Protein Thermal Shift Assays Made Easy with the Bio-Rad™ Family of CFX Real-Time PCR Systems

Protocol

Introduction

Protein thermal shift assays enable quick and easy buffer optimization for increased protein stability. Assays can be performed with higher throughput and more buffer system options than traditional circular dichroism detection. The Bio-Rad family of CFX Real-Time PCR Systems uses a simple protocol, described here, to measure protein thermal stability using SYPRO Orange Protein Gel Stain. SYPRO Orange Stain binds to hydrophobic regions in denatured proteins, illuminating the presence of unstable proteins. In the following example, we adjusted pH and buffer salt concentration in order to optimize a Tris buffer used with β-galactosidase (from *Aspergillus oryzae*) and validated using thermal shift assays.

Materials

Sample Preparation

The following preparation applies to our proof-of-concept case. For your own experiments, use the dyes and buffers that are most appropriate.

- SYPRO Orange Protein Gel Stain (final reaction 5x)
- 50 mM Tris buffers (we made 12 within a pH range of 3.5–9, 50 mM NaCl)
- Multiplate 96-Well PCR Plates; low profile, unskirted, clear (catalog #MLL9601)
- Hard-Shell™ 96-Well PCR Plates; low profile, thin wall, skirted, white shell, clear wells (#HSP9601)
- Microseal™ 'B' PCR Plate Sealing Film; adhesive, optical (#MSB1001)
- Protein resuspended in deionized (DI) water (we prepared β-galactosidase to a final concentration of 1 mg/ml)

Equipment

- CFX96 Touch Real-Time PCR System (#1855195)
- CFX Opus 96 Real-Time PCR System (#12011319)

Procedure

Note: Each experimental condition was prepared in triplicate, per best practices. Sample data are also shown.

1. Protein Preparation

1.1 Resuspend protein of choice in DI water to an appropriate concentration.

1.2 Make a 50x stock solution of SYPRO Orange Protein Gel Stain by diluting 2.5 µl of 5,000x concentrate into 250 µl of DI water.

1.3 Aliquot 10 µl of protein solution into each well of a 96-well plate (this gave us a final protein mass of 10 µg of β-galactosidase).

1.4 Aliquot 2.5 µl of 50x SYPRO stock solution into each well of the same 96-well plate.

1.5 Aliquot 12.5 µl of each pH of buffer solution to the appropriate replicate wells of the 96-well plate for a final SYPRO concentration of 5x in each well.

1.6 Repeat steps 1.1–1.5, but this time create a range of buffer and salt concentrations (50, 100, and 200 mM NaCl) for each of the different pH buffers.

Note: Protein thermal shift assays run on the CFX 96 Touch System used Multiplate 96-Well PCR Plates (#MLL9601) and assays run on the CFX Opus 96 System used Hard-Shell 96-Well PCR Plates (#HSP9601).

2. Melt Curve Analysis

Using the established thermal shift assay protocol (see section 3), β-galactosidase was first melted with increased pH and then with increases to both pH and buffer salt concentration using both the CFX96 Touch and CFX Opus 96 Systems to generate protein melt curves.

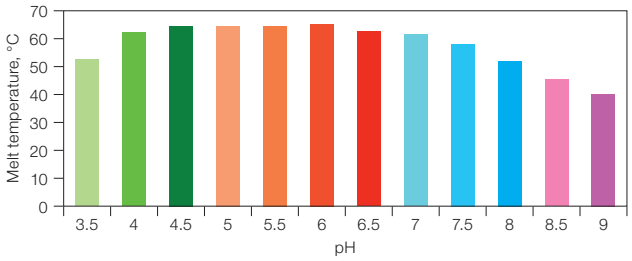
2.1 Melt curves were constructed for pH values between 3.5 and 9.0, in 0.5 intervals. Protein stabilization was observed between pH 4.5 and 6.0, with peak stability observed at pH 6.0 (67°C melt temperature) (Figure 1).

2.2 Exposing β-galactosidase to basic buffers past pH 6.0 caused destabilization of the protein and consequently lowered its melting temperature (Figure 1).

2.3 Varying salt concentration over a similar pH range showed a similar trend in the melt curve shift and was also seen with only pH change (Figure 2).

