MyiQ™ Single-Color Real-Time PCR Detection System

Instruction Manual

Catalog Number
170-9740

BIO-RAD

Safety Information

Important: Read this information carefully before using the MyiQ Real-Time PCR Detection System.

Grounding

Always connect the MyiQ Optical Module Power Supply to a 3-prong, grounded AC outlet using the AC power cord and external power supply provided with the MyiQ Real-Time PCR Detection System. Do not use an adapter to a two-terminal outlet.

Handling

Handle the MyiQ Optical Module with care when mounting or removing it. The optical system makes use of mirrors and lenses that may shatter if the unit is struck or dropped. If the unit is damaged, such that any internal wires or components are exposed, do not attempt to turn on the instrument. Contact Bio-Rad and return the instrument for repair if necessary.

Servicing

The only user-serviceable parts of the MyiQ are the lamp and filters. When replacing the lamp or filters, open ONLY the outer casing of the MyiQ Optical Module for lamp and filter replacement. Call your local Bio-Rad office for all other service.

Power Switch

The external power supply must be placed so that there is free access to its power switch.

Temperature

For normal operation the maximum ambient temperature should not exceed 28°C (see Appendix A for specifications).

There must be at least 4 inches clearance around the sides of the iCycler to adequately cool the system. Do not block the fan vents near the lamp, as this may lead to improper operation or cause physical damage to the MyiQ Detector.

Do not operate the MyiQ Optical Module in extreme humidity (>80%) or where condensation can cause a short of internal electrical circuits or fog optical elements.

The camera lamp may get extremely hot during operation. To prevent skin burns and fire hazards, do **not** operate with the cover open. See Section 9.2 of this manual for instructions on replacing the lamp.

Notice

This Bio-Rad instrument is designed and certified to meet EN-61010 safety standards.

EN-61010 certified products are safe to use when operated in accordance with the instruction manual. This instrument should not be modified in any way. Alteration of this instrument will:

- Void the manufacturer's warranty.
- Void the EN-61010 safety certification.
- Create a potential safety hazard.

Bio-Rad is not responsible for any injury or damage caused by the use of this instrument for purposes other than those for which it is intended, or by modifications of the instrument not performed by Bio-Rad or an authorized agent.

The MyiQ and iCycler™ are intended for laboratory research applications only.

This real-time thermal cycler is licensed under U.S. Patent No. 6,814,934 and corresponding claims in any counterpart Canadian patent thereof owned by Applera Corporation, solely in research and all other applied fields except human or veterinary in vitro diagnostics. No rights are conveyed expressly, by implication or estoppel to any patents on real-time methods, including but not limited to 5' nuclease assays, or to any patent claiming a reagent or kit.

Table of Contents

Section 1
Introduction

The Polymerase Chain Reaction (PCR) has been one of the most important developments in molecular biology. PCR has greatly accelerated the rate of genetic discovery, making critical techniques relatively easy and reproducible. Furthermore, the availability of technology for kinetic, real-time measurements of a PCR in progress greatly expands the benefits of this technique. Real-Time analysis of PCR enables truly quantitative analysis of template concentration, reduces contamination opportunities, and speeds time to results, because traditional post PCR steps are no longer necessary. A wide range of fluorescent chemistries may be employed to monitor the PCR reaction.

The iCycler thermal cycler provides optimum performance for PCR and other thermal cycling techniques. Incorporating a Peltier-driven heating and cooling design, the iCycler delivers rapid heating and cooling performance. Rigorous testing of thermal block temperature accuracy, uniformity, consistency and heating/cooling rates ensures reliable and reproducible results.

The MyiQ Optical Module, builds upon the strengths of the iCycler thermal cycler, and provides an easy and efficient means for single-color experimentation. The CCD detector allows for simultaneous imaging of all 96 wells. This results in a comprehensive data set illustrating the behavior of the reactions during each cycle. Simultaneous image collection ensures that well-to-well data may be compared reliably. The MyiQ system reports data on the PCR in progress in real-time, allowing immediate feedback on reaction success. All of these features found in the MyiQ system hardware were built to promote reliability and flexibility.

The MyiQ Optical System Software includes the features that make software easy and useful. The software is designed for convenience – offering fast setup and analytical results. The functions are presented graphically to minimize navigating through menus. Tips on usage are available as your mouse glides over the buttons. The MyiQ software automatically analyzes the collected data at the touch of a button, yet also allows for significant optimization of results based on your analysis preferences.

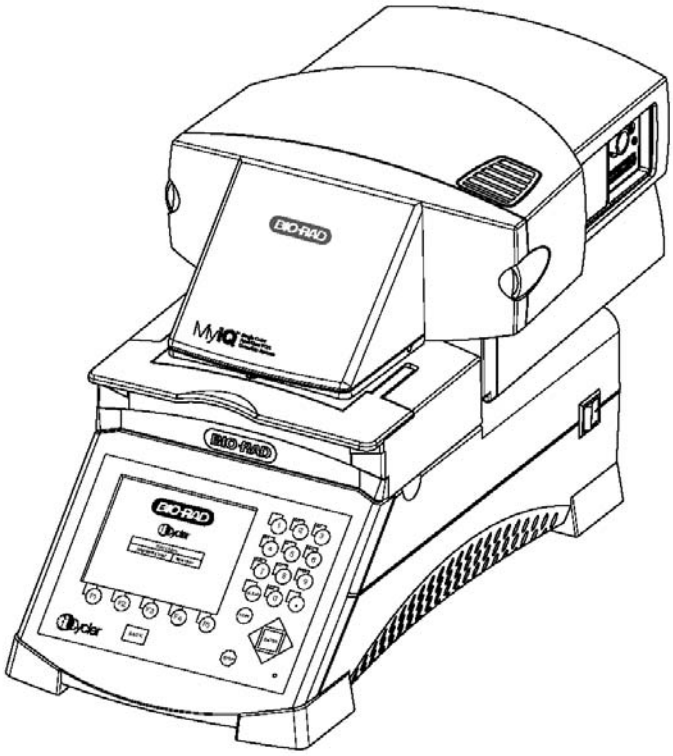


Fig. 1.1. The MyiQ Optical Module Upgrade for the iCycler Thermal Cycler.

1.1 MyiQ System Description

The optical module houses the excitation system and the detection system. The excitation system consists of a fan-cooled, 50-watt tungsten halogen lamp, a heat filter (infrared absorbing glass), an optical filter for excitation that may be shuttered closed, and a dual-mirror arrangement that allows for the simultaneous illumination of the entire sample plate. The excitation system is physically located on the right front corner of the optical module, with the lamp shining from right to left, perpendicular to the instrument axis. Light originates at the lamp, passes through the heat filter and the excitation filter (if open), and is then reflected onto the 96-well plate in the thermal cycler by a set of mirrors. This light source excites the fluorescent molecules in the wells.

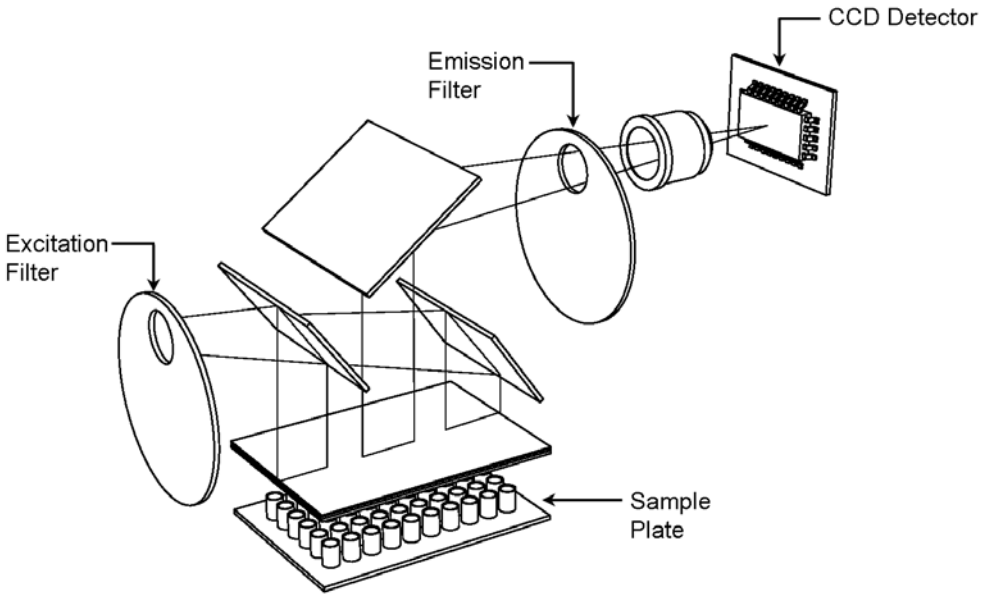


Fig. 1.2. Representation of Optical Detection System layout.

The detection system occupies the rear two-thirds of the optical module housing. The primary detection components include a single optical filter for emission and a CCD detector. The 350,000 pixel CCD allows discrete quantitation of the fluorescence in each well. Fluorescent light from the wells passes through the emission filter and is then detected by the CCD.

Note: Suggested computer specifications for running the system software are given in Appendix B.

Avoid getting any dust or fingerprints on the mirror within the underside of the unit.

WARNING: Do not attempt to clean any of the mirrors or lenses found inside the MyiQ Optical Module, as they are extremely sensitive and fragile. Following the recommended guidelines for operation, the camera optics should not require cleaning. Contact Bio-Rad Technical Support if you suspect a problem with the optical system. Do not attempt to disassemble or clean the optical system yourself.

1.1.1 MyiQ System Cable Connections

There are four cable connections that need to be made when setting up the MyiQ (see Figure 1.3):

- Round 9-pin power connector: This provides power to the optical module via the optical system power supply.

WARNING: **To avoid shorting the camera circuitry, always turn the the power supply switch to the OFF position (the "0" should be down) before making this connection. Also, to avoid the risk of an electric shock, make sure your hands are dry.**

- Parallel-port connector: This uses a cable that is 25 pin male-to-male and connects to the computer. The computer requires an IEEE 1284 compatible, 8-bit bi-directional, or EPP type, parallel port. Data are transferred to the computer via this cable.

- Miniature phone plug connector: This connector senses when the reaction module is opened, and triggers the closing of the camera's excitation shutter, protecting the CCD detector from excess light. The miniature phone plug, which is attached to the power cord, connects to the port found just behind the power connector, on the right side of the optical module. Additionally, a second internal connection must be made inside the casing of the camera. To access this internal connection, gently unlatch and slide the camera casing back. Now connect the internal phone plug, which is found on the lower right side of the camera, to the port found at the right, rear corner of the reaction module lid.
- Serial connector: The MyiQ software directs the operation of the iCycler thermal cycler via this cable connector. It is located at the left, rear corner of the iCycler thermal cycler. Connect the other end to the computer.

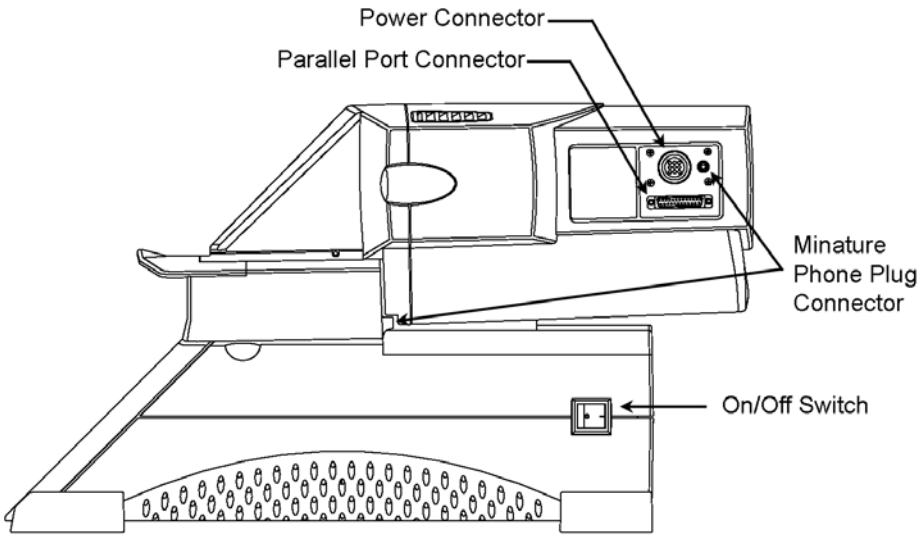


Fig. 1.3. Side View of MyiQ Real-Time Detection System showing cable connections.

Section 2
The MyiQ Real Time PCR Detection System for Single-Color Experimentation

2.1 Introduction

The MyiQ Detector can simultaneously collect light in all 96 wells. Fluorescent light from each monitored well is measured at each data collection step, and the software then processes these data to create amplification plots, point by point in real time. After an experiment has run to completion, the software offers additional tools for analyzing your experiment, such as analyzing replicates, calculating a standard curve and determining unknown concentrations.

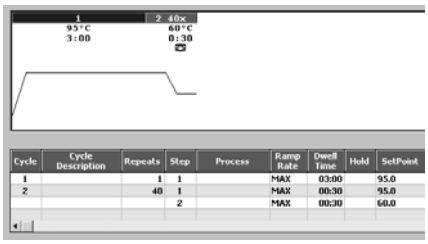
An experiment on the MyiQ system is defined by a **Protocol** file and a **Plate Setup** file. These files have the extensions .tmo and .psm, respectively. The output file of fluorescent data, to which the amplification, melting curve, and standard curve data for an experiment are saved, is called an **Optical Data** file, and has the extension .odm.

In addition, every experiment on the MyiQ system requires **well factor** data in order to account for any well-to-well variation generated by either the user or the system. Although well factor data may be generated prior to every run, it isn't always necessary to do so. Well factor data may be generated, saved, and applied to subsequent experiments of the same volume and media type. This concept is introduced and presented in detail in Section 5.4.2. Understanding well factor collection will make it possible to rapidly optimize experimental protocol development and to collect the best possible optical data.

