

LightCycler® 480 Instrument Quick Guide

Protein Melting

Protein Melting Analysis Principle:



Protein Melting is monitored using hydrophobic binding dyes, like SYPRO® Orange, and is used to determine the stability of protein in a given condition. The stability is determined by the T_m of the protein melting.

The change in T_m from a reference condition to the test condition can infer a change in stability. Often the test condition is used to assess different buffer conditions, or the interaction of the protein of choice with compounds, ligands, or other proteins in the test condition.

Different solutions may have an effect on this stability, increasing or decreasing the determined protein T_m . Interaction of the protein of choice with another molecule or protein could have a stabilizing effect, increasing the T_m of the protein of choice proportional to the concentration of the ligand and the binding affinity of the ligand (Matulis et al, (2005) Biochemistry 44, 5258-5266; Vedadi et al, (2006) PNAS, In Press; “Amazing LightCycler® 480 System Applications: High-Throughput Screening of Buffers for Optimal Protein Stability”, Bertram H.A. Ong and Ralf Jauch, direct communication to Roche).

Run Protein Melting

Detector Setup:

1. In the LightCycler® 480 software, enter the tools menu by clicking .
2. Click to select the Detection Formats from the bottom of the list on the left.
3. Click  to create another detection format. Name the Detection Format intuitively using the name of the dye to be used (e.g., “SYPRO® Orange”).
4. From the LightCycler® 480 System I and System II settings examples below, program the appropriate detection format.

Notes: *The examples shown are for SYPRO® Orange. If you choose to use another dye such as SYPRO® Red, please use the appropriate wavelengths for excitation and detection (emission) for your dye of choice.*

A Light Cycler System II states the name on the front of the instrument covering. A System I will only state LightCycler® 480. The wavelengths are not the same between the systems. Use the wavelengths which agree with the LightCycler® 480 instrument you are using.

Protein Melting

Run Protein Melting, continued

System I Format

Filter Combination Selection

Emission

E	500	533	568	610	640	670
x	450	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c	483	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
i	523	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
t	558	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
a	615	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Clear

Selected Filter Combination List

Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
483	568	SYPRO Orange	1	10	1

System II Format

Filter Combination Selection

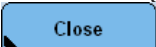
Emission

E	488	510	580	610	640	660
x	440	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
c	465	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
i	498	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
t	533	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
a	618	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Clear

Selected Filter Combination List

Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
465	580	Sypro Orange	1	10	1

- Choose the appropriate wavelength for Excitation and Emission by clicking the corresponding checkbox.
- Click the name field and change the name to the dye of choice.
- Click the Melt Factor field to enter the melt factor of 1.
- Click the Quant Factor field to change the Quant Factor to 10. This should have no effect on melting assays.
- Click the Max Integration Time field to set it to 1 second.
- Click .

Melting Program Setup

This Run Protocol is used for all future analyses, but may be modified anytime. Program the Protein Melting to have the following settings, as shown in the picture below:

Note: The Ramp Rates of 4.8 and 2.5 are the default for the 384 MWP setup. If you are using the 96 MWP setup, the expected default/maximum Ramp Rates are 4.4 and 2.2, and should not be changed.

The screenshot displays the 'Protein Melting' program setup in a software interface. The interface is organized into several sections:

- Setup:** Contains fields for 'Detection Format' (set to SYPRO Orange), 'Block Size' (384), 'Plate ID', 'Reaction Volume' (10), 'Color Comp', 'Lot No', and 'Test ID'. A 'Customize' button is also present.
- Programs:** A table with columns 'Program Name', 'Cycles', and 'Analysis Mode'. The 'Protein Melting' program is listed with 1 cycle and 'Melting Curves' analysis mode.
- Temperature Targets:** A table with columns: Target (°C), Acquisition Mode, Hold (hh:mm:ss), Ramp Rate (°C/s), Acquisitions (per °C), Sec Target (°C), Step Size (°C), and Step Delay (cycles).

Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
20	None	00:00:15	4.8				
85	Continuous		0.06	10			
20	None	00:00:15	2.5				
- Overview:** A graph showing 'Temperature (°C)' on the y-axis (0 to 100) and 'Estimated Time (h:mm:ss)' on the x-axis (0:00 to 18:38). The graph shows a green line representing the temperature profile, which starts at approximately 40°C, ramps up to 85°C, holds, and then ramps down.