CFX96 Touch System

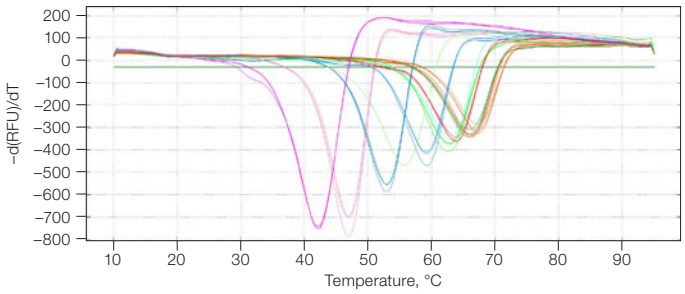
Melt Temperature vs. pH Change



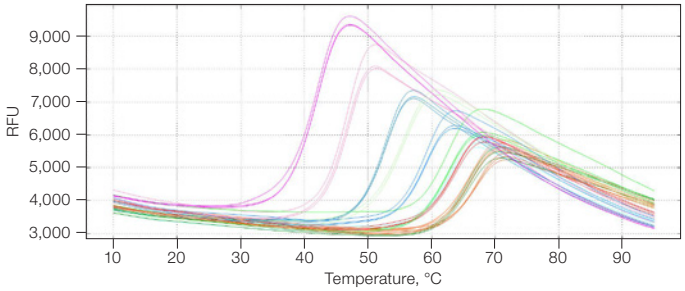
Sample	Average Melt Temperature, °C
pH 3.5	54.0
pH 4	63.0
pH 4.5	66.0
pH 5	66.0
pH 5.5	66.0
pH 6	67.0
pH 6.5	64.0

Sample	Average Melt Temperature, °C
pH 7	63.0
pH 7.5	60.0
pH 8	53.0
pH 8.5	47.0
pH 9	42.0

Melt Peak

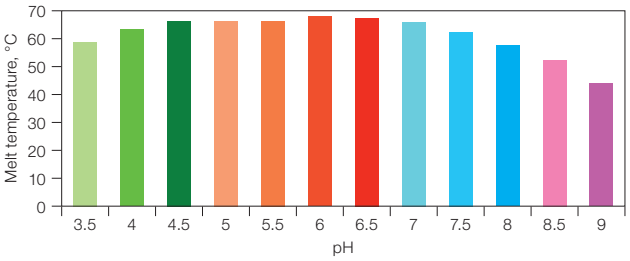


Melt Curve



CFX Opus 96 System

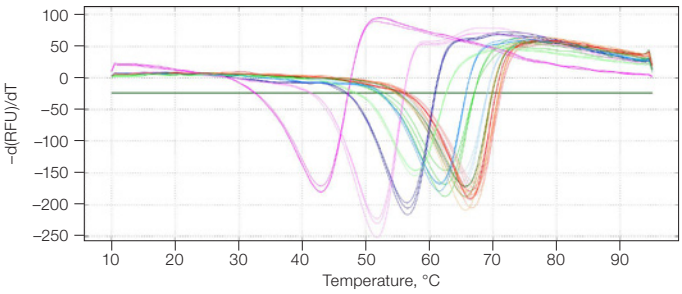
Melt Temperature vs. pH Change



Sample	Average Melt Temperature, °C
pH 3.5	57.8
pH 4	62.5
pH 4.5	65.5
pH 5	65.5
pH 5.5	66.5
pH 6	67.0
pH 6.5	66.5

Sample	Average Melt Temperature, °C
pH 7	65.0
pH 7.5	61.5
pH 8	56.5
pH 8.5	51.5
pH 9	43.0

Melt Peak



Melt Curve

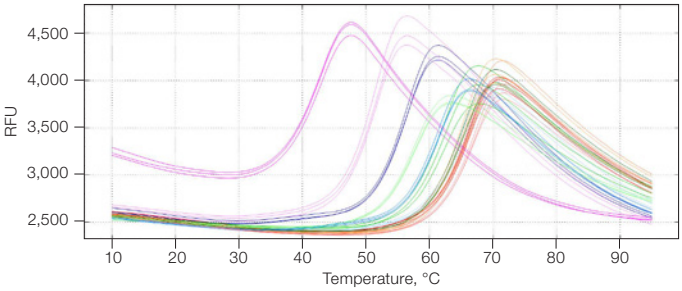
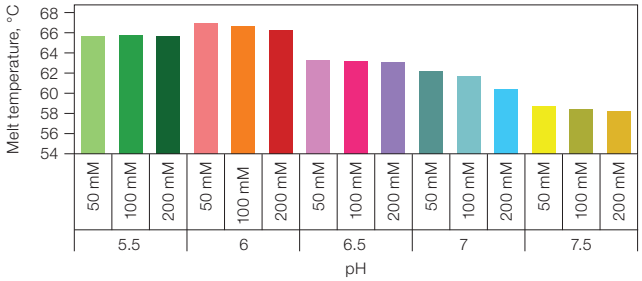


Fig. 1. Melt curve analyses over a range of pH levels. RFU, relative fluorescence units.