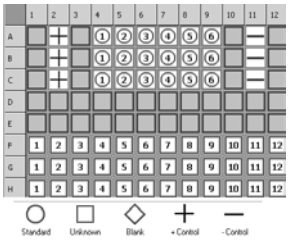
2.2 MyiQ Software Quick Guide

2.2.1 Overview

1. Turn on the MyiQ Optical module, iCycler base and MyiQ Software. Warm up MyiQ Optical module for a minimum of 30 minutes before collecting data.
2. Select or Create a Protocol



3. Select or Create a Plate Setup



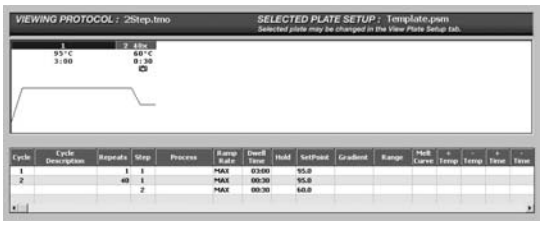
4. Run Protocol with Selected Plate Setup

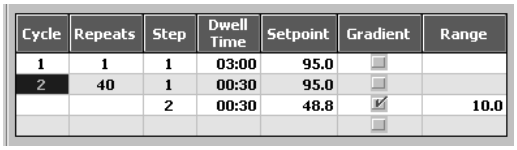
Use Well Factors from experimental plate if using:
- Bio-Rad iQ SYBR Green Supermix
- SYBR Green spiked with 10 nM Fluorescein
- Probes at same concentration in all wells

Use Persistent Well Factors if using:
- SYBR Green without fluorescein spike
- Probes at different concentrations
- End Point Only Analysis Run
- Probes of different fluorescent intensity

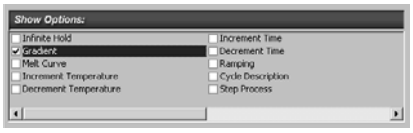
Note: Reaction Vessel Setup
Prepare the experimental PCR reactions in a 96-well Thin Wall plate (Catalog #223-9441). Place a sheet of Optical Quality sealing tape (Catalog #223-9444) on the top of the 96-well plate. Use the tape applicator (flat plastic wedge) to smooth the tape surface and tightly seal the tape to the plate. Avoid touching the surface of the sealing tape with gloved fingers. Tear off the white strips that remain on the sides of the tape. If individual sample tubes or strips of tubes are to be used, you must seal the tubes with the appropriate caps. Note that a minimum of 8 sample

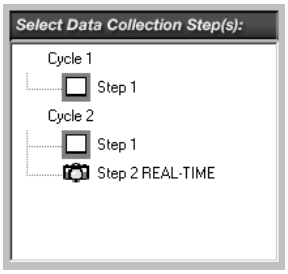
tubes is required to prevent tube crushing when using the green anticondensation ring. If the ring is not present, a minimum of 14 sample tubes must be present.





Cycle	Repeats	Step	Dwell Time	Setpoint	Gradient	Range
1	1	1	03:00	95.0	☐	
2	40	1	00:30	95.0	☐	
		2	00:30	48.8	☑	10.0
					☐	

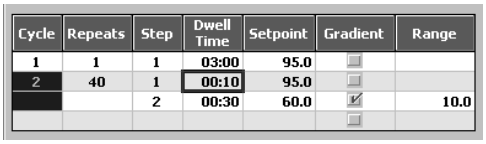




2.2.2 Select or Create a Protocol

View Protocol

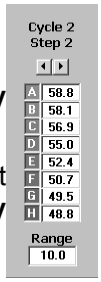
1. Click on the **View Protocol** tab in the **Library** module to view saved protocols.
2. Click on desired protocol under **Protocol Files** to display the selected protocol.
3. Click **Run with Selected Plate Setup** to use this protocol and plate setup for an experimental run.

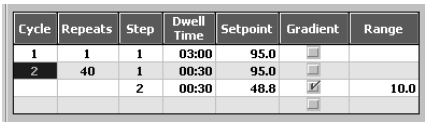


Cycle	Repeats	Step	Dwell Time	Setpoint	Gradient	Range
1	1	1	03:00	95.0		
2	40	1	00:10	95.0		
		2	00:30	60.0	☑	10.0

Editing and Creating a Protocol

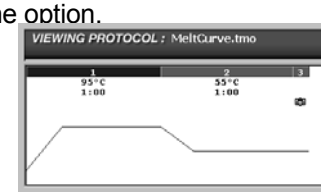
1. Begin editing and creating a protocol in the **Workshop** module in any one of the following ways:
   - Click **Edit This Protocol** in the **View Protocol** tab in the **Library** module
   - Click **Create a New Protocol** in t **View Protocol** tab in the **Library** module
   - Click on the **Workshop** module, then select the **Edit Protocol** tab





Cycle	Repeats	Step	Dwell Time	Setpoint	Gradient	Range
1	1	1	03:00	95.0		
2	40	1	00:30	95.0		
		2	00:30	48.8	☑	10.0

2. Click on **Insert Cycle**, **Delete Cycle**, **Insert Step**, or **Delete Step** to edit the default or selected protocol.
   - This option stays active until you deselect the option.
3. Click in the spreadsheet on the **Cycle** or **Step** cell, then enter values for **Dwell Time** and **Setpoint** temperature.



4. Select protocol options in the **Show Options** box to display in the protocol spreadsheet, such as **Gradient** or **Melt Curve**.

Cycle	Repeats	Step	Dwell Time	Setpoint	Melt Curve	+ Temp	- Temp
1	1	1	01:00	95.0			
2	1	1	01:00	55.0			
3	80	1	00:10	55.0	☑	0.5	

5. Select data collection step(s) by clicking twice on the step designated for **REAL-TIME** data collection.

6. Enter a name in the **Protocol Filename** box and click **Save this Protocol**.
7. Click **Run With Selected Plate Setup** to run this protocol and selected plate setup.

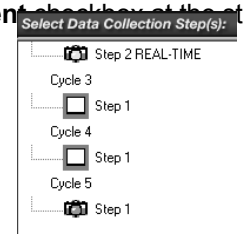
Cycle	Repeats	Step	Dwell Time	Setpoint	Melt Curve	+ Temp	- Temp
1	1	1	03:00	95.0			
2	40	1	00:10	95.0			
		2	00:45	55.0			
3	1	1	01:00	95.0			
4	1	1	01:00	55.0			
5	80	1	00:10	55.0	☑	0.5	

Creating a Gradient Protocol

1. Check the box for **Gradient** in the **Show Options** box. This will expand the protocol spreadsheet to include

   gradient columns.

2. In the protocol spreadsheet, click the **Gradient** checkbox at the step you want to begin the temperature gradient.



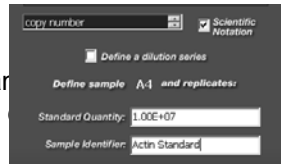
3. In the protocol spreadsheet, type in the **Setpoint** temperature (lowest temperature) and the temperature **Range** for the thermal gradient. The default range is 10°C.
4. Alternatively, you may type in a temperature in a selected row in the gradient display box and a temperature **Range**, and the **Setpoint** temperature will be updated in the spreadsheet.
5. Enter a name in the **Protocol Filename** box and click **Save This Protocol**.

Creating a Melt-Curve Protocol

1. Click **View Protocol** in the **Library** module to view saved protocols.
2. Under **Protocol Files**, select one of the following:
   - MeltCurve.tmo for a melt-curve protocol only

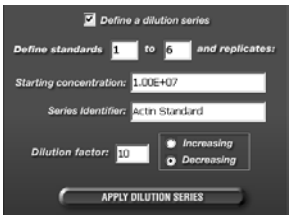
   - **2StepAmp+Melt.tmo** for an amplification and melt-curve protocol

3. To edit the protocol, click **Edit this Protocol** to get to the Workshop module.



4. Click on the appropriate cell to change values for any parar **Dwell Time** and **Setpoint** temperature.You may do this to annealing temperature or melt-curve starting temperature.

5. Data collection for the melt-curve step is automatically indicated with a green camera.



6. Enter a name in the **Protocol Filename** box and click **Save this Protocol**.

2.2.3 Select or Create a Plate Setup

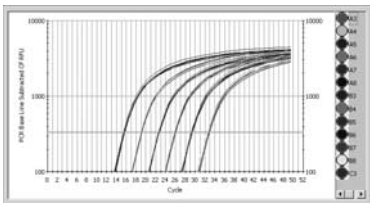
Viewing Plate Setup

1. Click on the **View Plate Setup** tab in the **Library** module to view saved plate setup files.
2. Click on the desired plate setup under **Plate Setup Files**.
3. Click the **View Quantities/Identifiers** tab to view sample types, quantities, and identifiers (sample names) for wells.
4. Click **View Plate Setup** tab, then click **Run with Selected Protocol** to initiate a run with the selected plate setup and indicated protocol.

Editing and Creating a Plate Setup

1. Begin editing and creating a plate setup in the **Workshop** module in one of the following ways:
   - Click **Edit this Plate Setup** in the **View Plate Setup** tab in the **Library** module
   - Click **Create a New Plate Setup** in the **View Plate Setup** tab in the **Library** module
   - Click the **Workshop** module button and on the **Edit Plate Setup** tab

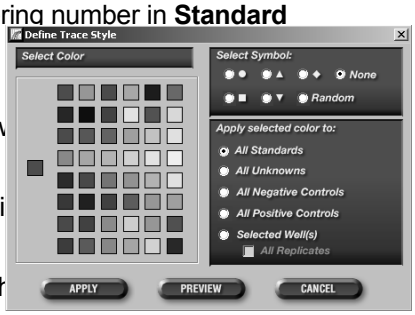
2. Select fluorophore from the **Fluorophore** Selection box



3. Click on an icon for the sample type.
4. Select wells for sample and replicates.

5. You may type the name of the sample in the **Sample Identifier** box and press Enter to update information.
6. To define standards, click on the **Standard** icon
   - Use the cursor to select wells on the plate for each standard and its replicates
   - Select units
   - Check the **Define a dilution series** box, and enter **Starting concentration** and **Series Identifier**

   - Enter a number for **Dilution factor** and select **Increasing** or **Decreasing**
   - Click on **Apply Dilution Series**
   - Alternatively, define standards individually by entering number in **Standard Quantity** and name in **Sample Identifier** for each replicates

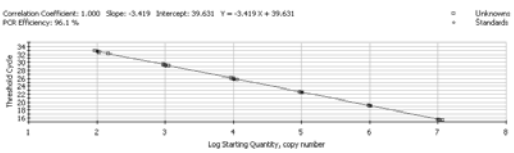


7. With the **Cursor** icon selected, click on any well to viev **Quantity**, and **Units** for that well.
8. Name the file in the **Plate Setup Filename** box and cli **Setup**.
9. Click **Run with Selected Protocol** to initiate a run with setup and indicated protocol.

2.2.4 Data Analysis – PCR Quantification

The **PCR Quantification** screen is displayed during PCR run and presents data as they are being collected in real time. This screen is also enabled post-run when you open your data file.

1. Select the **Library** module and click on the **View Post-Run Data** tab.
2. Open the desired file by double clicking on the file name.
3. The data file will open in the **PCR Quantification** tab of the **Data Analysis** module. The default view is PCR Base Line Subtracted Curve Fit.
4. Threshold cycle calculation is automatically calculated or can be user defined.
   - **Auto Calculated** — The software automatically defines baseline cycles and a threshold position
   - **User Defined** — When selected you may define the baseline cycles and threshold position. You may also click and drag the threshold bar directly on the amplification plot. click recalculate Threshold Cycles to update threshold cyucle values



5. Threshold Cycle (C_T) values and sample name identifiers are displayed in the spreadsheet to the right of the amplification plot.
6. Check the **Select Wells** box to select or deselect wells for analysis.

Graph Options

1. To Toggle between the normal and log views of the amplification plot click **Log View**
2. For other options right-click on the amplification plot to view the context menu.
3. Select **Define Trace Style** to customize the colors and symbols of the sample well traces.
    - Choose the type of trace to be modified (e.g., all standards) or click selected wells to modify on a well-by-well basis.
    - If you choose to apply the selected color to selected wells(s), the plate layout with colors is displayed so that you can click on the wells to change the well color.
    - Click **Preview** to see changes to the traces in the graph.
    - Click **Apply** to apply changes to the traces in the graph.
4. You may choose options to copy and print data and graphs from this menu.

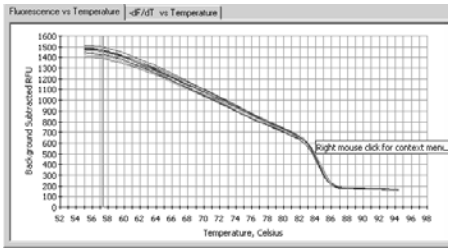
Reports in PCR Quantification

1. Click **Reports** to obtain customized reports of the PCR quantification data.
2. You may choose to sort the data by threshold cycle, well, sample identifier, or gradient setpoint.
3. Save or print reports containing information about the run.
4. Close **Report Viewer** to continue data analysis.

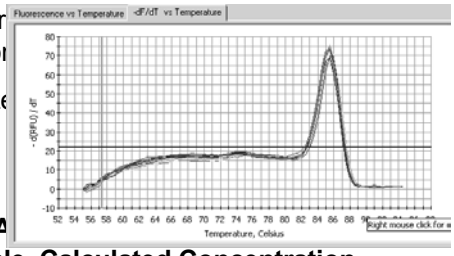
Standard Curve

When there ar.e standards present in the amplication run, the **PCR Standard Curve** tab is available.

1. Click on the **PCR Standard Curve** tab of the **Data Analysis** module to view the standard curve plot containing the correlation coefficient, the slop of the line, and the PCR efficiency.



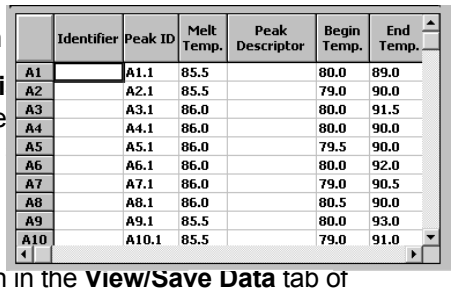
2. A data spreadsheet displays all information for quantities, threshold cycles, and calculated co



3. Click **Reports** on this page to obtain customize data or the PCR quantification plot.

Save Data

1. Click on the **View/Save Data** tab of the **Data A** plate view of results based on **Threshold Cycle**, **Calculated Concentration**, or **Standard Quantities**.



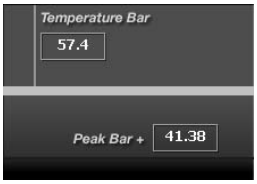
	Identifier	Peak ID	Melt Temp.	Peak Descriptor	Begin Temp.	End Temp.
A1		A1.1	85.5		80.0	89.0
A2		A2.1	85.5		79.0	90.0
A3		A3.1	86.0		80.0	91.5
A4		A4.1	86.0		80.0	90.0
A5		A5.1	86.0		79.5	90.0
A6		A6.1	86.0		80.0	92.0
A7		A7.1	86.0		79.0	90.5
A8		A8.1	86.0		80.5	90.0
A9		A9.1	85.5		80.0	93.0
A10		A10.1	85.5		79.0	91.0

2. Click **Reports** to obtain summarized results in

3. Click on **Save ODM File** to view **Data Analysi** and **Melt Peak Data**. Notes may also be writte Check the **Autosave to ODM** box to save the file is closed.

You may modify some attributes of a well post-run in the **View/Save Data** tab of the **Data Analysis** module.

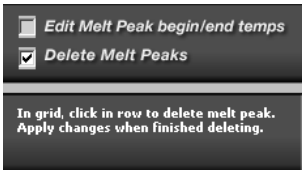
1. Click on a well to bring up information on that well and modify its contents. You may change the sample type, replicate number, starting concentration, and sample identifier.



2. Click **Apply Changes** to complete changes. To undo modifications to data file clilck **Restore Original Definitions**. Information on the modified wells is recorded in the reports.

2.2.5 Data Analysis – Melt Curve

The **Melt Curve** feature of the **Data Analysis** module is displayed during the melt-curve run and presents the data as they are being collected in real time. This screen is also enabled when you open a data file containing melt-curve data.