1. Change the Detection Format to the newly created SYPRO® Orange format.
2. The Block Size is determined at start-up by the LightCycler® 480 according to what block is in the instrument. This cannot be changed unless the instrument is powered OFF and the block is changed. The LightCycler® 480 detects which block is present when performing its Self-Test after being powered ON.
3. Set the reaction volume to the desired volume.
 - a. For the 96 well plate/block, a volume of 15 or 20 µL is suggested. A minimum of 10 may be used, but may not yield optimal results.
 - b. For the 384 well plate/block, a volume of 5 to 15 µL is suggested. A volume less than 5 µL may be attempted, but may not yield optimal results. The minimum instrument setting is 3 µL.
 - c. A single Program is typically used, and the program is renamed to Protein Melting or another name for user clarity.
 - This program should have the Analysis Mode of Melting Curves.
 - d. Program three temperature targets as follows:
 - The first target is 20°C, with a Hold of 15 seconds. All other fields are left at the default.
 - The second target is typically 85°C, with an Acquisition Mode of Continuous, and 10 acquisitions per degree C. All other fields are left at the default.
 - The third target is 20°C, with a Hold of 15 seconds. All other fields are left at the default.

Protein Melting

Melting Program Setup, continued

Notes: If it is determined by the researcher that this temperature is not acceptable, this temperature should be changed. The maximum temperature which can be programmed into the LightCycler® 480 is 99°C.


No hold time is necessary to see an effect at this maximum temperature.

The ramp rate is determined by the number of acquisitions per degree and the exposure time of the detection system. This cannot be edited, except by increasing or decreasing the number of Acquisitions per degree C.

4. Save the Run Program as a “Run Template” by clicking the down arrow next to **Apply Template**, then select **Save As Template**.
5. Give this template an intuitive name which includes intention of the run, the detection format, and the size of the block.
6. Save the template into your desired folder to be applied to New Experiments in the future.

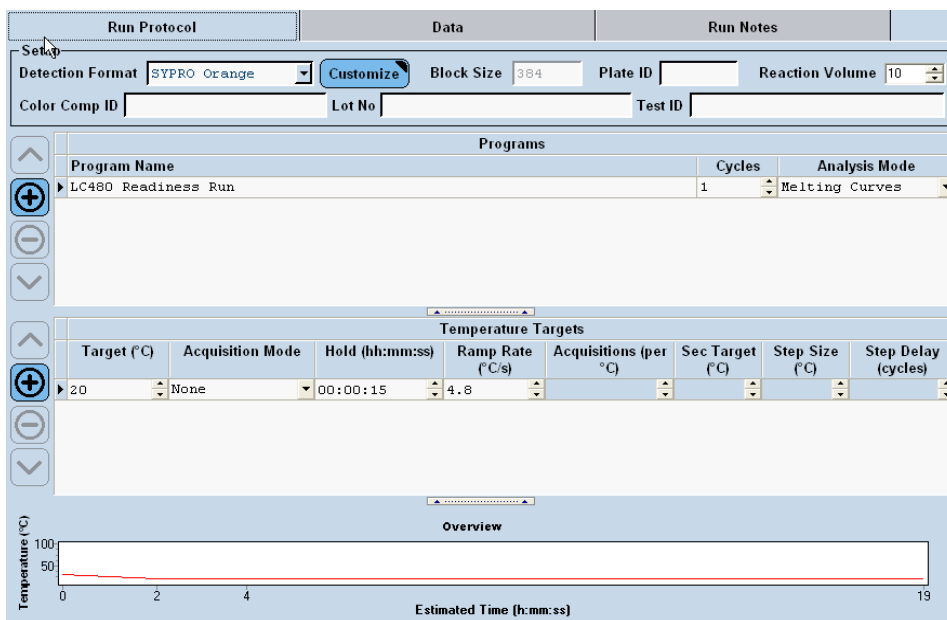
Readiness Program Setup

1. This Run Protocol is used to “ready” the LightCycler® 480 instrument for all future plate runs. Program the LC480 Readiness Run to have the following settings, as shown in the picture below:

Note: The LC480 Readiness Run only needs to be used when the instrument has been idle for more than 10 minutes. The LightCycler 480° lamp will have powered off to save lamp hours. This run activates the lamp so successive runs do not have a warm-up period of waiting after loading the plate and pressing .