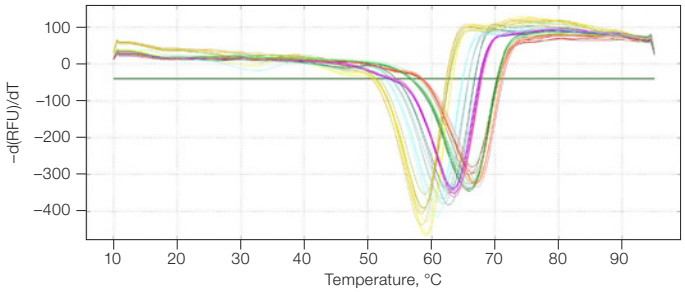
CFX96 Touch System

Melt Temperature vs. pH and Salt Concentration Changes

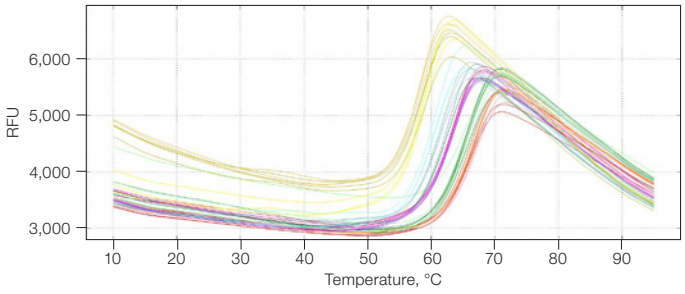


	Sample	Average Melt Temperature, °C
pH 5.5	50 mM	66.0
	100 mM	66.0
	200 mM	66.0
pH 6	50 mM	67.2
	100 mM	67.0
	200 mM	66.5
pH 6.5	50 mM	63.7
	100 mM	63.5
	200 mM	63.5
pH 7	50 mM	62.5
	100 mM	62.0
	200 mM	60.7
pH 7.5	50 mM	59.0
	100 mM	58.8
	200 mM	58.5

Melt Peak

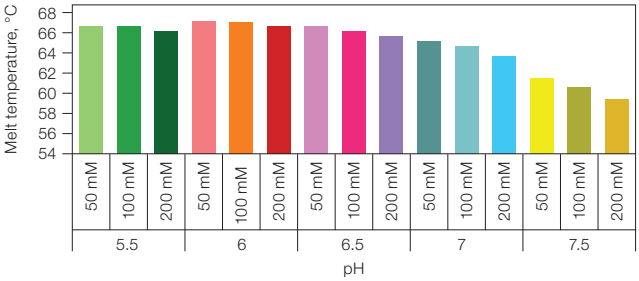


Melt Curve



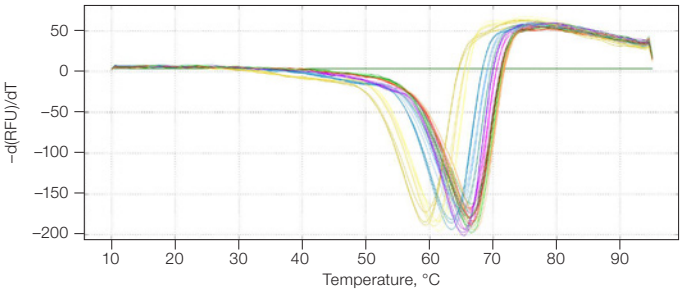
CFX Opus 96 System

Melt Temperature vs. pH and Salt Concentration Changes



	Sample	Average Melt Temperature, °C
pH 5.5	50 mM	66.5
	100 mM	66.5
	200 mM	66.0
pH 6	50 mM	67.0
	100 mM	66.8
	200 mM	66.5
pH 6.5	50 mM	66.5
	100 mM	66.0
	200 mM	65.5
pH 7	50 mM	65.0
	100 mM	64.5
	200 mM	63.5
pH 7.5	50 mM	61.5
	100 mM	60.5
	200 mM	59.3