1. Click on the **View Post-Run Data** tab in the **Library** module.
2. Open the desired file by double clicking on the file name.
3. If the data file also contains PCR data, the file will open in the **PCR Quantification** tab of the **Data Analysis** module. Click on the **Melt Curve** tab.
4. Data are displayed as **Fluorescence vs Temperature** or **-dF/dT vs Temperature**.

- **Fluorescence vs Temperature** plots fluorescent data in real time as temperature increases or decreases

- **-dF/dT vs Temperature displays** the negative first derivative of the **Fluorescence vs Temperature** plot. The software identifies peaks and assigns melting temperatures from this plot.

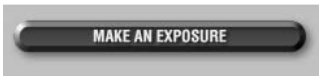
5. Peaks from the **-dF/dT vs Temperature** plot are identified and displayed in the **Peak Description** spreadsheet with **Identifier**, **Peak ID**, **Melt Temp.**, **Peak Descriptor**, **Begin Temp.**, and **End Temp.** for each sample.

6. The number of peaks displayed is dependent on the location of the blue peak bar. Only peaks above this bar are displayed in the spreadsheet.
7. Click and drag the blue peak bar to move it to a new location, for example, above the background noise. The exact positions of the temperature and peak bars are displayed in a box to the right of the plot.

8. Click **Apply Changes to Melt Peaks** to update the data spreadsheet. To undo, click **Undo All Melt Peak Changes**.

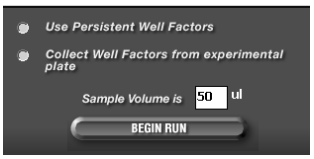
9. The delete feature may be used delete individual peaks. Check the **Delete Melt Peaks** box.

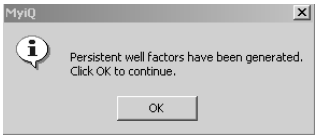
   - Click in the row to delete peaks. This may be done only for wells with more than one peak.
   - Click **Apply Changes to Melt Peaks** when you are finished deleting.


MAKE AN EXPOSURE


OPTIMIZE MASK POSITIONS
SAVE OPTIMIZED MASKS


COLLECT PERSISTENT WELL FACTORS


Use Persistent Well Factors
Collect Well Factors from experimental plate
Sample Volume is
50
ul
BEGIN RUN


MyiQ
Persistent well factors have been generated.
Click OK to continue.
OK

10. To edit the beginning and ending temperature of a peak, check the **Edit Melt Peak begin/end temps** box.
    - Click the **Begin Temp.** or **End Temp.** column in the data spreadsheet.
    - Click and drag the vertical orange temperature bar to the desired temperature on the plot.
    - Click **Apply Changes to Melt Peaks** to update **Begin Temp.**or **End Temp.** in the dataspreadsheet.
11. Click **Reports** to obtain customized reports for the melt-curve data.

2.2.6 Generating Persistent Well Factors

Persistent Well Factors Need to be Generated:

for Endpoint Runs
When well factors cannot be collected from the experimental plate eg SYBR Green I without fluorescein spike or fluorophore at different concentrations (see Section 2.3)
If using Persistent Well Factors and optical path (eg tape to caps, plate to tubes, reaction volumes) is changed.
If using Persistent Well Factors and the MyiQ optical module is moved

Requires

Bio-Rad iCycler iQ External Well Factor solution (Bio-Rad Catalog #170-8794)
96-well PCR plate (catalog #223-9441) or preferred reaction vessel – subsequently referred to as "plate"
Optical quality sealing tape (catalog #223-9444) of preferred sealing method
Reaction Vessels and Sealing Mechanism (eg plate and Optical tape) for all 96 wells
Microplate Tape Applicator (catalog #170-8736)

Aligning the Mask

1. Dilute 10x Well Factor solution 1 part to 9 with ddH_2O
2. Fill **ALL** 96 wells of plate, with the same volume and in the same media type (e.g. plate with tape) as the experiment uses. Seal the plate in the same mechanism as experiment uses.
3. Place the plate in the MyiQ and click **Imaging Services** in the **Run-Time Central** module.
4. Click **Optimize Bias**.
5. Click **Calibrate** then click the **Open** radio button.

6. Click **Make an Exposure**. Examine the image for pink pixels. If pink pixels are present reduce the exposure time and click **Make an Exposure**. Repeat as necessary.

   (If no pink pixels in first exposure,increase the exposure time until pink pixels are detected, then reduce exposure time to the longest time that does not result in pink pixels.)

7. Click on the **Show Masks** button. While holding down the Shift key, left click on the top left well of your plate image.
8. Click **Optimize Mask Positions**. After masks successfully optimized, click OK to clear the message box
9. Click **Save Optimized Masks**.

Collecting Persistent Well Factors

10. Click the **Collect Persistent Well Factors** button. The program will transfer to the **Run-Prep** tab.
11. Specify the sample volume of the well factor plate in the **Sample Volume** box.
12. Click on **Begin Run**. The program will transfer to the **Thermal Cycler** tab.
13. A message will be displayed, indicating that the persistent well factors were generated.

2.3 Well Factors

Well factors are used to compensate for any system or pipetting nonuniformity in order to optimize fluorescent data quality and analysis. Furthermore, well factor data must be applied to every experiment run on the MyiQ. New well factor data may either be generated from the experimental plate at the beginning of every run (Dynamic Well Factors), or alternatively, well factor data may be generated once from an external source plate and saved, and this well factor data may then be applied to experiments run afterwards (Persistent Well Factors). See Figure 2.2 for guidelines in choosing the appropriate well factors.

In order to collect well factors from the experimental plate, each monitored well must contain the same concentration of fluorophore. For example, you can collect well factors from an experimental plate that contains 200nM of a FAM labelled probe in every monitored well. However, if you have an experimental plate containing 100nM FAM in one monitored well and 200nM FAM in another, you would have to use persistent well factors instead (See Section 5.4.2C).

The collection of well factors from the experimental plate is a completely automated process (See Section 5.4.2B), and the well factor collection process begins as soon as the **Begin Run** button is clicked and the file name saved. When collecting well factors from the experimental plate, it is held at 95°C for one and a half minutes prior to the first cycle with a setpoint temperature of 90.0°C or higher. You may choose to adjust the dwell time/temperature for the first step of your thermal protocol accordingly.

In most experiments using DNA-binding dyes, like SYBR® Green I or ethidium bromide, well factors may not be collected from the experimental plate. When the template DNA is denatured, the fluorescence of these dyes is not sufficiently high to calculate statistically valid well factors from the experimental plate. There are three solutions to this problem: (1) use iQ™ SYBR Green Supermix (Catalog # 170-8880) which already includes fluorescein (2) use persistent well factors see Section 6.2.5 (3) for experiments with SYBR Green I, spike the master mix with a small volume of dilute fluorescein solution (see Section 5.4.2B). The addition of fluorescein provides sufficient fluorescence at 95°C for the collection of well factors from the experimental plate, and at the same time will not interfere with the PCR.

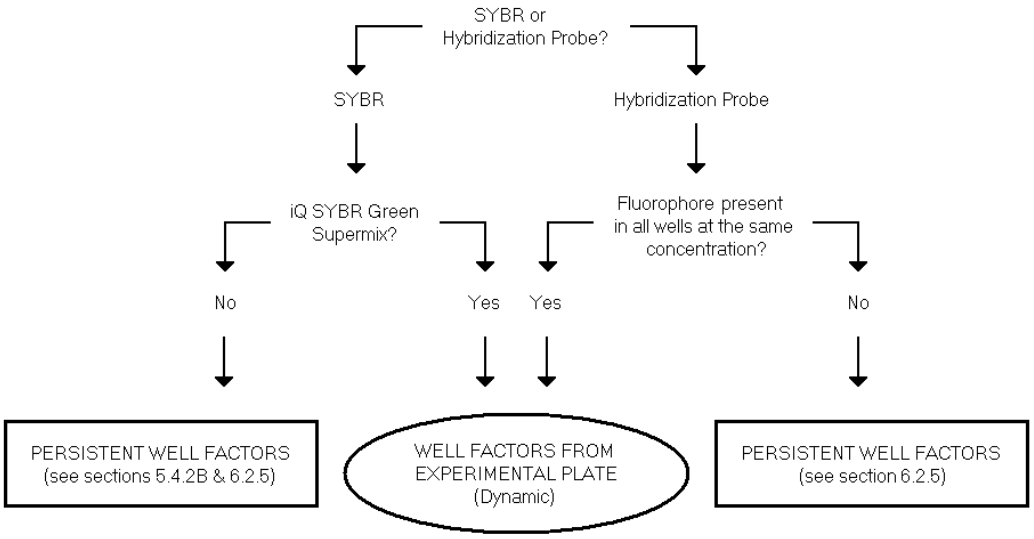


Fig. 2.2. Well Factor guidelines.

Section 3
Introduction to the MyiQ Software

3.1 Organization of the Software

The MyiQ software allows you to create and run thermal cycling programs in the iCycler thermal cycler, and to simultaneously collect and analyze fluorescent data captured by the MyiQ optical module. Customized files, called 'Protocols', direct the operation of the iCycler, and also specify when optical data will be collected during the thermal cycling run. MyiQ Protocol files are stored with the extension, '.tmo', and are mutually compatible with Protocol files for the iQ™ Real-Time PCR Detection System, which also employs Protocol files with the extension '.tmo'. The details of setting up Protocol files are described in Section 5.1.

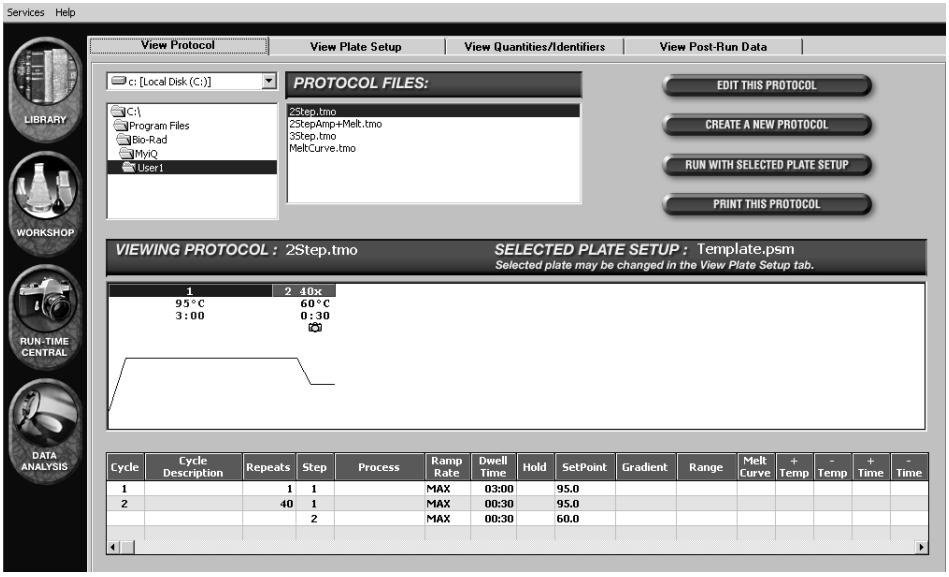


Fig. 3.1. Layout of a screen.

'Plate Setup' files determine the wells that the optical module will monitor for any run. Plate Setup files allow you to select the sample wells for which data is to be collected, the type of sample in each well (e.g., standard, unknown, control, etc.), and the fluorophore to be monitored. Plate Setup files are stored with the extension, '.psm', and are incompatible with Plate Setup files for the iQ Real-Time PCR Detection System. The details of setting up Plate Setup files are described in Section 5.2. In order to run a thermal cycling program and collect fluorescent data, both a Protocol file and a Plate Setup file must be specified.

Since Protocol and Plate Setup files are written and saved separately, you may "mix and match" different Protocols with different Plate Setup files in new experiments.

The MyiQ software is organized into four different sections, called 'modules'. These are the Library Module, the Workshop Module, the Run-Time Central Module, and the Data Analysis Module. Icons representing each of the modules are always shown on the left side of the screen. The module that is currently active is displayed with a highlighted border, while the icons for the other modules have plain borders. Figure 3.1 shows the first screen you see when you open the MyiQ

software: the names of all of the modules are listed on the left side of the screen with the Library Module icon highlighted, and the Library Module window is displayed on the entire right side of the screen. Each module has a different function, described below.

3.1.1 The Library Module

The Library Module contains Protocol, Plate Setup, and data files. In the Library, you may

- View Protocol files
- View Plate Setup files
- View Quantities and Identifiers for a Plate Setup file
- Select Data files to view in the Data Analysis Module
- Select Protocol files to edit in the Workshop Module
- Select Plate Setup files to edit in the Workshop Module
- Initiate a run using stored Protocol and Plate Setup Files
- Initiate the creation of a new Protocol or Plate Setup File

3.1.2 The Workshop Module

The Workshop Module allows you to work with Protocol and Plate Setup files. Use this module to

- Edit existing Protocol files
- Edit existing Plate Setup files
- Create new Protocol files
- Create new Plate Setup files
- View Quantities and Identifiers for a Plate Setup file
- Save new and edited Protocol files
- Save new and edited Plate Setup files
- Initiate a run using new or edited Protocol and Plate Setup files.

3.1.3 The Run-Time Central Module

Once settings are confirmed in the Run Prep section of the Workshop Module, the MyiQ software transfers you directly into the Run-Time Central Module. From this module you may

- View the start time and the estimated time of completion for a running Protocol
- View the current cycle, step, repeat number, and dwell time of a running Protocol
- View the current thermal activity of the reaction block
- Pause, stop, or resume an active Protocol
- Add repeats to, or skip steps or cycles for a running Protocol

- View and Save images of your plate at different exposure times (except during a run)
- Collect Persistent Well Factors
- Optimize well mask positions

3.1.4 The Data Analysis Module

The Data Analysis Module opens automatically from the Run Time Central Module when the MyiQ optical module begins collecting fluorescence data in real time. Saved real-time data from previous experiments may also be viewed and analyzed in the Data Analysis Module. This module allows you to

- View Amplification and Melt Curve data from an experiment running in real time
- View and analyze saved data from a previous experiment
- Optimize, track, and save data analysis parameters
- Construct standard curves
- Conduct End Point Analyses
- Conduct statistical analyses
- Determine threshold cycle values
- Determine starting concentrations of unknowns
- Edit the labeling and configuration of a data file's plate setup post-run
- Generate, view, and print reports describing the results of an experiment
- Add textual descriptions to data files for easy archiving of experimental results

3.2 Organization of the Manual

The four sections that follow this one describe each of the four modules. Within each section, the manual is organized by the separate windows of the module.

3.3 Definitions and Conventions

The following customs have been adopted in the text of this instruction manual:

- A "window" refers to an independently movable display screen found within the MyiQ software.
- Active buttons across the top of a window are referred to as 'tabs'.
- A text box refers to a field that you can type in, within a window.
- A field box refers to a region in the window that you cannot type in, but provides information about the program.
- A dialog box refers to a region in the window that allows you to make a selection.
- All active buttons are printed in bold type in the text descriptions and figure legends. For example, the **Edit** button is always printed in bold since selecting this will result in some action by the MyiQ software.