The intention of this run is to prevent the 105°C heated lid from having an effect on the user’s protein melting analysis plate. Although this is a preventative measure, no effect was seen from not performing this LC480 Readiness Run.

The Ramp Rates of 4.8 and 2.5 are the default for the 384 MWP setup. If you are using the 96 MWP setup, the expected default/maximum Ramp Rates are 4.4 and 2.2, and should not be changed.



Program Name	Cycles	Analysis Mode
LC480 Readiness Run	1	Melting Curves

Target (°C)	Acquisition Mode	Hold (h:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)
20	None	00:00:15	4.8				

Readiness Program Setup

- a. Change the Detection Format to the newly created SYPRO® Orange format. This is done for consistency, although any detection format works for this run.
 - b. The Block Size is determined by the LightCycler® 480 instrument according to which block is in the instrument. This cannot be changed, unless the instrument is powered OFF and the block is changed. The LightCycler® 480 instrument detects what block is present when performing its Self-Test after being powered ON.
 - c. Set the reaction volume to the desired volume. This is done for consistency, although any reaction volume works for this run.
 - For the 96 well plate/block, a volume of 15 or 20 µL is suggested. A minimum of 10 may be used, but may not yield optimal results.
 - For the 384 well plate/block, a volume of 5 to 15 µL is suggested. A volume less than 5 µL may be attempted, but may not yield optimal results. The minimum instrument setting is 3 µL.
 - A single Program is typically used, and the program is renamed to LC480 Readiness Run or another name for user clarity.

This program should have the Analysis Mode of Melting Curves.
 - d. Program one temperature targets as follows:
 - The first target is 20°C, with a Hold of 15 seconds. All other fields are left at the default.
2. Save the Run Program as a “Run Template” by clicking the down arrow next to **Apply Template**, then select **Save As Template**.
 3. Give this template an intuitive name which includes intention of the run, the detection format, and the size of the block.
 4. Save the template into your desired folder to be applied to New Experiments in the future.

Protein Melting

Protein Melting Experimental Conditions Setup and Determination

Determination of optimal Protein and Dye concentrations:

Note: *The protein and dye concentrations may need to be optimized for each protein. Protein size and hydrophobic domain content impact the association with the dye, and thus the total fluorescence obtained in the analysis. Typical protein concentrations range from 0.5 μ M to 10 μ M. Typical SYPRO® Orange dye concentrations range from 5x to 20x, although some may find lower concentrations useful. Typical Reaction sizes range from 5 μ l in a 384 well plate to 20 μ L in a 96 well plate.*

1. Prepare the protein in to a 20 μ M stock in a suitable screening buffer. Other dilutions can be made for the ease of pipetting.
 - a. Suggested buffers if another is unavailable:
 - 50 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 2 mM DTT, pH 7.4.
 - Phosphate Buffered Saline (PBS)
 - Standard HEPES screening buffer (100 mM HEPES, 150 mM NaCl, pH 7.5), Vedadi et.al.

Note: *It is not necessary to use these buffers; however, it is suggested to avoid buffers containing BSA, DMSO, or other compounds that may cause high background or effect the stability of the protein as this hinders the assay. Many compounds used with the protein are dissolved in DMSO. In small amounts this may not harm the assay, but a vehicle control should be used to ensure the effects on T_m seen are due to the compound of interest.*

2. Prepare a 100x working stock solution from SYPRO® Orange 5000x Stock in the buffer of choice from 1.1. Further dilutions can be made for ease of pipetting if desired.
3. Combine into wells described in Table 1 to determine the optimal concentrations using a standard 10 μ L reaction size. Adjust appropriately if another final volume is used. The final concentrations are shown in Table 2.