Melt Peak



Melt Curve

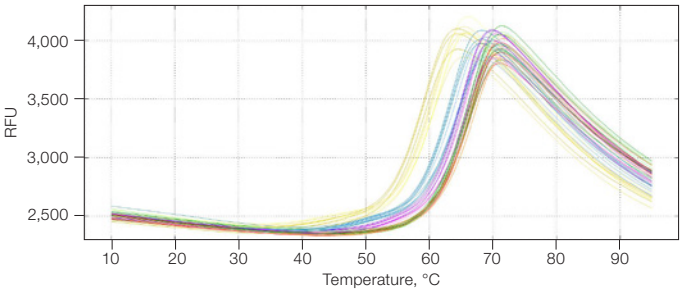


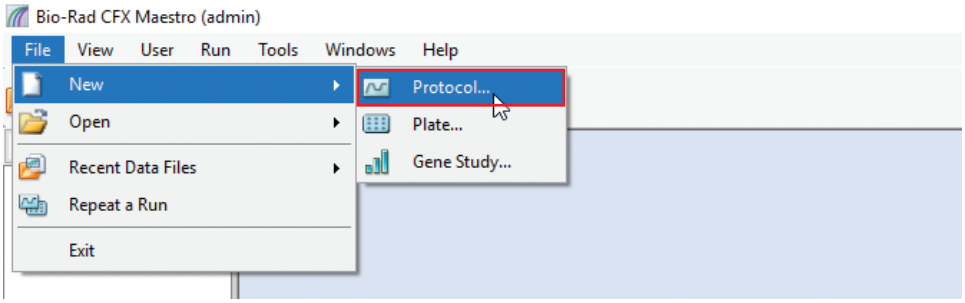
Fig. 2. Melt temperature analyses for increasing salt concentration over a range of pH levels. RFU, relative fluorescence units.

CFX Maestro Software Procedure

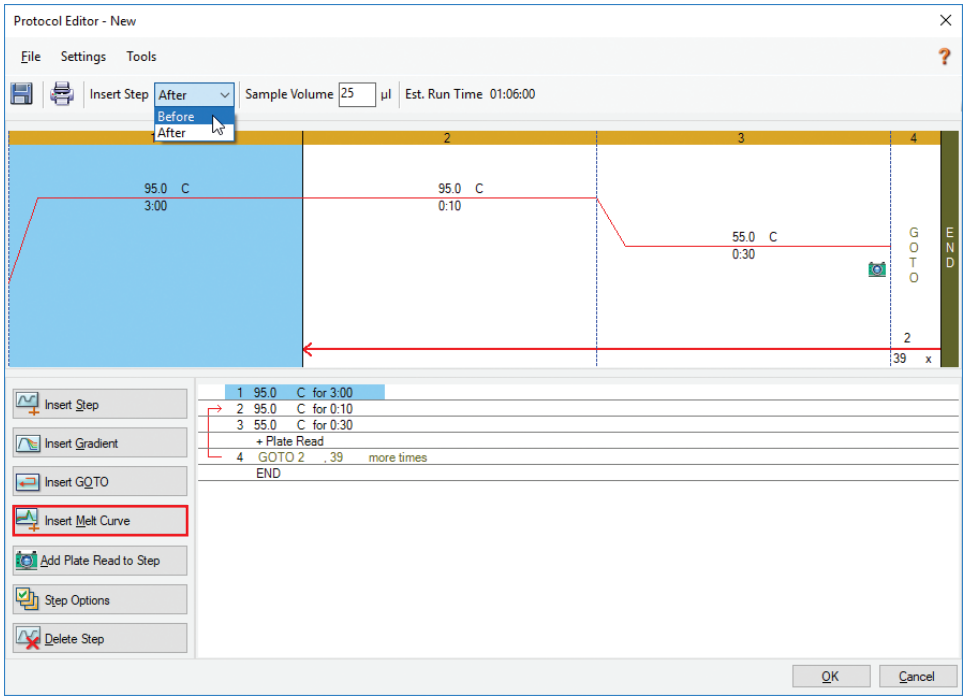
Note: A walkthrough of how to use your CFX Maestro Software to analyze melt temperatures is provided in sections 3 and 4.