3.4 Thermal Cycling Parameters

Protocol files contain the information necessary to direct the operation of the iCycler thermal cycler. A protocol is made up of as many as nine cycles, and a cycle is made up of as many as nine steps. A step is defined by specifying a set point temperature and the dwell time at that temperature. A cycle is defined by specifying the times and temperatures for all steps and the number of times the cycle is repeated. Cycles may be repeated up to 600 times.

3.4.1 Temperature and Dwell Time Ranges

Temperatures between 4.0 and 100.0°C may be entered for any set point temperature.

Finite dwell times may be as long as 99 minutes and 59 seconds (99:59), or as short as 1 second (00:01).

Zero Dwell Times: When the dwell time is set to 00:00, the iCycler will heat or cool until it attains the set point temperature and then immediately begin heating or cooling to the next set point temperature.

3.4.2 Programming Options

Many advanced options are available for thermal protocols. They are listed below and detailed in the section describing the Edit Protocol window of the Workshop (Section 5.1).

- Infinite Hold: Holds a defined temperature indefinitely until user intervention.
- Gradient: Allows a reproducible gradient of between 1 and 25°C to be programmed down-the-block during any single step.
- Melt Curve: Enables the collection of melting curve data over a specified temperature range.
- Increment/Decrement Temperature: Defines a periodic incremental increase or decrease in temperature during a repeated cycle.
- Increment/Decrement Time: Defines a periodic incremental increase or decrease in dwell time during a repeated cycle.
- Ramping: Allows specification of the rate of heating and cooling for a particular step
- Cycle Description: Names the cycles of the protocol.
- Step Process: Names the steps of a cycle.

Section 4
The Library Module

From the **Library** module, you may open saved Optical Data files, initiate experiments using saved Protocol and Plate setup files, and also view Protocol and Plate Setup files. The Quantities and Identifiers of the currently selected plate setup file and any notations about the currently selected optical data file are also available in the Library. Protocol and Plate Setup files viewed in the Library may be selected for editing in the Workshop, or you may choose to create a new Protocol or Plate Setup instead.

The Library module consists of four windows; each is accessed by its associated tab.

- **View Protocol**: Allows the navigation of stored protocol files. Provides information on the thermal parameters for the specified protocol and indicates when data will be collected and analyzed. Saved protocols may be selected for initiation of a new run, or opened for editing, or a new protocol may be created (see Figure 4.1 and 4.2).
- **View Plate Setup**: Allows the navigation of stored plate setup files. Provides information about the location of sample wells, the sample type, and the fluorophore that will be analyzed. Saved plate setup files may be selected for the initiation of a new run, or opened for editing, or a new plate setup may be created (see Figures 4.2 and 4.3).
- **View Quantities/Identifiers**: Displays the layout and information of the plate setup file currently selected in the **View Protocol** window. (see Figure 4.4).
- **View Post-Run Data**: Allows the navigation of stored data files. Displays any notes entered by the user. Permits opening of stored data files (see Figure 4.5). Stored data files may be opened by double clicking the file name or clicking **Analyze Data**.

4.1 View Protocol Window

The **View Protocol** window of the **Library** module is the first window that appears when the MyiQ software is opened (see Figure 4.1). In this window, you can navigate the directory of protocol files stored on your computer. When a protocol file name is highlighted in the directory tree, the details of the protocol are displayed in the View Protocol window.

The upper part of the window displays the following protocol file information:

- The Drive Location: The Protocol files shown here are stored on the C drive.
- The Directory Tree: The Protocol files shown here are stored in the User1 folder.
- The Protocol Files menu: A list of protocol file names in the current directory. All protocol filenames have a '.tmo' extension.
- The Viewing Protocol field: The file name of the selected protocol is displayed in this window.
- The Selected Plate Setup field: The currently loaded plate setup file.

The right side of the window has the following active buttons:

- **Edit this protocol**: Transfers the selected Protocol file to the Edit Protocol window of the Workshop; this allows you to edit the protocol displayed on the screen.
- **Create a new protocol**: Transfers to the Edit Protocol window of the Workshop for creation of a new protocol file.
- **Run with selected plate set up**: Transfers the selected protocol file and the selected plate setup file to the Run Prep window of the Workshop for initiation of an experiment.
- **Print this protocol**: Prints the spreadsheet section of the selected Protocol.

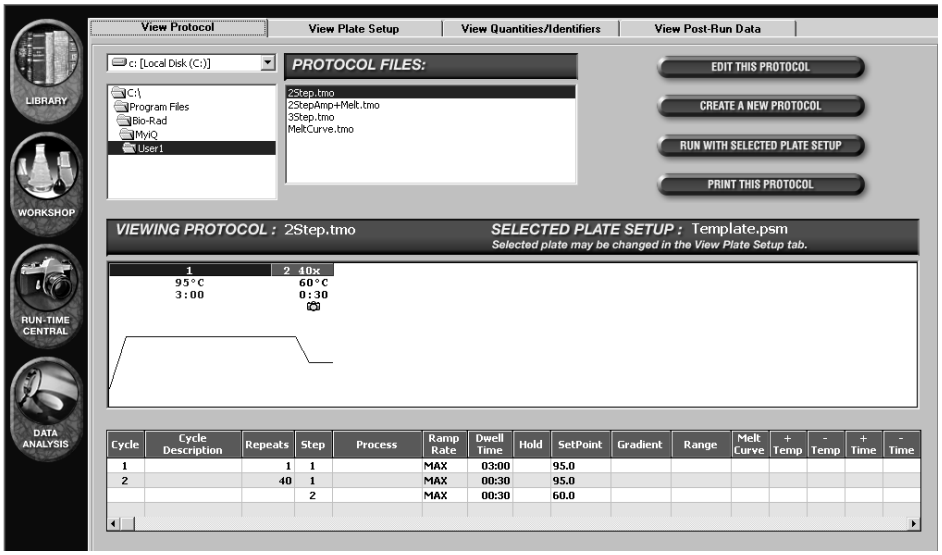


Fig. 4.1. Library / View Protocol Window.

The lower half of the window displays the protocol file identified in the Viewing Protocol field, both graphically and in spreadsheet format (see Figure 4.1). The graphical display shows the reaction temperature (y-axis) as a function of time (x-axis). If you would like to print the graphical display (or copy-and-paste the entire protocol) to another application, such as a word processing program, simply right click on the graphical display and select the desired option. In the graphical display:

- The bar across the top of the graphical display shows the cycle number;
- The numbers below the bar indicate the set point temperature for each step in the cycle (i.e., the y-axis) and the dwell time specified for that step (i.e., the x-axis).
- The presence of a camera icon on a particular cycle of the graphical display indicates that optical data will be collected at that step. A yellow camera icon indicates that amplification data will be collected, while a green camera icon indicates collection of melt curve data.
- The presence of a thickened trace in the graphical display indicates a gradient step (see Figure 4.2). To view the set point temperatures of specific rows, view the gradient temperatures chart, which appears next to the spreadsheet.

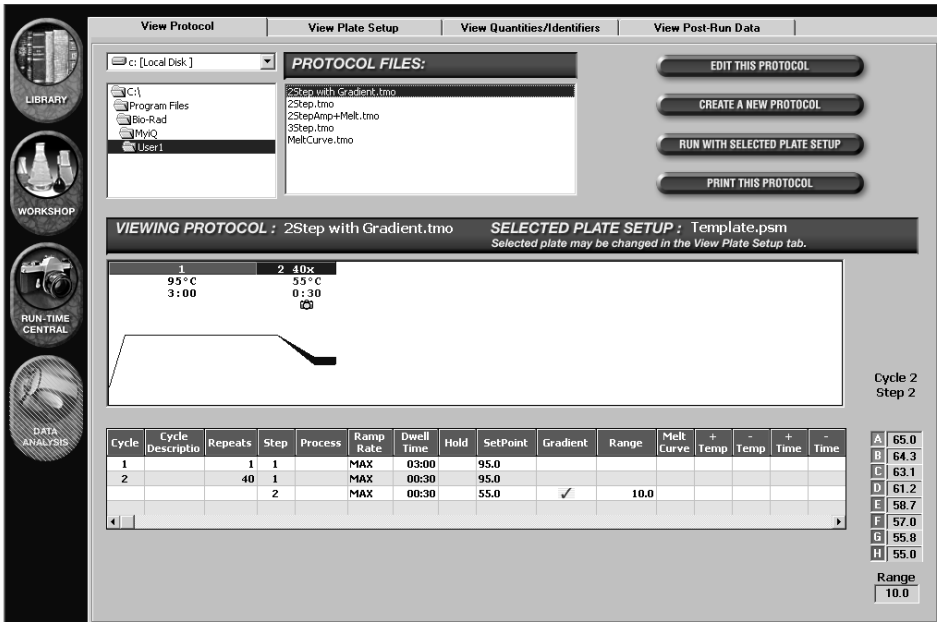


Fig. 4.2. Library / View Protocol window showing a gradient protocol.

The protocol spreadsheet, which is found just below the graphical display, provides a detailed, textual description of the protocol. Details of specialized options, such as temperature gradients and automatic incrementing and decrementing of temperature or dwell time, are provided in the spreadsheet but not in the graphical display. Customized cycle descriptions and step process descriptions are also displayed in the spreadsheet.

In addition, if the selected protocol contains a gradient step, a color-coded chart containing the set point temperatures of each row of the gradient step will appear to the right of the protocol spreadsheet. If there is more than one gradient step in the selected protocol, you may scroll through the gradient temperatures for each gradient step via a horizontal scroll bar which appears above the gradient temperatures chart.

4.2 View Plate Setup Window

In this window, you can navigate the directory of plate setup files. When a plate setup file name is highlighted in the directory tree, the details of the plate setup, including the monitored wells, the sample types loaded in each well, and the fluorophore, are displayed in the View Plate Setup window (Figure 4.3).

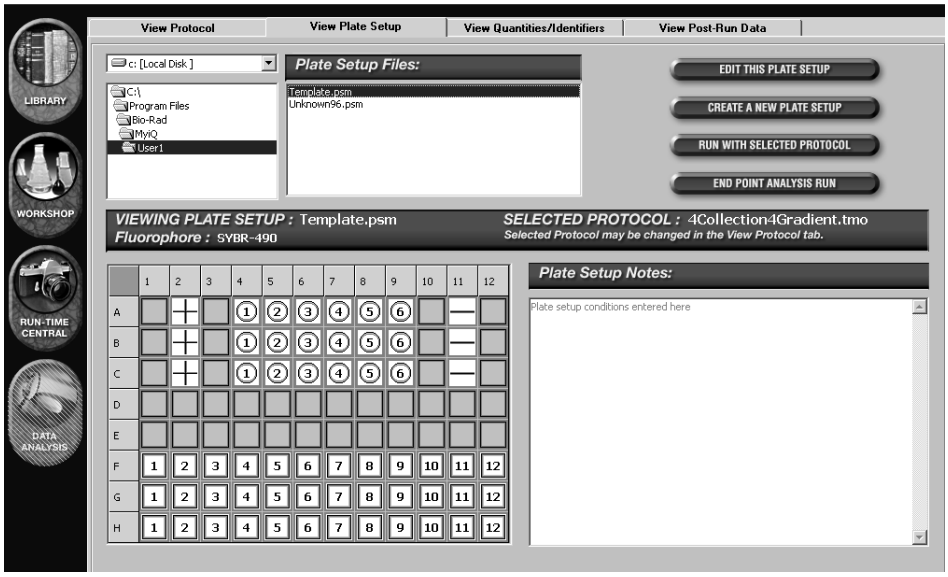


Fig. 4.3. Library / View Plate Setup Window.

The upper part of the **View Plate Setup** window is similar to the upper part of the **View Protocol** window (compare Figures 4.1 and 4.3.) The **View Plate Setup** window displays the following plate setup file information:

- The Drive Location: The Plate setup files shown here are stored on the C drive.
- The Directory Tree: The plate setup files shown here are stored in the User1 folder.
- The Plate Setup Files menu: A list box of all plate setup file names in the current directory; all plate setup filenames have a '.psm' extension.
- The Viewing Plate Setup field: The filename of the selected plate setup is displayed in this window
- The Selected Protocol field: The filename of the currently selected protocol file.

The **View Plate Setup** window has the following active buttons on the right side:

- **Edit this plate setup**: Transfers the selected Plate Setup file to the Edit Plate Setup window of the Workshop; this allows you to edit the plate setup displayed on the screen.
- **Create a new plate setup**: Transfers you to the Edit Plate Setup window of the Workshop; this allows you to create a new plate setup beginning with a blank plate layout.

- **Run with selected protocol**: Transfers the selected protocol file and the selected plate setup file to the Run Prep window of the Workshop for initiation of an experiment.
- **End Point Analysis Run:** Transfers the selected plate setup file to the Run Prep window of the Workshop for initiation of an End Point Analysis experiment.

To the right of the plate setup grid is the Plate Setup Notes display, which displays any notes written about the plate setup (see Figure 4.3). Plate Setup Notes may not be altered directly in the View Plate Setup window. To edit the Plate Setup Notes from the View Plate Setup window, click on the **Edit this Plate Setup** button to open the Edit Plate Setup window, and then make the desired changes in the Enter Plate Notes text box.

4.3 View Quantities/Identifiers Window

The View Quantities/Identifiers window displays standard quantities and identifiers for every well of the plate setup currently selected in the View Plate Setup Window. From this window you may view the fluorophore, monitored wells, sample types, standard quantities, and units of standards all in one glance. This display provides an easy means of proofreading your Plate Setup files prior to running experiments. If you would like to print the plate setup display click **Print Spreadsheet**. To copy-and-paste the entire plate setup display to another application, such as a word processing program, click the **Copy to Clipboard** button. Alternatively, you may choose to copy-and-paste only a portion of the plate setup by clicking and dragging your mouse over the desired wells, pressing **Control–C** in the MyiQ software, and pressing **Control–V** in the desired application.

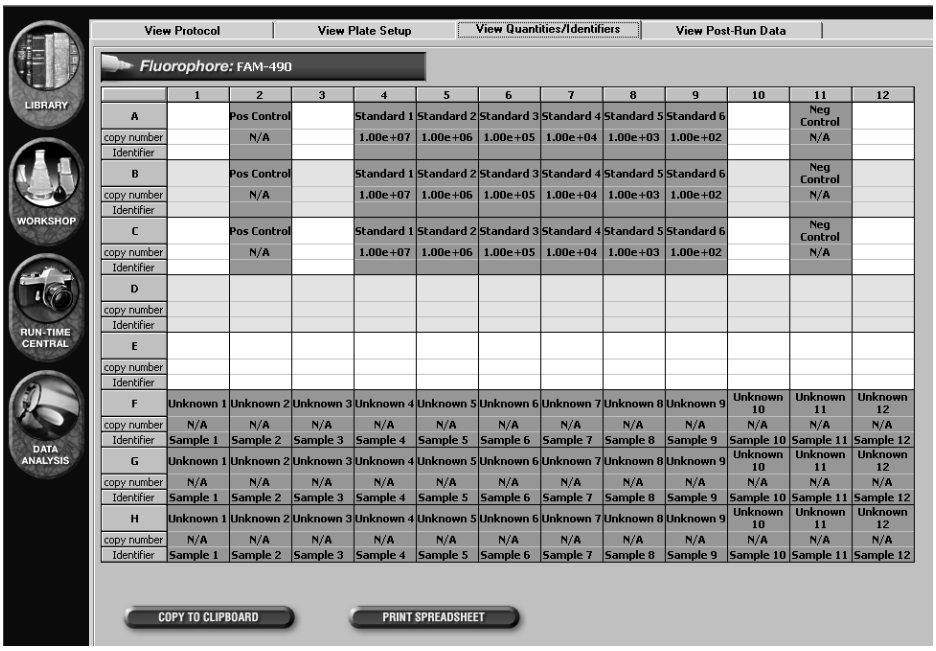


Fig. 4.4. Library / View Quantities/Identifiers window.