Protein Melting Experimental Conditions Setup and Determination, continued

Table 1	Protein from 100 μ M Stock	SYPRO® Dye from 100x Stock	Buffer
Well A1	4.0 μ L	0.5 μ L	5.50 μ L
A2	4.0 μ L	0.67 μ L	5.33 μ L
A3	4.0 μ L	1.0 μ L	5.00 μ L
A4	4.0 μ L	2.0 μ L	4.00 μ L
B1	2.0 μ L	0.5 μ L	7.50 μ L
B2	2.0 μ L	0.67 μ L	7.33 μ L
B3	2.0 μ L	1.0 μ L	7.00 μ L
B4	2.0 μ L	2.0 μ L	6.00 μ L
C1	1.0 μ L	0.5 μ L	8.50 μ L
C2	1.0 μ L	0.67 μ L	8.33 μ L
C3	1.0 μ L	1.0 μ L	8.00 μ L
C4	1.0 μ L	2.0 μ L	7.00 μ L
D1	0.5 μ L	0.5 μ L	9.00 μ L
D2	0.5 μ L	0.67 μ L	8.83 μ L
D3	0.5 μ L	1.0 μ L	8.50 μ L
D4	0.5 μ L	2.0 μ L	7.50 μ L
E1	0.25 μ L	0.5 μ L	9.25 μ L
E2	0.25 μ L	0.67 μ L	9.08 μ L
E3	0.25 μ L	1.0 μ L	8.75 μ L
E4	0.25 μ L	2.0 μ L	7.75 μ L

Table 1: This table is an example of the concentration matrix for optimization.
The actual concentrations, amounts, rows and columns may be chosen by the user.

Protein Melting

Protein Melting Experimental Conditions Setup and Determination, continued

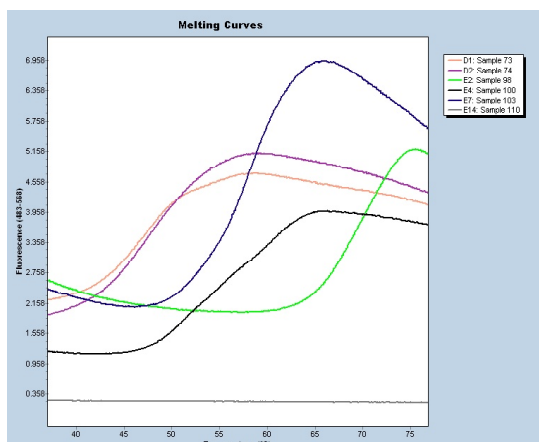
Table 2	Column 1	Column 2	Column 3	Column 4
Row A	8.0 μM Protein 5x SYPRO [®]	8.0 μM Protein 6.67x SYPRO [®]	8.0 μM Protein 10x SYPRO [®]	8.0 μM Protein 20x SYPRO [®]
Row B	4.0 μM Protein 5x SYPRO [®]	4.0 μM Protein 6.67x SYPRO [®]	4.0 μM Protein 10x SYPRO [®]	4.0 μM Protein 20x SYPRO [®]
Row C	2.0 μM Protein 5x SYPRO [®]	2.0 μM Protein 6.67x SYPRO [®]	2.0 μM Protein 10x SYPRO [®]	2.0 μM Protein 20x SYPRO [®]
Row D	1.0 μM Protein 5x SYPRO [®]	1.0 μM Protein 6.67x SYPRO [®]	1.0 μM Protein 10x SYPRO [®]	1.0 μM Protein 20x SYPRO [®]
Row E	0.5 μM Protein 5x SYPRO [®]	0.5 μM Protein 6.67x SYPRO [®]	0.5 μM Protein 10x SYPRO [®]	0.5 μM Protein 20x SYPRO [®]

Table 2: This table is an example of the concentration matrix for optimization. The actual rows and columns may be chosen by the user.

Choice of optimal Protein and Dye concentrations:

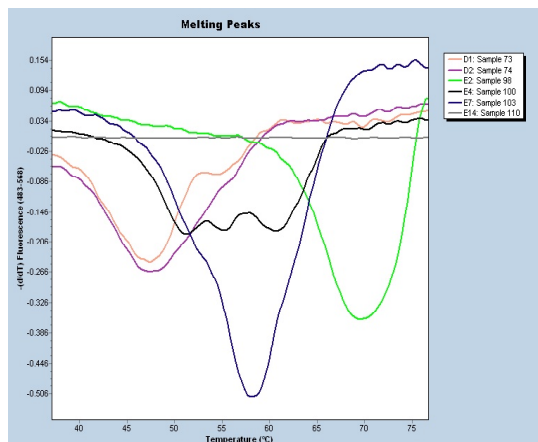
Note: The choice of the optimal concentration is a decision made by the researcher. The optimal concentration differs slightly depending on the goals of the researcher. We suggest the best curves are those giving high fluorescence, giving a nicely shaped curve with a single peak/valley when inspected in the LC480 Tm Calling Analysis, having a middle concentration of protein of those giving nicely shaped, high fluorescence curves, and use the lowest SYPRO[®] dye concentration. If volume is also a factor, you may wish to assay the lowest or most appropriate volume to give the results above for future assays.

