
Total volume for each control reaction	**20.0 µL**

IMPORTANT! Make sure that the arrangement of reactions in the reaction plate corresponds exactly with the well assignments in the experiment file.

3. Pipet each reaction up and down 10 times to mix well.
4. Seal the plate with MicroAmp® Optical Adhesive Film, spin it at 1000 rpm for 1 minute, then place it on ice until you load the instrument.

Run the protein melt reactions

IMPORTANT! Keep the protein melt reactions on ice until you load the instrument.

Load and run the reactions

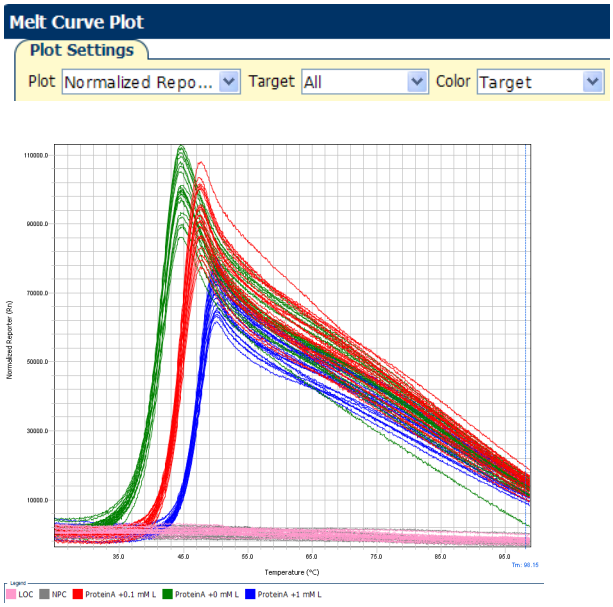
Load and run the protein melt reactions on a supported Applied Biosystems Real-Time PCR System.

1. If necessary, transfer the experiment file that you created for the run to the computer that is connected to the instrument.
2. Using the Real-Time PCR System Software on the computer that is connected to the instrument, open the experiment file for the run.
3. Load the reaction plate into the instrument, then start the run.

Review the melt curves

Using the Real-Time PCR System Software, open the experiment file from the completed instrument run, analyze and save the experiment file, then review the melt curves.

For detailed troubleshooting information, see the *Protein Thermal Shift™ Studies User Guide*.



- Do you see fluorescence signals in all of the sample wells?

  No fluorescence signals in the sample wells may indicate missing dye or protein or an instrument problem.
- Do you see flat fluorescence levels in the NPC wells?

  High fluorescence levels in the NPC wells may indicate protein contamination in the wells or protein melt reactions; or it may indicate that the dye interacts with a component in the buffer.
- Do you see flat fluorescence levels in the LOC wells?

  High fluorescence levels in the LOC wells but not in the NPC wells may indicate protein contamination in the ligand or ligand-dye interactions. Fluorescence from ligand-dye interactions may mask the protein-dye interactions.
- Do the replicates have similar melt curves?

Set up the analysis

Create and set up the study

1. Using the Protein Thermal Shift™ Software, click **Create Study** on the Home screen.
2. Complete the Setup screens, then save the study:

Setup screen	Tasks
Properties	1. Enter a Study Name that is than 100 characters and does not contain these characters: / \ * ‟ ? < > | . , 2. For the instrument, select the instrument type that you used to run the protein melt reactions and generate the experiment files.
Conditions	Define the conditions, condition values, and analysis groups for the study.
Experiment Files	1. For each experiment file you ran for the study, click **Import**, then select the experiment file (*.eds) from the completed instrument run. 2. For each experiment file that you import, assign the conditions, task, and analysis group to the wells that contained protein melt reactions. **IMPORTANT!** Make sure that the condition assignments correspond exactly with the contents of the reaction plate. Setup errors may result in an incorrect grouping of the data and incorrect Tm statistics. 3. For each analysis group, select the wells for the reference replicate group, click **Assign**, then select **Reference** as the Task.

Review the analysis settings

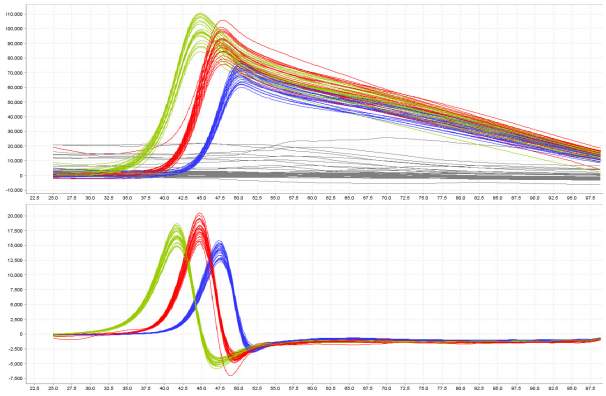
1. Click **Analysis Settings** in the toolbar.
2. In the Positive Hit tab, specify the ΔTm-Boltzmann and ΔTm-Derivative values to indicate a positive hit.
3. In the Flags tab, specify settings for applying flags.
4. Apply the new analysis settings and analyze the study.