4.4 View Post-Run Data Window

The **View Post-Run Data** window is used to navigate the directory tree of saved optical data files. When an optical data file name is highlighted, the following information is displayed:

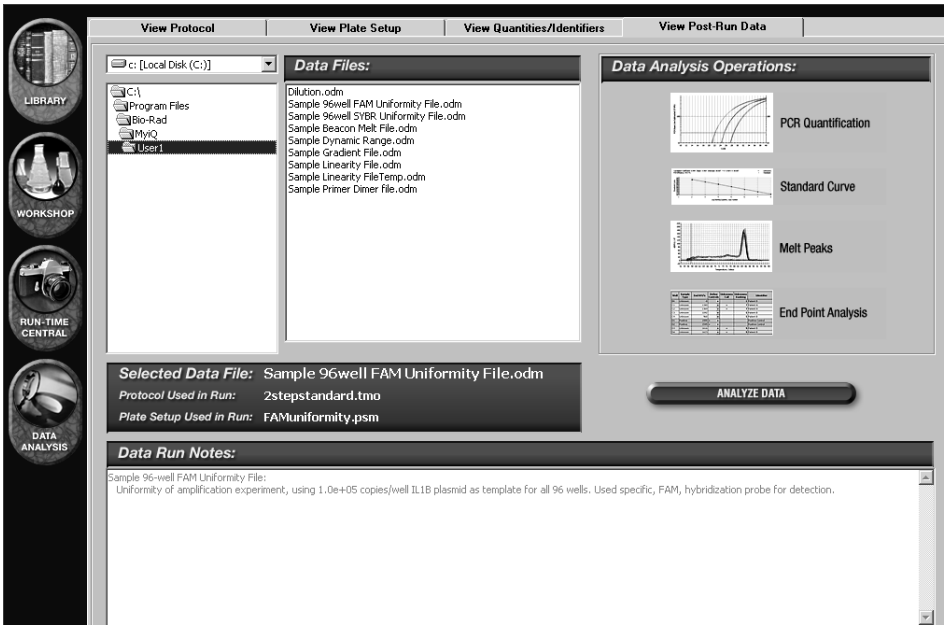


Fig. 4.5. Library / View Post-Run Data Window.

- The Drive Location: The data files shown here are stored on the C drive.
- The Directory Tree: The data files shown here are stored in the User1 folder.
- The Data Files menu: A list of data file names in the current directory. All optical data filenames have an '.odm' extension.
- The Selected Data File field: The name of the selected data file is displayed here.
- The Protocol Used in Run field: The name of the thermal protocol file used for the selected experiment is displayed here.
- Plate Setup Used in Run field: The name of the Plate Setup file used for the selected experiment is displayed here.
- Data Run Notes: These are text notes that the user may add to the data file in the View and Save Analysis Settings window, which can be accessed via the **Save ODM File** button in the View/Save Data window of the Data Analysis module.

4.4.1 Opening Stored Optical Data Files

To open an optical data file from any amplification, melt curve, or end point analysis experiment, select its name in the Data Files menu and click **Analyze Data** or double-click on the filename.

Section 5
The Workshop Module

The **Workshop** module is where Protocol and Plate Setup files are created and edited. Section 5.1 describes the layout of the **Edit Protocol** window and instructions for creating and editing protocol files. Section 5.2 describes the organization of the **Edit Plate Setup** window and explains how to create and edit plate setup files. There are four windows in the **Workshop**:

- **Edit Protocol**: Protocol files are created, edited, and saved in this window. From this window, you can initiate an experiment with the currently selected Plate Setup file and the displayed protocol, once it is saved.
- **Edit Plate Setup**. Plate setup files are created, edited and saved in this window. From this window, you can initiate an experiment with the currently selected Protocol file and the displayed Plate Setup file, once it is saved.
- **View Quantities/Identifiers**: Displays the layout and information of the plate setup file currently selected in the **Edit Plate Setup** window.
- **Run Prep**: When you choose to **Run with Selected Protocol** or **Run with Selected Plate Setup** from either the Workshop or the Library, this is the window in which you confirm the protocol file, plate setup file, sample volume, and method of well factor application. The experiment begins after you click **Begin Run** from this window and specify a file name for your data.

5.1 Edit Protocol Window

New protocols are created and existing protocols are edited in the Edit Protocol window (Figure 5.1).

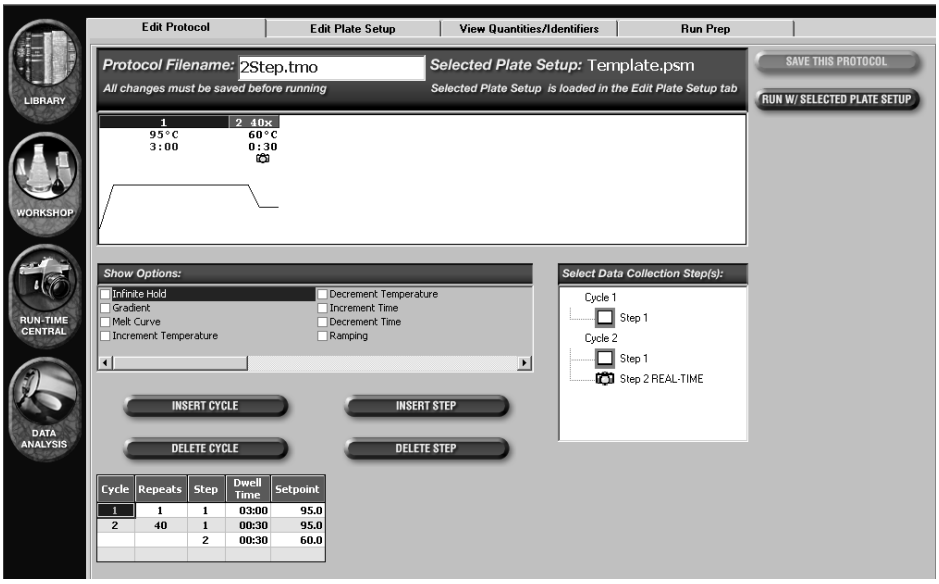


Fig. 5.1. Workshop / Edit Protocol Window. This shows the minimum programming spreadsheet.

However, the thermal protocol is actually edited within the adjustable spreadsheet at the bottom of the window. The Edit Protocol window contains the following:

- The Protocol Filename text box: The file name of the currently selected protocol is displayed in this text box. You may save the protocol under a new name by typing a new name into this text box and clicking **Save this Protocol**. If the name you specify does not include the extension '.tmo', it will automatically be appended to the protocol file name.
- The Selected Plate Setup field: This field displays the file name of the currently selected Plate Setup file.
- The **Save this Protocol** button: Click this button to save changes to the protocol (see Section 5.1.6).
- The **Run w/ Selected Plate Setup** button is used to run the displayed protocol, after it has been saved, with the currently selected plate setup (see Section 5.4).
- Graphical Display of the Protocol: Displays the currently loaded protocol, showing reaction temperatures (y-axis) and dwell times (x-axis). The graphical display does not contain any editing options, and merely depicts the protocol as it is programmed in the Protocol Spreadsheet.
- The Show Options box: Lists advanced options that can be applied to the protocol (see Section 5.1.5).
- The Select Data Collection Step(s) box: Used to specify the step at which the data are collected (see Section 5.1.4).
- The Protocol Spreadsheet: The thermal cycling conditions for the protocol are edited in this spreadsheet (see Section 5.1.2).
- The **Insert Cycle** button: Click this button to insert new cycles into the Protocol Spreadsheet.

- The **Insert Step** button: Click this button to insert new steps into the Protocol Spreadsheet.
- The **Delete Cycle** button: Click this button to delete full cycles from the Protocol Spreadsheet.
- The **Delete Step** button: Click this button to delete a step from the Protocol Spreadsheet.

The graphical display is described in Section 5.1.1. Programming protocols in the spreadsheet and specifying optical data collection are described in detail in Sections 5.1.2–5.1.5, and the saving of protocol files is detailed in Section 5.1.6.

5.1.1 Graphical Display

The graph at the top of the Edit Protocol window shows a display of the temperature cycling program (Figure 5.1). The bar above the graphical display indicates the cycle number for each section of the protocol; the active cycle (i.e., the one being edited) is highlighted. The set point temperature and the dwell time for each step are displayed below the bar. Note that occasionally space limitations do not permit display of all temperature and time settings. You can expand the time axis in the graphical display by holding down the **Control** key while dragging the cursor over a section of the graph. When the left mouse button is released, the time axis will expand. To zoom back out, left click anywhere on the graph. When optical data collection is specified (see Figure 5.1 and Section 5.1.6), a camera icon is shown above the temperature at the step(s) the data will be collected. A yellow camera indicates that optical data will be collected for quantitative analysis, while a green camera indicates that optical data will be collected for melt curve analysis. Placing the cursor anywhere over the graphical display and pressing the right mouse button displays the following menu:

Fig. 5.2. Context menu for protocol graph.

- **Copy Graph**: Copies the graph to the clipboard so it can be pasted into another application such as a text or a spreadsheet program.
- **Print Graph**: Prints a copy of the graph.

5.1.2 Programming Dwell Times and Temperature in the Spreadsheet

The bottom third of the Workshop / Edit Protocol window displays a spreadsheet that shows each cycle and step in the protocol. There are five columns that are always present for any protocol. These are:

Cycle	Repeats	Step	Dwell Time	Setpoint
1	1	1	03:00	95.0
2	40	1	00:30	95.0
		2	00:30	60.0

Fig. 5.3. Protocol spreadsheet.

- Cycle: A group of up to 9 steps (numbered 1–9) that are repeated; there may be up to 9 cycles (numbered 1 to 9) in a protocol;
- Repeats: The number of times a cycle is repeated; cycles may be repeated up to 600 times; the repeat number is displayed only on the first line of a multi-step cycle.
- Step: An individual temperature or dwell time event; each cycle may have up to 9 steps;
- Dwell time: The time the step is maintained at the specified temperature; this may vary from 0 sec (00:00) to 99 min, 59 sec (99:59)
- Set point temperature: The specified temperature that the reaction step will achieve; this may be within the range of 4.0° to 100.0°C.

5.1.3 Editing Cycles and Steps in the Spreadsheet

Fig. 5.4. Insert/delete cycles or steps.

Cycles and Steps in the thermal cycling program may be inserted and deleted using the **Insert Cycle**, **Insert Step**, **Delete Cycle**, and **Delete Step** buttons above the spreadsheet. While the Insert Cycle or Insert Step button is active, the cursor icon will be a plus sign when over the spreadsheet (except over check boxes which can be edited as usual). While the Delete Cycle or Delete Step button is active, the cursor will be an "X" when over the spreadsheet (except over check boxes which can be edited as usual). When all these buttons are inactive the cursor will have the arrow icon. A new cycle may consist of one, two, or three steps.

- To insert a cycle:
  a) Right mouse click **Insert Cycle** and an active box will appear showing **1-step**, **2-step**, and **3-step**. A 1-step cycle is the default setting.
  b) Select the desired number of steps; the active box will disappear and the **Insert Cycle** button will become highlighted.
  c) In the spreadsheet, select a cell within a cycle that will follow the inserted cycle; a new cycle will be inserted into the protocol.
  d) Deselect **Insert Cycle** by clicking the button again.
- To delete a cycle:
  a) Click **Delete Cycle**. The button will become highlighted.
  b) In the spreadsheet, select a cell within the cycle to be deleted; all of the steps in the cycle will be deleted.
  c) Deselect **Delete Cycle** by clicking the button again.

- To insert a step:
  a) Right mouse click **Insert Step**. Choose **Before** or **After** from the list box (the default is Before). The Insert Step button will become highlighted.
  b) In the spreadsheet, click at the point where the new step is desired, keeping in mind whether it will be inserted before or after the highlighted step.
  c) Deselect **Insert Step** by clicking the button again.
- To delete a step:
  a) Click **Delete Step**. The button will become highlighted.
  b) In the spreadsheet, select a cell in the step to be deleted and click on it.
  c) Deselect **Delete Step** by clicking the button again.

5.1.4 Select Data Collection Step(s) Box

The Select data collection step(s) box at the right side of the Edit Protocol window allows you to specify the step(s) in which data will be collected for real time analysis or for post-run analysis. Identify the step(s) at which data will be analyzed as follows:

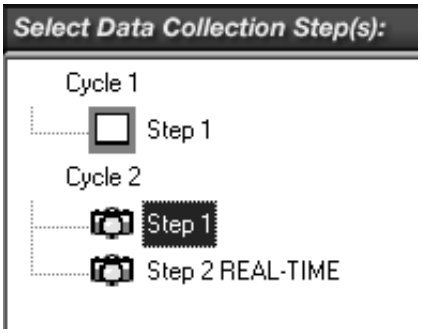


Fig. 5.5. Select Data Collection Step(s).

- Click once on a camera box at a step in the cycle at which data are to be collected; a yellow camera icon will appear in the box to indicate that collected data are to be saved for possible post-run analysis.
- Click on the same camera box a second time; **REAL-TIME** will appear next to the yellow camera icon to indicate that data are to be analyzed and displayed in real time.
- Clicking on the camera box a third time will clear the box of any camera icons.
- If you program a melt curve cycle, a green camera icon will appear in the Select data collection step(s) box. It appears and disappears as you check and uncheck the melt curve option in the Protocol Spreadsheet.

In addition to a camera icon appearing in the Select data collection step(s) box, a camera icon will also appear at the appropriate step in the graphical display of the Edit Protocol window. Data may be collected during one or more steps in any one cycle. However, data may not be collected in more than one cycle. Also, only one REAL-TIME yellow camera is allowed for any protocol.

5.1.5 Programming Protocol Options in the Spreadsheet

A number of advanced options are available for any thermal protocol. To enable the options, click on them in the Show Options box. Each time you click an option, one or more additional columns may open up in the Protocol Spreadsheet. Define the options by filling in the appropriate information in the spreadsheet.

Note: To defeat an option in the Show Options box, you must clear the entries for that particular option from the columns in the Protocol Spreadsheet before unchecking the option in the Show Options box. You cannot deselect an option by unchecking it in the Show Options box alone.

The following list describes the available options. The details of programming the options are in the following section.