1. Visual inspection of Protein Melting curves.
 - a. The curves should be nicely shaped with a good amount of fluorescence. Refer to the blue and green curves below for examples of good Protein Melting curves.



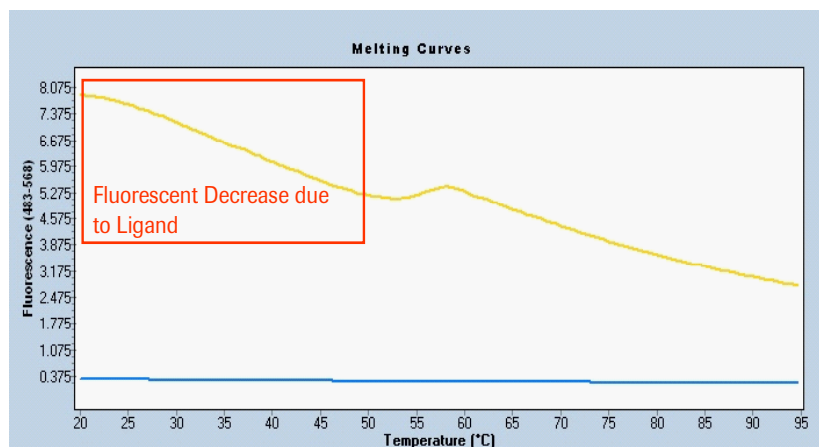
Protein Melting Experimental Conditions Setup and Determination, continued

- b. Use the T_m Calling analysis to help show the curves have a good shape. The negative first derivative drawing of the curves should show curves with a single valley or negative peak. If the curve shows multiple valleys, this is not an optimal curve. Refer to the blue and green curves below for examples of good Protein Melting curves.



Protein Melting Plate Setup and Run

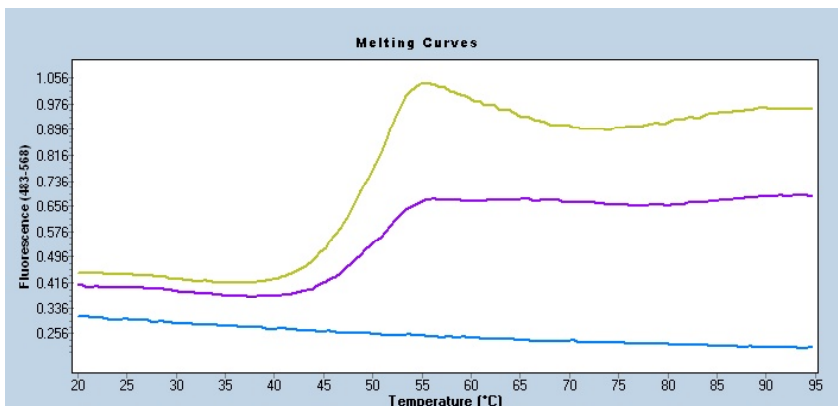
1. Use the protein and dye concentrations determined from the test run above.
2. The Change of buffer or addition of Compound, Ligand, or interacting protein should be performed at this time.
3. A reference well (or wells) containing only the protein of interest in determined optimal conditions without change should be maintained on plate as a reference..
4. Changes in T_m from the reference wells is used to determine how the changes in buffer or addition of compounds has effected the protein stability.
5. Further setup is determined by the researcher.
6. Ligand fluorescence may effect the shape of the curve. This was not found to effect the T_m resulting from the test.