3. Protocol Setup

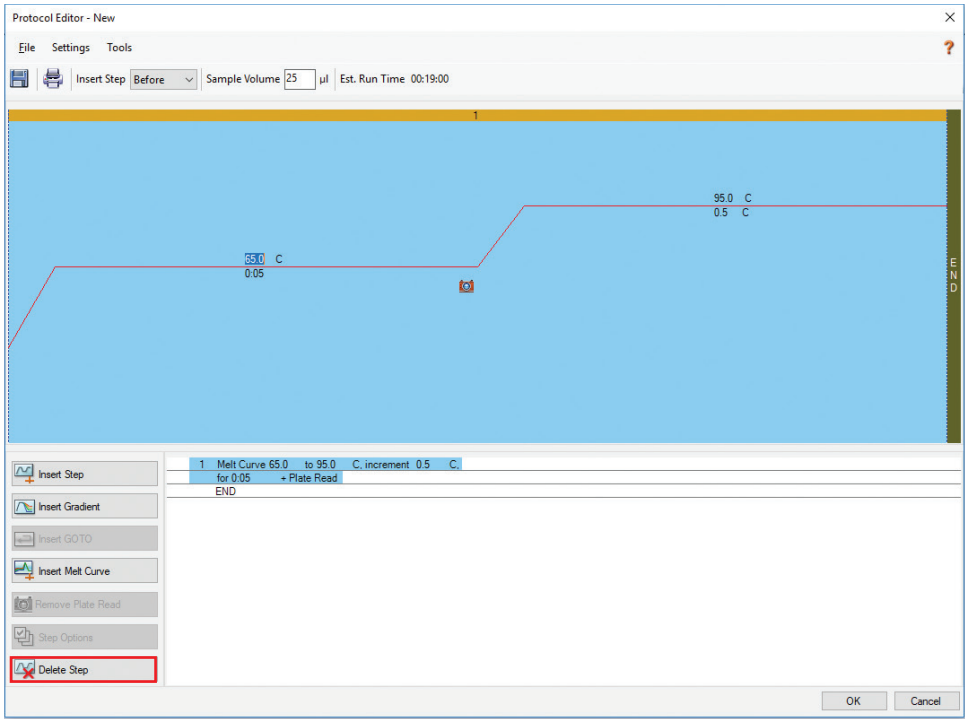
3.1 Open CFX Maestro Software and go to File > New > **Protocol…**



3.2 In Protocol Editor, select **Before** in the Insert Step dropdown menu and select **Insert Melt Curve**.



3.3 Select **Delete Step** to remove steps 2–4, leaving only melt curve step 1.



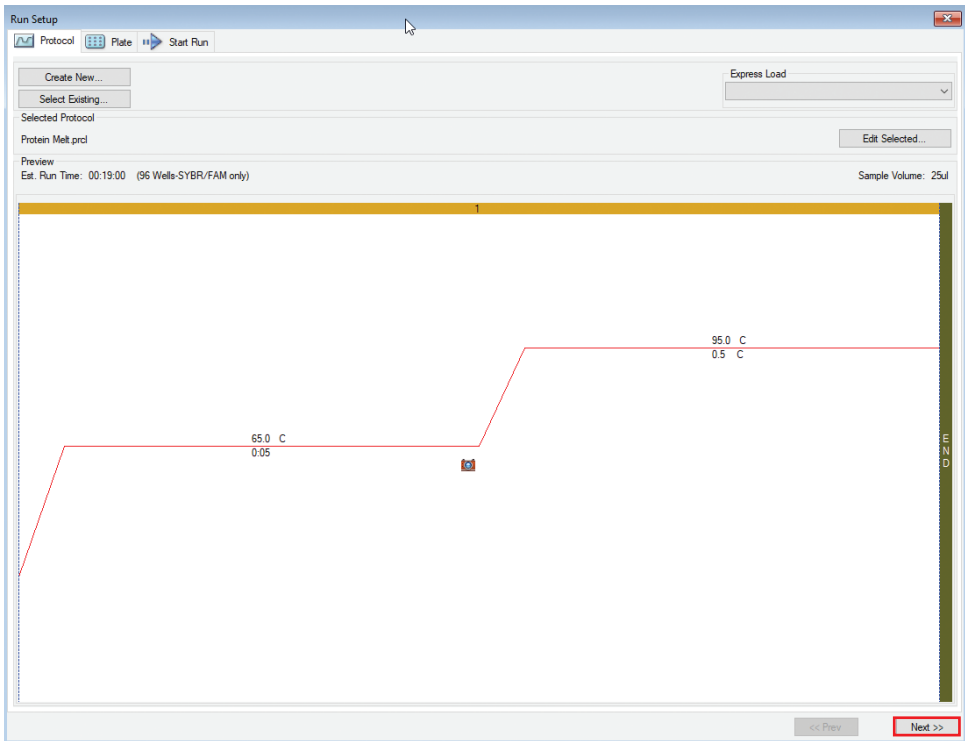
3.4 Change Melt Curve range to 10.0°C to 95.0°C in increments of 0.5°C for 10 seconds + Plate Read.