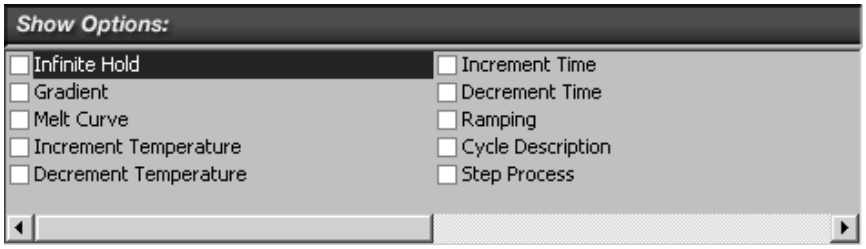


Fig. 5.6. Protocol options.

A. **Infinite Hold**: When a cycle is not repeated, the dwell time at any step in that cycle may be specified as infinite by using the Infinite Hold option. This means that the instrument will maintain the specified temperature until the user intervenes. When an infinite dwell time is programmed within a protocol at some step other than the last step, the instrument will go into Pause mode when it reaches that step, and will hold that set point temperature until either the **Continue Running Protocol** button or the **End Protocol** button is clicked in the Thermal Cycler tab of the Run-Time Central Module.

   An infinite hold may be programmed in the following way:

   1. Click **Infinite Hold** in the Show Options box. A new column titled “Hold” will appear in the spreadsheet.
   2. Check the **Hold** box for the step that you want to maintain at a constant temperature, and enter the desired temperature in the set point temperature cell of the spreadsheet.

B. **Gradient**: A thermal gradient may be programmed down the reaction block at any step of a protocol. The gradient runs, with the lowest temperature in Row H and the highest temperature in Row A. All wells in each respective row are at the same temperature, so at any time during a gradient step, there will be eight different temperatures across the block, with 12 wells at each temperature. The gradient may be as large as 25°C or as small as 1°C, and the resulting temperatures must fall between the range of 40°C and 100°C. The gradient is not linear, but is highly reproducible. Ramp rates for steps involving a gradient have been optimized such that all wells reach set point at the same time, irrespective of temperature, and therefore may not be adjusted.

The gradient may be programmed in the following way:

1. Click **Gradient** in the Show Options box. Two columns will appear in the Protocol Spreadsheet and a representation of the gradient will appear on the right side of the window.
2. Click the Gradient check box in the spreadsheet for the desired step.
3. The temperature listed in the Setpoint temperature cell of the spreadsheet will be the lowest temperature on the block during the gradient step (Row H). Enter the desired difference between the lowest and highest temperatures of the temperature gradient into the Range cell of the spreadsheet. The Gradient Temperatures chart will update with the temperatures at each row.

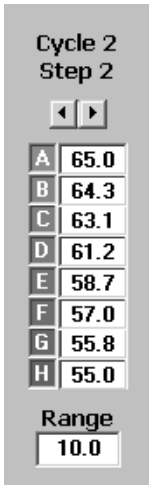


Fig. 5.7. Gradient display.

4. You can change the range in the spreadsheet or you can make a direct entry of the range in the gradient display. Press **Enter** and the display will update with the new calculated temperature for each row.
5. If you want to obtain a specific temperature at any one row, you can enter that temperature into that row on the gradient display and after you press **Enter**, the temperatures for the other rows will be calculated based on the input desired temperature and the range. You cannot specify the exact temperature on more than one row at a time.

C. **Melt Curve**: Melt curve analysis is a dynamic tool used to measure the melting temperature (Tm) of double stranded DNA molecules. DNA duplexes can be visualized by either the incorporation of DNA-binding dyes (e.g.SYBR Green I) or by hybridization with fluorescently labeled probes. In the case of DNA-binding dyes and non-cleavable hybridization probes, fluorescence is brightest when the two strands of DNA are annealed. As the temperature is raised towards the Tm of the duplex, the fluorescence will decrease at a constant rate (constant slope). At the Tm, there is a dramatic reduction in the fluorescence with a noticeable change in slope. The rate of this change is determined by plotting the negative first derivative (-dF/dT) versus temperature. The greatest rate changes yield visible peaks, representing the Tm of the double-stranded DNA complexes. Melt curve analysis can be used in peak identification (number of amplified products) and the characterization of molecular beacons. These applications are typically used as a guide for improving real-time PCR assay results. Melt curve analysis applications are discussed in detail in Appendix J.

   Melt curve cycles are programmed and processed separately from amplification cycles. When programming a melt curve cycle, the temperature is programmed to increase or decrease incrementally with each repeat of the cycle. The cycle may be repeated as many as 600 times. The increase or decrease, combined with the number of repeats, may not result in a temperature that is below 4°C or above 100°C at any time during the protocol. Although melt curve data may be collected in the same protocol in which amplification data are collected, melt curve data may also be collected independently of the amplification. Melt curves may be programmed in the following manner:

   1. Insert a cycle into the protocol at the point that you want the melt curve.
   2. Enter 95°C for the set point temperature, and enter 1:00 for the dwell time. This will be the initial denaturation step.
   3. If the beginning temperature of the melt curve is any temperature other than 95°C, insert a new cycle by selecting **Insert Cycle** and clicking on the next available blank line. Enter the temperature at which you wish to begin your melt curve and enter 1:00 minute for the dwell time. This step serves to adjust all DNA products to the desired start temperature at the beginning of the melt curve.
   4. Insert another new one-step cycle in the next available blank line. This cycle will be used to generate the melt curve data. In the Show Options box, click **Melt Curve**. This will add three additional columns to the spreadsheet entitled Melt Curve, + Temp, and – Temp.

      Enter an appropriate number of repeats under the Repeats column of this cycle (2–600 repeats), based on the desired beginning and end temperatures and the temperature increments/decrements. For example, a desired starting temperature of 95°C and ending temperature of 25°C at 1.0°C increments would require 70 repeats.
   5. Check the box under the Melt Curve column only at the cycle where you intend to collect melt curve data (the cycle you last added). This will automatically place a green camera under this cycle number in the Set Data Collection Step(s) Box, and the software will collect data for melt curve analysis at this cycle when the protocol is run.

Note: Melt curve data collection may only be programmed for cycles containing only one step and more than one repeat. Furthermore, only one melt curve cycle is allowed in a protocol.

6. Enter the temperature at which you wish to begin the melt curve in the setpoint temperature cell (4–100°C).
7. Enter the increment (+ Temp) or decrement (- Temp) temperature value. Temperature increments and decrements as low as 0.1°C may be programmed.

   Note: typical increment values are 0.3–0.5°C for SYBR Green I.
8. If an inappropriate number of repeats is entered based on the starting temperature and increment/decrement values, the + Temp or – Temp box will be highlighted in yellow. This is followed by an error message when attempting to save the protocol. You must then change the number of repeats, the set point temperature, or the increment/decrement value before the protocol can be properly saved.
9. Enter an appropriate dwell time for data collection under the Dwell Time column. The minimum dwell time for data collection is 10 seconds. We recommend using a slightly higher dwell time than the minimum values, such as 15 seconds, so that more data points are collected at each repeat.
10. If desired, insert a final hold cycle. Click **Insert Cycle**, click on the next blank line, enter the desired hold temperature under the set point column, and finally, check the box under the Hold column.

    Note: a dwell time is not necessary for a hold cycle.
11. Save the protocol by entering a file name in the text box labeled Protocol Filename, and clicking **Save**. The new protocol will be saved under this file name to the directory you specify. You can bring up this protocol at any time by clicking the View Protocols tab in the Library.

D. **Increment/Decrement Temperature**: You may program an automatic periodic increase or decrease in the step temperature (Increment Temp or Decrement Temp) in a repeated cycle. Temperature increments or decrements may be as little as 0.1°C per repeat. You may make the increase or decrease as frequently as every cycle, and the increase or decrease can begin following any repeat. The temperature increment or decrement may be as large as desired, as long it does not result in temperatures that are outside the temperature limits of 4–100°C. A temperature increment or decrement may be programmed in the following way:

   1. Click **Increment Temperature** or **Decrement Temperature** in the Show Options box. Three new columns will appear in the spreadsheet.
   2. For the repeated step you want to affect, enter the incremental change desired in the +Temp column (for Increment Temperature) or in the –Temp column (for Decrement Temperature).
   3. Enter the repeat in which you want the change to occur for the first time in the Begin Repeat column. Usually it's repeat 2, but it can be any cycle.

4. Enter the frequency that you want the change to occur in the How Often column. Usually you will want the change to occur every repeat, so enter 1 in this column.

E. **Increment/Decrement Time**: You may program an automatic periodic increase or decrease in the step dwell time (Increment Time or Decrement Time) in a repeated cycle. Time increments or decrements may be as little as 1.0 sec per cycle. You may make the increase or decrease as frequently as every cycle, and the increase or decrease can begin following any repeat. The time increment or decrement may be as large as desired, as long it does not result in dwell times which are outside the limits of 00:00 and 99:59. A time increment or decrement may be programmed in the following way:

1. Click Increment Time or Decrement Time in the Show Options box. Three new columns will appear in the spreadsheet.
2. For the repeated step that you want to affect, enter the incremental change desired in the +Time column (for Increment Time) or in the –Time column (for Decrement Time).
3. Enter the repeat in which you want the change to occur for the first time in the Begin Repeat column. Usually it's cycle 2, but it can be any cycle.
4. Enter the frequency that you want the change to occur in the How Often column. Usually you will want the change to occur every repeat, so enter 1 in this column.

F. **Ramping**: The ramp rate is the speed with which the iCycler thermal cycler changes temperatures between the steps of a cycle, or between cycles. The default condition is for the iCycler to adjust temperatures at the maximum ramp rate. However, you may change temperatures to a fixed rate less than the maximum. Ramp rates are adjustable to 0.1°C /sec and must fall within the range of 0.1 to 3.3°C per second for heating, and 0.1 to 2.0°C per second for cooling. Invalid ramp rate entries are adjusted to the nearest valid entry. A ramp rate may be programmed in the following manner:

1. Click **Ramping** in the Show Options box. A new column titled Ramp Rate will appear in the spreadsheet.
2. Click in the Ramp Rate column on the line of the spreadsheet containing the temperature toward which you wish to control the ramp rate. Use the pull down menu to select MIN or MAX or make a direct entry into the field. If an invalid ramp rates is input, it is adjusted to the nearest valid ramp rate automatically.

Note: The ramp rate to and from a gradient step must always be set to maximum. Ramp rates for steps involving a gradient have been optimized so that all wells reach set point at the same time, irrespective of temperature, and therefore may not be adjusted.

G. **Cycle Description/Step Process**: You can choose from a list of descriptive names or enter one of your own to describe cycle or step processes. A cycle description or step process may be entered in the spreadsheet in the following manner:

1. Click **Cycle Description** or **Step Process** in the Show Options box.
2. Click the cell of the spreadsheet for the cycle or step you wish to name, and either choose one of the listed names from the pull down menu or enter your own description. Note that a cycle description may only be added to the first line of the cycle.

Note: If an error is made in programming, at least one of the fields in the spreadsheet is highlighted in yellow. For example, if a step with 30 cycle repeats and a dwell time of 40 sec is programmed for a time decrement of 10 seconds beginning on the fifth cycle and decreasing by 10 seconds every other cycle, the field in the "- Time" column containing 00:10 will be highlighted in yellow since by the 13th cycle the dwell time of that step would be less than zero. A problem such as this could be corrected by reducing the decrement time to 3 seconds or less, by changing the cycle to begin the decrement to 24 or higher, or by changing the time of the occurrence to every sixth cycle.

5.1.6 Saving the Protocol

After you have finished editing the protocol, you must save it before it can be run.

- Edited protocols may be saved with the existing name by clicking Save this protocol. The old protocol will be overwritten with the new protocol.
- Edited and new Protocols may be saved with new names as follows:

1. To rename an existing protocol or to name a new protocol, click with the left mouse button in the Protocol Filename text box.
2. Type a new name in the text field.
3. Click **Save this protocol**; a Saving a Protocol dialog box will appear on the screen with '.tmo' added to the filename. You may then choose a directory in which to save the protocol.
4. Click **Save** to save the protocol with the given name or select **Cancel** to return to the Workshop.

5.2 Edit Plate Setup Window

The plate setup file contains information about the samples in the experimental plate. In the Workshop/Edit Plate Setup window, existing plate setups may be modified and new plate setups may be created. A plate setup includes the identification of the wells to be monitored, the fluorophore in all wells, the type of sample in each well, and the concentration of any standards.

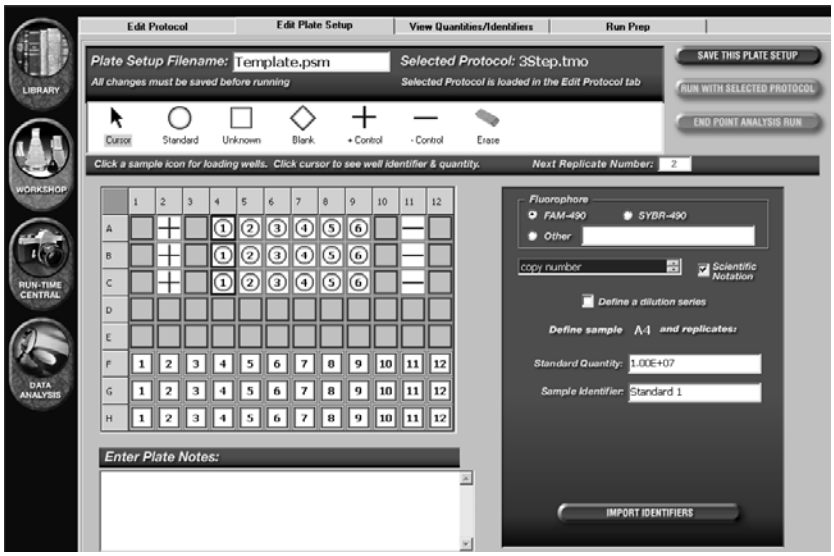


Fig. 5.8. Workshop / Edit Plate Setup Window.

The lower portion of the window shows the Plate Layout. If you are creating a new plate setup, the Plate Layout will be blank. If you are editing an existing plate setup, the well positions that have been previously defined will be indicated on the layout. Near the top of the window are the following:

- The **Plate Setup Filename** text box displays the name of the plate setup file.
- The name of the currently selected protocol file is displayed in the **Selected Protocol** field.
- The **Save this Plate Setup** button may be used to save changes to the plate setup.
- The **Run with Selected Protocol** button may be used to start running the currently selected protocol with the displayed plate setup file.
- The **End Point Analysis Run** button may be used to initiate an End Point Analysis Run. The End Point Analysis thermal protocol must be used.
- The **Toolbar Menu** contains different icons and tools for defining your plate setup.

5.2.1 Edit Plate Setup/Samples

The Workshop / Edit Plate Setup window lets you select the fluorophore to monitor, identify which wells have been used in the sample plate, where the standards, unknowns, blanks, and controls have been placed, and the quantities of the standards used. The icons for each sample type are chosen from the Toolbar menu.

Fig. 5.9. Sample icons.