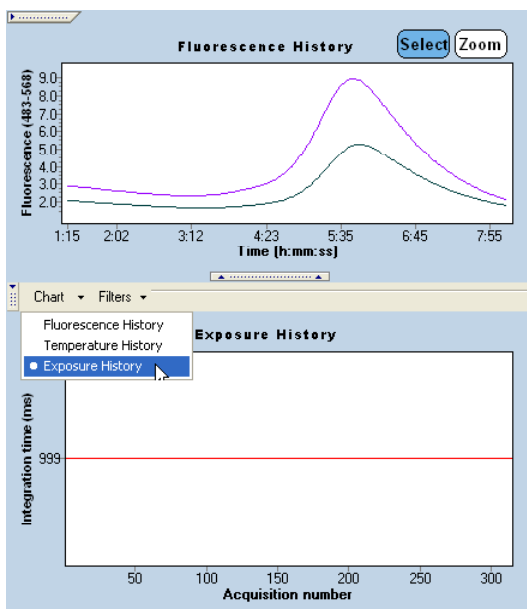
Protein Melting

Protein Melting Plate Setup and Run, continued

7. Curves with too low of a concentration can have a poor shape, possibly giving multiple peaks in the analysis. Although these curves are not optimal and should have the protein concentration increased, the Roche LightCycler® Protein Melting Analysis only generates a T_m for the greatest peak/melting event.




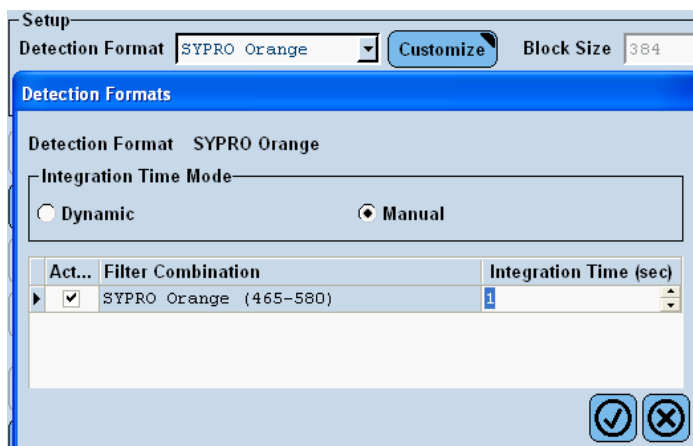
8. The best way to ensure plate-to-plate consistency in your LightCycler® 480 plate runs for protein analysis is to control the exposure time. This is especially applicable if you wish to use very short melting runs, that are less than 8 minutes run time.
 - a. Find run time from run used to determine the optimal protein and dye concentrations.
 - In the Experiment section, choose the **Data** tab.
 - Click and expand the hidden menu bar on one of the charts displayed. See illustration below:



- Change the Chart type to Exposure History.
- The exposure time is displayed as Integration time (in milliseconds).
- Round the exposure time up to a reasonable time. In the example above, the exposure/integration time would be rounded to 1 sec (or 1000 ms).

Protein Melting Plate Setup and Run, continued

- When starting your future runs, use this exposure time by changing the exposure time to this time. Click the **Customize** button, choose manual, and enter the exposure time in the Integration Time (sec) field. Click  to confirm the changes. See illustration below.



- To ensure these changes are used in future analyses, a template may be created. Save the Run Program as a Run Template by clicking the down arrow next to **Apply Template**, then select **Save As Template**.
- Give this template an intuitive name that includes intention of the run, the detection format, and the size of the block.
- Save the template into your desired folder to be applied to New Experiments in the future.

Setup of Protein Melting Analysis Tool

Note: *The Roche Protein Melting software may be obtained from a Roche Representative or Roche Technical Support.*




1. Installation of the Roche Protein Melting software.
 - a. The CD with the Roche Protein Melting Software contains a ReadMe.doc file that describes the installation and setup of the software.
 - b. Install the software by running the setup.exe file.
 - This installs the Roche Protein Melting Software and may update your computer to the proper level Microsoft .Net Framework, if necessary.
 - c. Run the LightCycler® 480 Protein Melting software.
 - The first run requires you to configure the directory watcher. The Roche.LC480.ExportWatcher watches a specific “IXO export directory” (folder) chosen by you, allowing the Roche Protein Melting Software to immediately analyze any LightCycler® 480 .ixo file exported into this directory.
 - Choose a CSV target directory (folder) you want to save the resulting .csv analysis file.
 - From the LightCycler® 480 Basic software, you may choose to Export a specific protein melting .ixo run file, or Batch Export a group of these files to be analyzed. Target this export to the specified folder for the “IXO export directory”, set above.
 - d. The output of the software is a .csv file, that can be viewed in any spreadsheet or text program. CSV stands for comma separated value, and is a standard file format recognized by most programs. The first line of the file is the header with the following columns:
 - Program: 0-based index of the LightCycler® 480 program
 - Channel: 0-based index of the LightCycler® 480 channel
 - Position: 0-based position on the MWP (0...95 or 0...383)
 - Call: Positive, Negative or Uncertain.
 - Negative: no results are listed in the following columns.
 - Uncertain: an additional message is listed in the Message column
 - Tm: calculated melting temperature (see the Roche Protein Melting software’s ReadMe.doc for further description)
 - Slope: slope of the fluorescence signal at the Tm
 - Initial: fluorescence at beginning of the curve (lowest temperature)
 - Low: minimum fluorescence below Tm
 - Peak: minimum fluorescence above Tm
 - Message: additional calculation information, if call is uncertain.