3.5 Set Sample Volume to 25 µl.

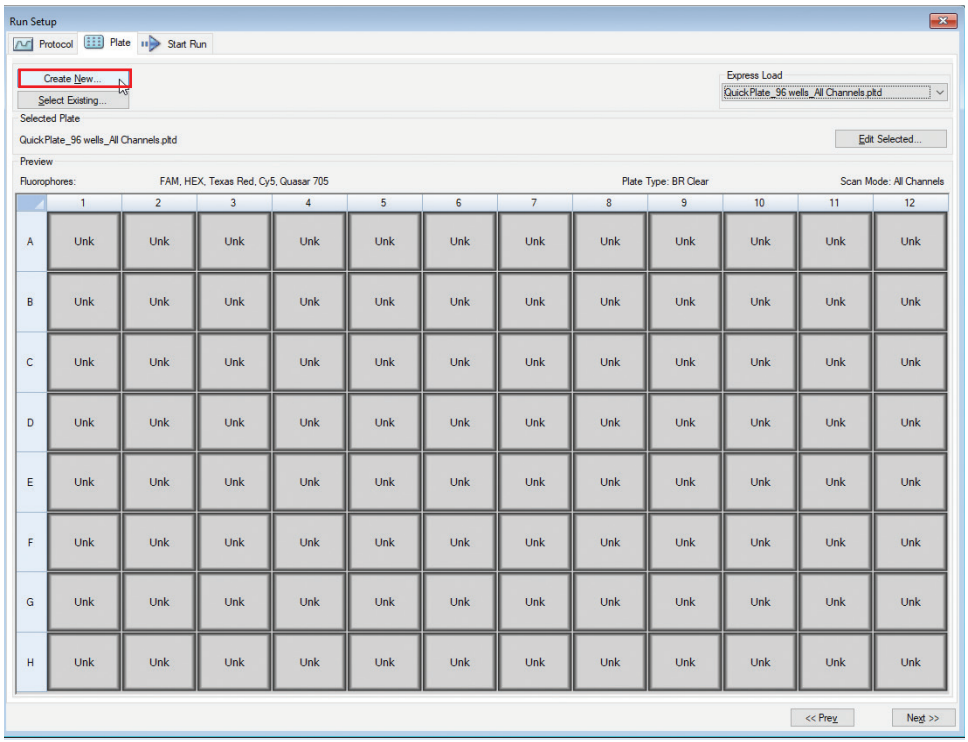
3.6 Click **OK** to save protocol.

4. Plate Editing

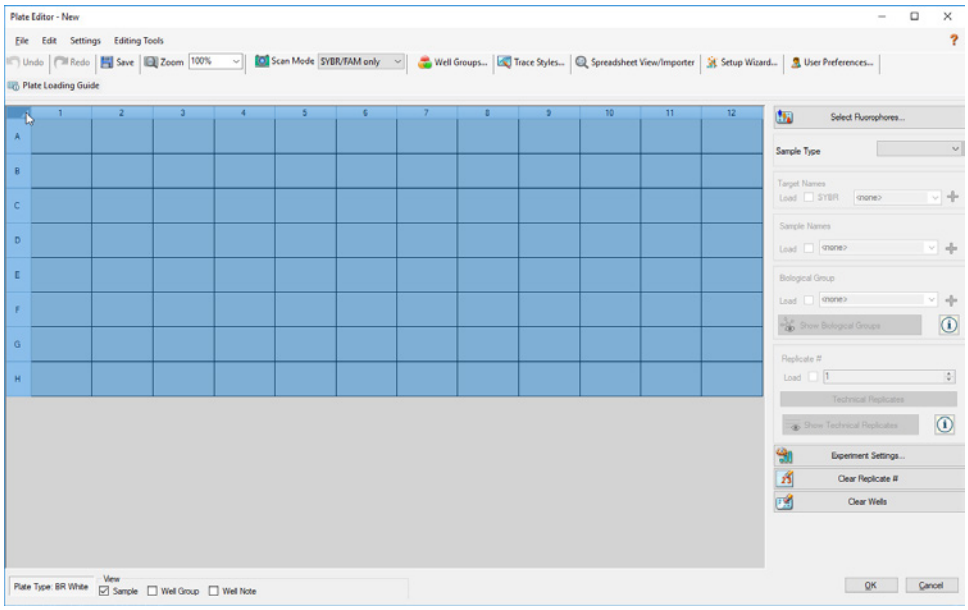
4.1 Select **Next >>** to move on to plate editing.