A. Icon Functions

The toolbar menu contains the following icons that may be used to identify the types of samples in each well of the Plate Layout (see Figure 5.9).

Icon	Description
Cursor	Use this tool to select an already defined well in the Plate Layout. You may find it useful to use the cursor when assigning or double-checking standard concentrations or sample identifiers for certain wells.
Standard	Use this tool to define standards with known template concentrations. Standards are given sequential numbers when they are defined, and replicate wells containing the same concentrations may be labeled with the same number. The starting quantity of each standard must be defined in the **Standard Quantity** text box, which becomes active whenever a standard is either selected or defined. The units of measure are specified in the **Units** scroll bar found above the Standard Quantity box. If multiple standards are defined on the same Plate Layout, a standard curve will be calculated post-run.
Unknown	Use this tool to label samples with an unknown quantity of template. Unknowns are given sequential numbers when they are defined, and replicates containing the same concentrations may be labeled with the same number. If standards are defined along with the unknowns, the concentration of the unknowns may be calculated in the PCR Standard Curve tab and then displayed in the View/Save Data tab post-run.
Blank	Use this tool to identify wells without any reaction mixture.
+Control	Use this tool to identify positive controls. Wells labeled +Control will be used as positive controls if an Endpoint Analysis is conducted.
-Control	Use this tool to identify negative controls. Wells labeled –Control will be used as negative controls if an Endpoint Analysis is conducted.
Erase	Use this tool to clear the definitions of selected wells.

B. Activating and Using Icons from the Toolbar Menu to Specify Well Sample Type

1. Select an **Icon** from the Toolbar menu to activate it (Figure 5.9); the icon will appear highlighted.
2. In the Plate Layout, specify a sample location by selecting the desired well(s); the appropriate selection will appear in the designated well(s). Multiple wells may be selected in several ways:
   - To specify the entire plate, click the top left corner.
   - To specify an entire column, click one of the numeric column headings at the top of the plate layout.

- To specify an entire row, click one of the alphabetic row designations on the left of the plate layout.
- To select a group of wells, click and drag the cursor over the desired wells.

When specifying replicates, it is important to use one of these methods to select the wells, otherwise they will not share the same replicate number. Note that defining a well for the second time will overwrite the previous definition, and that you may specify the replicate numbers for your next set of standards or unknowns in the **Next Replicate Number** text box, which appears underneath the Toolbar menu on the right side.

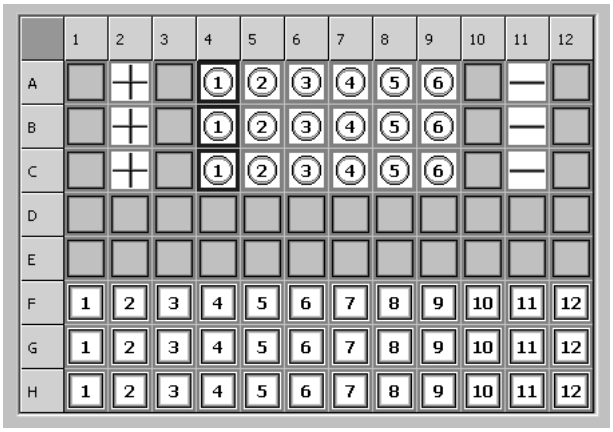


Fig. 5.10. Plate layout.

C. Assigning Standard Quantities, Identifiers and Units

Standard quantities may be defined at the same time each standard is assigned to the well, or they may be defined after all standards have been assigned to the wells. If the standard quantities are in a dilution series, you may also let the software calculate and assign the appropriate concentrations to the standards based on the starting concentration and the dilution factor.

- To define a dilution series of standards:

1. Select the fluorophore to be monitored in the **Fluorophore** box by clicking the appropriate radio button. To specify a fluorophore other than FAM or SYBR, select Other and input the name of the fluorophore into the text field. Only fluorophores with spectral characteristics similar to FAM and SYBR Green I may be successfully used.
2. Select the units of measure from the **Units** scrollbar. Note that all standards on the same plate setup must share the same units of measure.
3. Click the **Standards** icon and then click on a single well, or click and drag across a series of wells to define the first standard.
4. Repeat the previous step to define the remaining standard well locations. Note that the dilution series will be applied sequentially according to replicate numbers.
5. Select the **Cursor** icon after all standard well locations have been set.

6. Click the **Define a dilution series checkbox** to bring up the boxes for specifying a dilution series. The beginning and ending standard numbers will be displayed in the **Define Standards _ to _ and replicates** fields. You may edit these fields to leave some of the standards out of the dilution series calculation. No quantities will be calculated for standards manually excluded from this calculation.
7. Enter the concentration of Standard 1 in the **Starting Concentration** field. Click **Scientific Notation** to make the entries in that form. Click on the **Scientific Notation** check box to toggle scientific notation on and off. When this box is checked, input your standard quantities in scientific notation, with the exponent following the "E" and a plus or minus sign.
8. Enter a name for these standards in the **Series Identifier** box. This identifier will be applied to all standards defined in the same dilution series.
9. Enter a value in the **Dilution Factor** field. The default is 10 for a 10-fold dilution series.
10. Specify whether the dilution series is **Increasing** or **Decreasing** from Standard 1 onward via the appropriate radio button.
11. Click **Apply Dilution Series** to calculate and assign quantities to all standards defined in the dilution series.

Note on Defining a Dilution Series: If standards are specified to range from replicate group 1 through 5, the calculation assumes that the fold dilution is constant over the entire range. This means if you specify a 10-fold decreasing dilution factor, beginning with $1e^{10}$ for replicate 1, the software will assign values of $1e^{9}$ for replicate 2, $1e^{8}$ for replicate 3, $1e^{7}$ for replicate 4, and $1e^{6}$ for replicate 5. If you only define standards 1, 2, 3, and 5 (no standard 4), the software will still assign a value of $1e^{06}$ for replicate 5 as though replicate 4 were present.

- To define standard quantities one replicate group at a time:
  1. Select the fluorophore to be monitored in the **Fluorophore** box by clicking on the appropriate radio button. To specify a fluorophore other than FAM or SYBR, select Other and input the name of the fluorophore into the text field. Only fluorophores with spectral characteristics similar to FAM and SYBR Green I may be successfully used.
  2. Select the units of measure from the Units scrollbar. Note that all standards on the same plate setup must share the same units of measure.
  3. Click on the **Standards** icon and then click on a single well, or click and drag across a series of wells to define the first standard.
  4. Enter a value in the Standard Quantity box. Click on the **Scientific Notation** check box to toggle scientific notation on and off. When this box is checked, input your standard quantities in scientific notation, with the exponent following the "E" and a plus or minus sign.
  5. Enter any name for the standard in the **Sample Identifier** text box. You may also choose to leave this field blank.
  6. Repeat steps 3–5 to define each additional standard.

- **Alternatively**, you can specify the well locations of all standards first, and then define the standard quantities one replicate group at a time:

    1. Select the fluorophore to be monitored in the **Fluorophore** box by clicking on the appropriate radio button. To specify a fluorophore other than FAM or SYBR, select Other and input the name of the fluorophore into the text field. Only fluorophores with spectral characteristics similar to FAM and SYBR Green I may be successfully used.
    2. Select the units of measure from the Units scrollbar. Note that all standards on the same plate setup must share the same units of measure.
    3. Click the **Standards** icon and then click on a single well, or click and drag across a series of wells to define the first standard.
    4. Repeat the previous step to assign the well locations of all other standards.
    5. Click the **Cursor** icon.
    6. Click on any replicate well of the first standard.
    7. Enter a value in the Standard Quantity box. Click the **Scientific Notation** check box to toggle scientific notation on and off. When this box is checked, input your standard quantities in scientific notation, with the exponent following the “E” and a plus or minus sign. (e.g. to input $2x10^{-5}$, enter "2E-5")
    8. Enter any name for the standard in the **Sample Identifier** text box. You may also choose to leave this field blank.
    9. Click on a replicate well of the next standard to be defined and repeat steps 7–8.

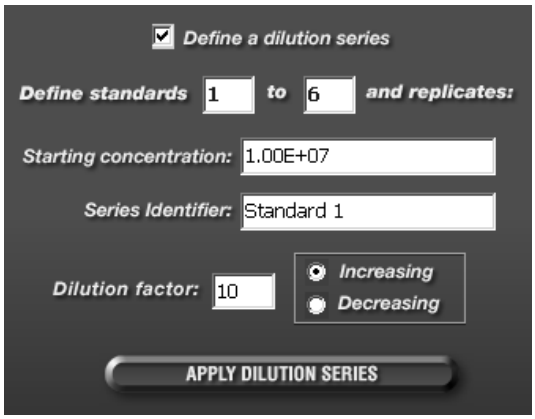


Fig. 5.11. Dilution series definition box.

D. Editing Standard Quantities, Identifiers and Units

Standard quantities may be edited after a plate setup has been saved.

- To change standard quantities one replicate group at a time:

    1. Click on a well in the standard replicate group to be edited.
    2. Select a new fluorophore or type of units for the standards if desired. If you change the fluorophore or units for one standard, they are changed for all samples on the plate.

3. Enter a new value in the **Standard Quantity** field if desired.
4. Enter a new name for the standard in the **Sample Identifier** field if desired. Alternatively, you may also use the **Import Identifiers** button to import identifiers from a separate spreadsheet (see Section G below).
5. Repeat steps 1–4 for each additional standard to be edited.

- To edit a dilution series of standards.

    1. Click on the **Define a dilution series checkbox** to bring up the boxes for specifying a dilution series. The beginning and ending standard numbers will be displayed in the **Define Standards _ to _ and replicates** fields. You may edit these fields to leave some of the standards out of the dilution series calculation. No quantities will be calculated for standards manually excluded from this calculation.
    2. Enter the concentration of Standard 1 in the **Starting Concentration** field. Click **Scientific Notation** to make the entries in that form. Click on the **Scientific Notation** check box to toggle scientific notation on and off. When this box is checked, input your standard quantities in scientific notation, with the exponent following the "E" and a plus or minus sign.
    3. Enter a name for these standards in the **Series Identifier** box. This identifier will be applied to all standards defined in the same dilution series.
    4. Enter a value in the **Dilution Factor** field. The default is 10 for a 10-fold dilution series.
    5. Specify whether the dilution series is **Increasing** or **Decreasing** from Standard 1 onward via the appropriate radio button.
    6. Click **Apply Dilution Series** to calculate and reassign quantities to all standards defined in the dilution series.

E. Editing Standard and Unknown Replicate Numbers

Wells in the Plate Layout identified as either standards or unknowns are given sequential numbers when they are selected. You may change the numerical designation of either of these sample types in the Plate Layout as follows:

1. Select the **Standard** or **Unknown** icon from the toolbar menu; the icon will become highlighted.
2. Enter the desired replicate number in the **Next Replicate Number** field.
3. Click on the desired well(s). Note that if additional wells are selected, they are subsequently identified as 1 unit higher than the number entered in the previous well.

F. Entering Plate Notes

You may input any comments or notes regarding the plate setup file in the **Enter Plate Notes** text box, which appears at the bottom of the Plate Setup layout. The entered plate notes will be displayed along with the plate setup layout whenever you browse this plate setup file in the **View Plate Setup** tab of the **Library**.

G. Import Identifiers

Although sample identifiers may be inputted individually as described above, they may also be imported from a separate spreadsheet, via the **Import Identifiers** button in the **Edit Plate Setup** window. To use the Import Identifiers function, you must first use the Microsoft Excel® Import Identifiers macro (supplied with the MyiQ software) to create a spreadsheet with sample identifiers listed row-by-row. Upon clicking the Import Identifiers button, a Select Import File dialog box will prompt you to load the desired identifier spreadsheet file.

When using the Import Identifiers function make sure of the following:

- The import file does not need to provide an identifier for every sample.
- The import file should not provide an identifier for an empty well.
- The import file should contain identical identifiers for well samples that are defined as replicates (same replicate number and sample type in the plate setup file).

After the import process is complete, click the **Save this Plate Setup** button. You may review the updated identifier information from the **Quantities/Identifiers** window.

5.3 View Quantities/Identifiers Window

This tab offers a view of the entire plate layout, including all loaded wells, sample types, quantities, identifiers, and fluorophore. No editing may be done in this tab. However, this tab is convenient for browsing and double-checking plate setup files created or edited in the **Edit Plate Setup** display.

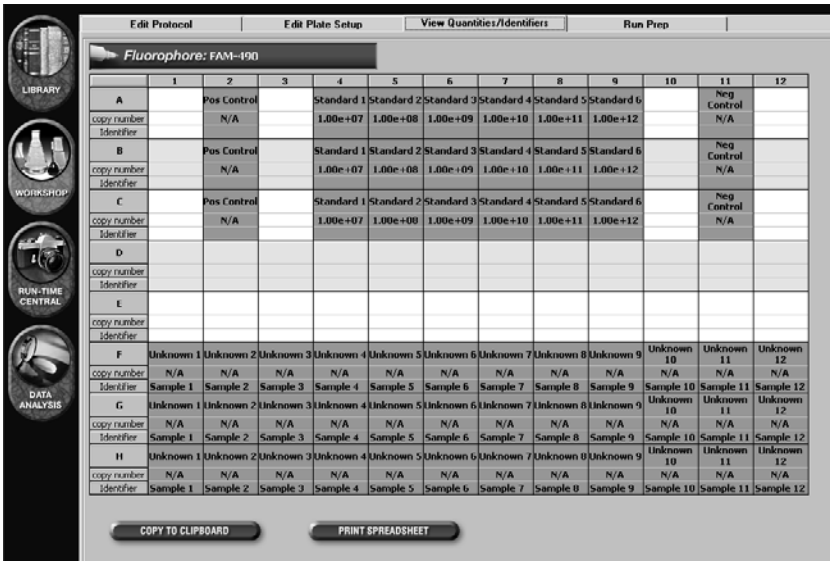


Fig. 5.12. View Quantities and Identifiers window.

5.4 Run Prep Window

Clicking **Run with Selected Plate Setup** or **Run with Selected Protocol** from either the **Library** or the **Workshop** opens up the **Run Prep** tab of the Workshop. The instrument will begin executing the experimental protocol once you click the **Begin Run** button and give the data file a name.