Use of Protein Melting Analysis



Export of LightCycler® 480 run and Protein Melting Analysis

Note: Runs may be exported individually, in a group as a Batch Export, or exported automatically every run with an automated or robotically controlled LightCycler® 480 setup.

Single run export

1. There are two ways to perform a single run export.
 - a. Export from within an open run.
 - b. With the run of choice open, click .
2. Choose the appropriate export folder, chosen in the Roche LightCycler® 480 Protein Melting software.
 - a. If setup properly, the Roche Protein Melting software analyzes the data instantly to produce the analysis *.csv result file.
3. Export from the LC480 Navigator.
4. Click , or select the Navigator from the Window dropdown menu in the LightCycler® 480 software.
5. Choose the experiment to export by left-clicking the experiment of choice.
6. Click . Choose the appropriate export folder, chosen in the Roche LightCycler® 480 Protein Melting software.
 - a. If setup properly, the Roche Protein Melting software analyzes the data instantly to produce the analysis *.csv result file.

Multiple run/Batch Export

1. Click , or select the Navigator from the Window dropdown menu in the LightCycler® 480 software
2. Click . Choose the appropriate export folder, chosen in the Roche LightCycler® 480 Protein Melting software.
 - a. If setup properly, the Roche Protein Melting software will analyze the data instantly to produce the analysis *.csv result files.
3. Automated Export
 - a. The automated export should be setup in your scheduling software to export the LC480 experiment .ixo file at the end of every run. The LC480 LIMS module accommodates this. Contact Roche Technical Support for further information, if needed.

Protein Melting Analysis Result Table Description

Roche LightCycler® 480 Protein Melting Analysis Result Table Description

1. The output of the software is a .csv file, that can be viewed in any spreadsheet or text program. CSV stands for comma separated value, and is a standard file format recognized by most of these programs.
 - a. The first line of the file is the header with the following columns:
 - Program: 0-based index of the LightCycler® 480 program
 - Channel: 0-based index of the LightCycler® 480 channel
 - Position: 0-based position on the MWP (0...95 or 0...383)
 - Call: Positive, Negative or Uncertain.
Negative: no results are listed in the following columns.
Uncertain: an additional message is listed in the Message column
 - Tm: calculated melting temperature (see the Roche Protein Melting Software's ReadMe.doc for further description)
 - Slope: slope of the fluorescence signal at the Tm
 - Initial: fluorescence at beginning of the curve (lowest temperature)
 - Low: minimum fluorescence below Tm
 - Peak: maximum fluorescence above Tm
 - Message: additional calculation information, if call is uncertain
2. The data points gathered by the LightCycler® 480 Protein Melting Analysis relate to information that could be helpful to the researcher.
 - a. The difference between the Initial and the Low fluorescence values relates to the affinity of the dye for the protein or ligand/compound.
 - b. The difference between the Low and Peak relates to the affinity of the protein hydrophobic domains to the dye.
 - c. The slope (taken by our software at the Tm exactly) is relative to the Kd determined by other software.

Protein Melting Reagents from other vendors:

Product Name	Cat. No.	Pack Size/Description
SYPRO® Orange	n/a	(Sigma-Aldrich)
Protein Analysis Buffer	n/a	This is an aqueous buffer determined by the researcher. This buffer should be free of BSA and nonspecific protein stabilizers, detergents, and other compounds which can/will have impact on the protein stability/folding or background fluorescence of the analysis.

Consumables:

Product Name	Cat. No.	Pack Size/Description
LightCycler® 480 MultiWell Plate 96 (White)	04 729 692 001	5 x 10 plates and sealing foils
LightCycler® 480 MultiWell Plate 384 (White)	04 729 749 001	5 x 10 plates and sealing foils
LightCycler® 480 Sealing Foil	04 729 757 001	50 foils

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