Review the well results

For detailed troubleshooting information, see the *Protein Thermal Shift™ Studies User Guide*.

Review the melt curves

1. In the Analysis > Well Results screen, select **Show in Plot**, then select to show:
   - Unselected Wells
   - Legend
2. Select **Color By**, then select **Ligand** to color the melt curves according to the ligand condition value assigned for the well.



3. For each replicate group, select all the wells in the replicate group, then review the fluorescence levels in the melt curves:
   - For the NPC wells, do you observe a rise in fluorescence? If so, the wells or protein melt reactions may be contaminated with protein or the dye may interact with a buffer component.
   - For the LOC wells, do you observe a rise in fluorescence? A rise in fluorescence in LOC wells but not in NPC wells may indicate protein contamination in the ligand or ligand-dye interactions.
   - For sample or reference wells, do you observe flat melt curves? If so, condition assignments may be incorrect or a component is missing from the protein melt reactions.
   - Within each replicate group, are the fluorescence melt curves similar to each other? Within each replicate group, are the derivative melt curves similar to each other? If the melt curves for the replicates are dissimilar, pipetting errors may have occurred during reaction setup or condition assignments may be incorrect.

4. If the derivative melt curves for the replicate group show multiple melt phases, set the analysis mode to Auto: Multiple Tm, then review the derivative melt curves:
   a. In the Well Table or in the melt curve plot, select the wells with multiple melt phases.
   b. Click **Auto Analysis Options**, then select **Auto: Multiple Tm**.
   c. Click **Analyze** to reanalyze using the Auto: Multiple Tm analysis mode.
   d. Review the number of melt phases in the derivative melt curves:
      - Do all replicate groups have the same number of melt phases?
      - For each replicate group, are there outliers with a different number of melt phases than the other samples in the replicate group? You may consider omitting outliers from analysis.

Review the region(s) of analysis (ROA)

1. In the Analysis > Well Results screen, confirm that each ROA meets the following criteria:
   - For melt curves with one melt phase, the curve within the ROA resembles a sigmoidal profile.
   - At the start temperature, the signal is relatively flat.
   - At the end temperature, the signal has already reached its maximum.
2. For each replicate group, edit the ROAs so that all of the wells in the replicate group have the same ROA(s):
   a. Select the replicates, click (Define ROA) in the toolbar above the melt curve plots, then click-drag an area in one of the plots to define a melt phase. Repeat for each melt phase you identify.
   b. If necessary adjust the start and end temperatures for each ROA: click-drag the ROA to move it or click-drag the ROA start or end line.
3. Click **Analyze** to reanalyze using the edited ROAs.

Note: After you edit ROAs, the analysis mode changes from Auto to Manual.

Review the flags and Tm values

1. In the Analysis > Well Results screen, select **Show in Plot**, then select to show:
   - Boltzmann Tm
   - Derivative Tm
   - Unselected Wells
   - Legend
2. Review the melt curves for flagged wells and the other wells in the replicate group, then omit wells from analysis as necessary:

Flag	Description
	Flag Indicator
RFU	Low Signal
Fit	Poor Fit
	Multiple Melts
RFU	High Background
NPC	High NPC
	Algorithm Failure
RPL	Replicate Mismatch
REF	Reference Mismatch

3. For each replicate group, select the wells in the replicate group, then review the Tm B (Boltzmann Tm, green dashed line) and the Tm D (derivative Tm, black dotted line) in the Well Table and in the melt curves:
   - Are the Tm B or Tm D values significantly different from the Tm values for other wells in the replicate group? Do any replicates have melt curves that are inconsistent with the other melt curves for the replicate group? If so, you may consider omitting wells from analysis.
   - Are the melt curves within the replicate group similar?
4. If you omitted any wells from the analysis, click **Analyze**.

Review the Boltzmann fit

1. In the Analysis > Well Results screen, select **Show in Plot**, then select to show:
   - Boltzmann Fit
   - Unselected Wells
   - Legend
2. For each well, compare the fluorescence melt curve to the Boltzmann fit curve (dark green thick curve) and review the value in the B Fit (Boltzmann Fit) column of the Well Table:
   - How well does the fluorescence melt curve correspond to the Boltzmann fit curve?
   - Is the B Fit value close to 1?
   - Is the B Fit value similar among wells in the replicate group?