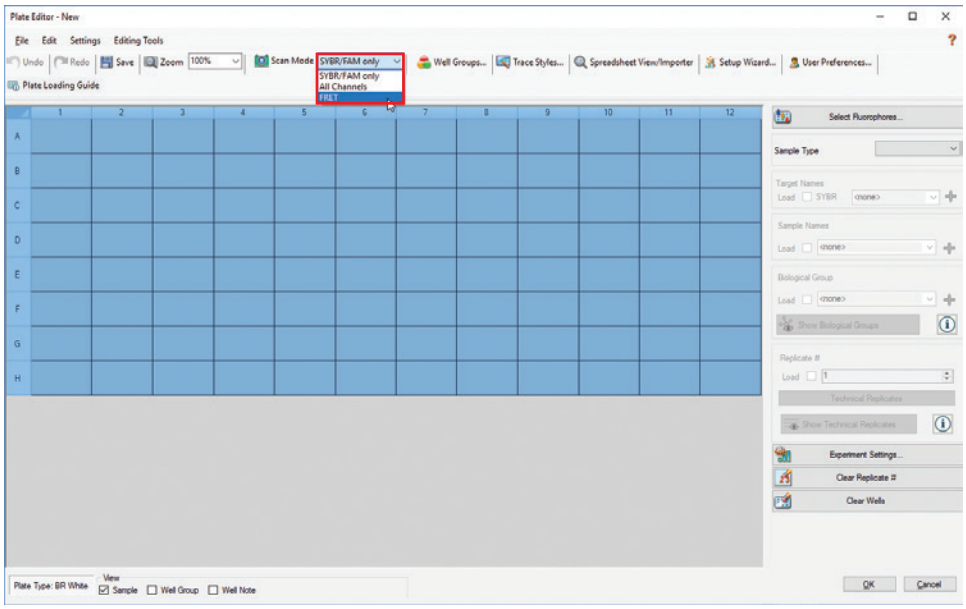
4.2 Select **Create New...** to begin editing the plate template.



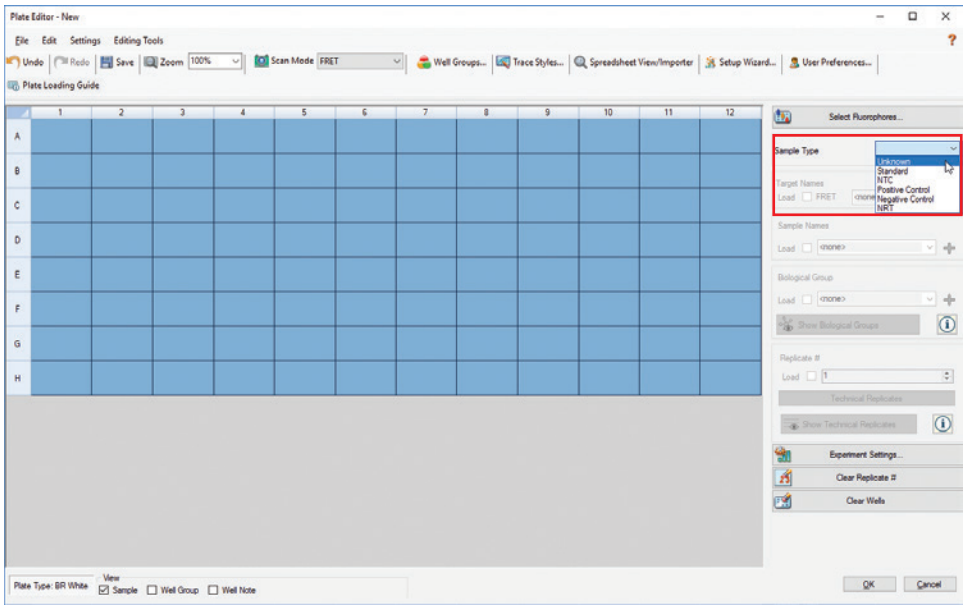
4.3 A Plate Editor window will open. Left click and drag to select wells. Alternatively, using your mouse, left click on the first desired well, press and hold down the Shift key, and left click on the last well to select a range of wells. Control select also works to highlight all the wells that you want the reader to analyze.