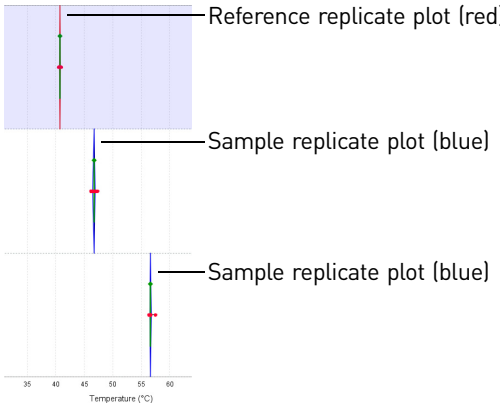
Review the replicate results

For detailed troubleshooting information, see the *Protein Thermal Shift™ Studies User Guide*.

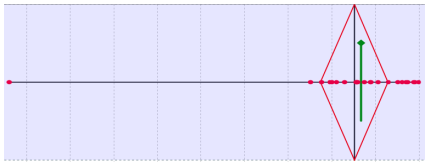
Review the Tm statistics

1. In the Analysis > Replicate Results screen, select **Plot by**, then select **Tm-Boltzmann** or **Tm-Derivative**.
2. Click **Condition Hierarchy** to group the replicate plots and change the order of conditions in the plot.

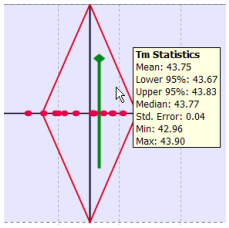
   The condition at the top level of the hierarchy is displayed in the far-right side of the plot.
3. Scan the Replicate Results Plot to review the conditions that affect the Tm values relative to the reference replicate group.



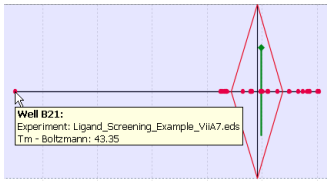
4. Review the Tm statistics for each replicate group:
   a. Zoom in: Click , then click-drag an area on the plot.
   b. Move the plot: Click , then click-drag the plot.



   c. View the Tm statistics tooltip for the replicate group.



   d. For outliers, view the datapoint tooltip.



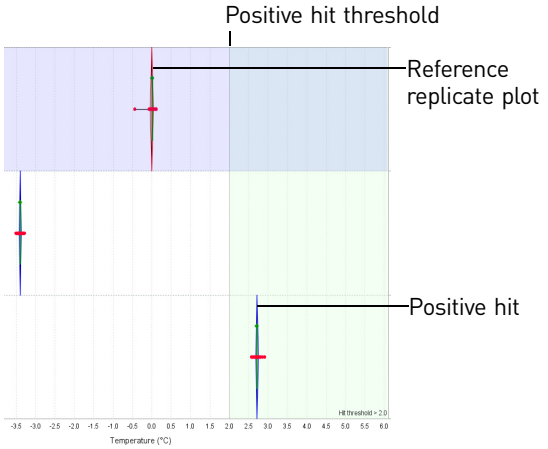
Note: Click to restore the default zoom.

5. In the Replicate Groups table, review the Tm statistics for each replicate group:
   - **Std. Error** (standard error of the mean for the Tm value): Is the value low? If the value is high, review the data for each replicate.
   - **Min** and **Max** (minimum and maximum Tm values for the replicate group): Is the range of Tm values for the replicate group within 1 degree? If the range of Tm values exceeds 1 degree, review the data for each replicate.
6. Omit outliers as necessary, then click **Analyze**.

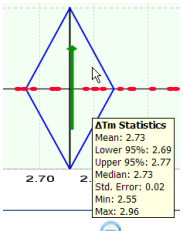
Review the positive hits

Note: You must specify the reference replicate group to calculate ΔTm values and to determine positive hits.

1. In the Analysis > Replicate Results screen, select **Plot by**, then select **ΔTm-Boltzmann** or **ΔTm-Derivative**.
2. Select **Show in Plot**, then select to show **Positive Hits**.
3. Scan the green shaded area of the Replicate Results Plot for positive hits.



4. Review the ΔTm statistics for each replicate group:
   a. Zoom in: Click , then click-drag an area on the plot.
   b. Move the plot: Click , then click-drag the plot.
   c. View the ΔTm statistics tooltip for the replicate group.



Note: Click to restore the default zoom.

5. In the Replicate Groups table, review the positive hits ( ) in the Hits B or Hits D column and review the ΔTm B or ΔTm D statistics.

Ordering information

Item	Catalog no.
Protein Thermal Shift™ Starter Kit	4462263
Protein Thermal Shift™ Dye Kit	4461146
Protein Thermal Shift™ Software v1.0 – Additional License	4466038
Protein Thermal Shift™ Software v1.0, 10 licenses	4466037
Protein Thermal Shift™ Studies User Documentation Set	4463373

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