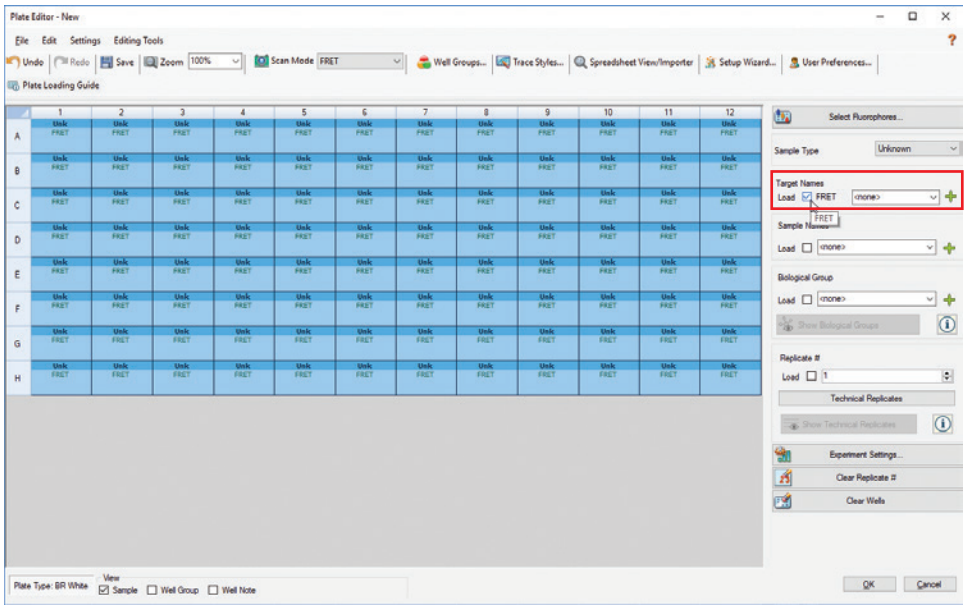
4.4 Go to the Scan Mode dropdown menu and choose the **FRET** channel.



4.5 In the Sample Type dropdown menu, choose from **Unknown**, **Standard**, **NTC**, **Positive Control**, **Negative Control**, or **NRT**.

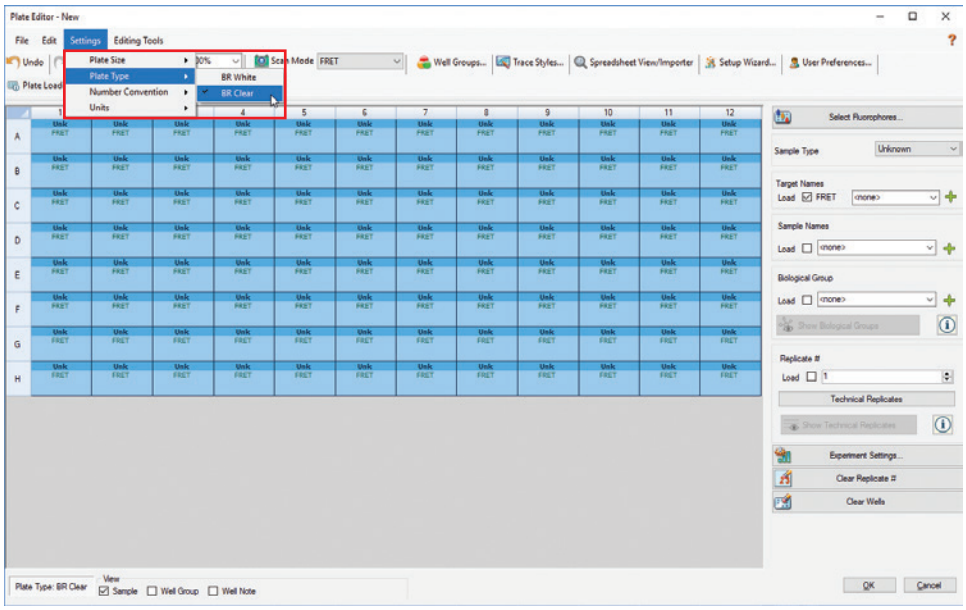


4.6 Make sure wells are still highlighted and check the **Load** box next to FRET under Target Names.



4.7 You can also add Sample Names and designate Replicate #s in this window.

4.8 Before clicking **OK**, ensure that you have chosen a clear plate by clicking Settings > Plate Type > **BR Clear**.



4.9 Click **OK** when you're done editing the plate template.

4.10 Click **Next** to get to the Start Run tab. Here you can add a description of the experiment and check the CFX System to use.

Summary

Instruments in the Bio-Rad family of CFX Real-Time PCR Systems accurately measure thermal shift, making them effective tools for buffer screening to find the ideal conditions that will maximize protein stability. All CFX Systems are compatible with CFX Maestro Software, which provides a straightforward interface for creating optimization protocols. For our examples, we were able to easily observe changes in the stability of our chosen protein while adjusting buffer conditions. The experimental setup and analytical approach we used can serve as an adaptable template for others. Multiplate 96-Well (#MLL9601; data not shown) and Hard-Shell 96-Well (#HSP9601) PCR Plates were tested for use with the protein thermal shift assays for CFX Opus 96 Systems. Both plate types showed comparable results and either can be used.

Visit **bio-rad.com/CFXOpus** for more information.

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