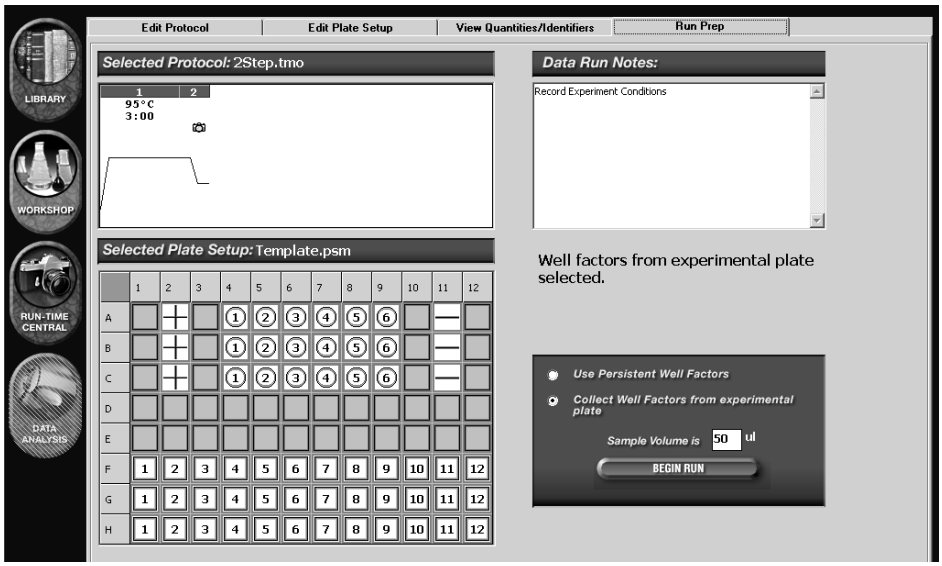


Fig. 5.13. Run Prep window.

5.4.1 Beginning a Run

1. Check alignment of the mask, as described in section 6.2.2, if you are uncertain about the current mask alignment.
2. Confirm that the desired protocol file and plate setup file are listed in the **Selected Protocol** and **Selected Plate Setup** fields. If the wrong files are listed, return to the **Library** and choose the correct protocol from the **View Protocol** tab and/or the correct plate setup from the **View Plate Setup** tab and then click **Run with Selected Plate Setup** or **Run with Selected Protocol**, respectively.
3. Input the volume of sample into the **Sample Volume is** field.
4. You may input any comments or notes regarding the experiment in the **Data Run Notes** text box, which appears in the upper right corner of the **Run Prep** tab. These notes will be displayed along with the **Protocol Used in Run** and the **Plate Setup Used in Run** whenever you browse this ODM data file in the **View Post-Run Data** tab of the **Library** module. You may also view and edit **Data Run Notes** within the **View and Save Analysis Settings** window, which is accessible from the **Save ODM File** button in the **Data Analysis / View/Save Data** tab.
5. Select the desired source of well factors from the box on the right of the plate layout. See the discussion on well factors below.

6. Insert the experimental plate in the instrument. Verify that the reaction mixture is sitting at the bottom of each well and contains no bubbles Surface bubbles may decrease overall fluorescence from a particular well. Thus, if these bubbles pop during data collection, sudden increases in signal may be observed, potentially interfering with base line calculations during data analysis.
7. Click **Begin Run**. A Save dialog box will open. Enter a name for the optical data file and specify a directory to save it to. Data are saved to the data file automatically during the course of the experiment if there is sufficient time at the end of each data collection step, otherwise they are saved only at the end of the experiment.

Note: If the computer loses power during execution of a protocol, the data collected up to that point can usually be found in the file created at the beginning of the run, but the last cycle of data will be lost. Depending on the point in the cycle at which power is lost, the data might not be found in the file created at the beginning, but instead will be found in a file named TEMP DATE TIME where DATE and TIME refer to the date and time of the experiment. The TEMP file will be in the USER1 folder.

5.4.2 Well Factors

Well factors are used to compensate for any system or pipetting non-uniformity and serve to optimize fluorescent data quality and analysis. Well factors must be applied to every experiment run. Well factor data may be generated from the experimental plate (**dynamic well factors**) at the beginning of a run, or alternatively **persistent well factors** may also be applied.

The better source of well factors, in terms of correcting non-uniformity, is the actual experimental plate. However, in order to collect well factors from the experimental plate, the plate must meet certain requirements and must also be cycled for approximately five minutes extra.

Persistent well factors may be applied to any experiment, as long as persistent well factor data have been generated beforehand, (see Section 6.2.5). Persistent well factors are collected from an external plate (not the experimental plate) filled with External Well Factor Solution (Catalog #170-8794). Persistent well factors are automatically written to the file PersistentWF.ini after they are generated and are good for at least 30 days under normal operation of the MyiQ (i.e., the instrument is not moved and no change has been made to any component of the optical path, including the filters and lamp). Persistent well factors may be applied to any experiment, as long as persistent well factor data have been collected beforehand.

The requirements for collecting well factors from the experimental plate are that each monitored well must contain the same concentration of fluorophore and the same reaction volume. For instance, if some of the wells have 100 nM fluorescein and others have 200 nM fluorescein, you may not collect well factors from the experimental plate and you must use persistent well factors.

Note: irrespective of well factor source, each experiment must have the same reaction volume in every well.

In experiments using DNA binding dyes, the fluorescence of these dyes is not sufficiently high to calculate statistically valid well factors from the experimental plate when the template DNA is denatured. For this reason persistent well factors should be used for experiments with intercalating dyes. However for SYBR Green experiments there are two additional options that permit the use of the experimental plate for well factor collection. The simplest is to use Bio-Rad iQ SYBR Green Supermix (Catalog # 170-8880 or 170-8882) which contains a small amount of fluorescein sufficient to give valid well factors, but which will not impact the amplification reaction. Another option is to spike the SYBR Green reaction mixture with a small amount of fluorescein (described below). The addition of small amounts of FAM or fluorescein has been shown to have no deleterious impact on the PCR reaction efficiencies. Figure 5.14 is a flow chart to aid in choosing appropriate well factors.

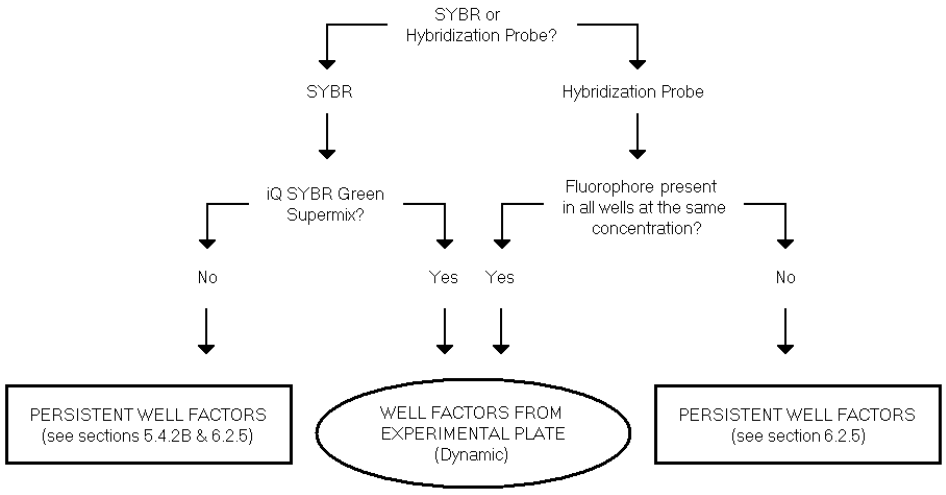


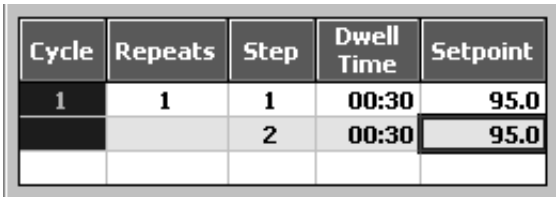
Fig. 5.14. Well factor decision tree.

A. Well Factor Source: Experimental Plate

Collection of well factors from the experimental plate is an automated process initiated by selecting the **Collect Well Factors from experimental plate** option from the **Run Prep** screen, clicking **Begin Run** and saving the data file name. Well factors are then collected the first time that the experimental plate is heated above 90°C. This is particularly important if the mode of detection employs a probe with any secondary structure (the secondary structure must be relaxed for proper calibration). Because the well factors are not collected until just before the plate is heated to 90°C, optical data collection cannot be specified in the thermal protocol until after a step in which the temperature is programmed to exceed 90°C.

B. Using the Experimental Plate for Well Factors

To use the experimental plate for well factor collection, click on the **Collect Well Factors from experimental plate** option from the **Run Prep** tab. When you select the experimental plate as the source of well factors, the software automatically inserts a short well factor collection protocol in front of the first step at which the temperature exceeds 90°C. This well factor collection protocol consists of a 90 second hold at 95°C. You may want to take this into consideration when creating your thermal protocol. For example, if you normally heat your reaction mixture to 95°C for 10 minutes prior to amplification, you could accomplish the same cycling conditions by programming an initial cycle of 8 minutes and 30 seconds at 95°C when using the experimental plate for well factors.



Cycle	Repeats	Step	Dwell Time	Setpoint
1	1	1	00:30	95.0
		2	00:30	95.0

Fig. 5.15. Protocol for collecting well factors from the experiment plate.

During the inserted well factor collection protocol, the excitation shutter is opened, optical data are collected from the plate, and the well factors are calculated. While the well factor data are being collected a message is displayed in the **Run Time Central / Thermal Cycler** screen.

Fig. 5.16. Collecting well factors from the experimental plate.

The Bio-Rad iQ SYBR Green Supermix (Catalog # 170-880 or 170-8882) is spiked with a small amount of fluorescein that permits the collection of well factors from the experimental plate. It is also possible to collect well factors from experimental plates with other commercial SYBR Green mixes or with home-brew mixes by adding sufficient fluorescein to bring the reaction mixture to 10 nM fluorescein. First make a 1 µM solution by a 1:1000 dilution of the 1 mM stock Fluorescein Calibration Dye (Catalog # 170-8780) in PCR buffer (10 mM Tris, pH 8.0, 50 mM KCl, 3 mM MgCl2). Then add 1 part of the 1 µM dilution to each 99 parts of master mix. For example, mix 10 µl of 1 µM fluorescein with 990 µl of master mix to yield a final concentration of 10 nM fluorescein. Once well factors are collected from the experimental plate, they are written to the odm file, and the software continues to execute the programmed protocol.

C. Well Factor Source: Persistent Well Factors

The persistent well factor approach must be employed whenever there are varying concentrations of the fluorophore in the individual wells of the plate, and for any other experiments for which well factors may not be collected from the experimental plate. Persistent well factors may be generated, saved to the PersistentWF.ini file, and then applied to future experiments performed on the same MyiQ System. In general, persistent well factors are good for about one month, but should be collected again anytime something pertinent to the optical system is changed, such as the optical filters or the camera lamp. A warning will occur when Persistent well factors are older than 30 days. Persistent well factors are not collected from the experimental plate, but rather from a separate, external well factor plate containing External Well Factor Solution (Catalog # 170-8794) at 1x concentration in all 96 wells (see below). Persistent well factors must be created using the same particular reaction vessel type, e.g. plate and tape, as the experimental plate. (see Section 6.2.5)

D. Using Persistent Well Factors

Once persistent well factors have been generated, they are quick and easy to apply. To use persistent well factors, select **Use Persistent Well Factors** from the **Run Prep** tab, before clicking the **Begin Run** button. When you select persistent well factors, well factor data from the current persistentWF.ini file is applied to the experiment. Note that your experimental protocol will begin running immediately after clicking the **Begin Run** button and saving the optical data file. There are no extra steps or cycles inserted into your protocol when using persistent well factors.

Section 6
The Run-Time Central Module

You will enter this module automatically after clicking **Begin Run** from the **Workshop / Run Prep** tab, or you may enter it by simply clicking the **Run-Time Central** icon. There are two tabs in this module: **Thermal Cycler** and **Imaging Services**.

6.1 Thermal Cycler Tab

All of the fields in this screen are for display purposes only and none are editable (Figure 6.1). Initially there are only three active buttons on the screen:

- **Pause/Stop**: Used to stop a running protocol.
- **Show Protocol Graph** and **Show Plate Setup Grid**: These two buttons allow you to toggle the display on the bottom half of the screen between one describing the thermal behavior of the iCycler and one showing the current plate setup with loaded with fluorophore. These displays are discussed in greater detail below.

Field boxes in the upper half of the display contain the following information:

- **Running Protocol**: Name of the running protocol
- **Running Plate Setup**: Name of the running plate setup
- **Start Time**: The time the run was started
- **Stop Time (est.)**: The estimated time the current protocol will stop
- **Dwell Time – Current**: Dwell time of current step or next step if temperature is ramping
- **Dwell Time – Remaining**: Time remaining in current step
- **Current - Cycle, Step and Repeat**: Displays the current cycle number, step number, and repeat number

In addition to monitoring the progress of a thermal protocol, it is also possible to modify a running protocol from this tab (see below).

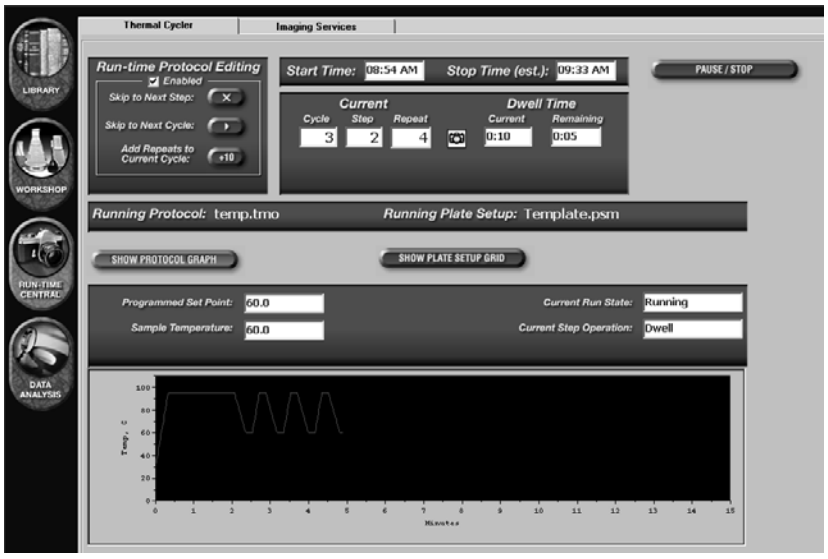


Fig. 6.1. Run Time Central window, Show Protocol Graph view.

6.1.1 Run-Time Protocol Editing

There are three choices available for modifying a protocol during a run: **Skip to Next Step**, **Skip to Next Cycle**, and **Add Repeats to Current Cycle**. During a run, these options are available by accessing the **Thermal Cycler** tab in the **Run Time Central** module. The options are listed in the **Run-time Protocol Editing** box and are accessed by clicking the **Enabled** check box (Figure 6.2).

Fig. 6.2. Run-Time Protocol Editing Options.

A. Skip to Next Step

- To skip the current step and to proceed to the next step, enable Run-Time Protocol Editing and click **Skip to Next Step** when the current step has reached its setpoint temperature. For example, for a two-step cycle of 37°C for 60 minutes and 95°C for 30 seconds, click **Skip to Next Step** anytime after 37°C has been reached to transfer the protocol immediately into the 95°C step.
- Refer to the iCycler LCD display of the protocol or the **Thermal Cycler** tab of the **Run-Time Central** Module to determine if the base unit is ramping to a temperature or if the instrument has reached the setpoint temperature.
- This feature is only available in the running state during a dwell. It is not available when a run is in pause mode or if the thermal cycler is ramping.

Note: The error message "**Action Denied**" will appear if you click this option when it a run is in pause mode or if the thermal cycler is ramping. This will NOT interrupt or terminate the run.

B. Skip to Next Cycle

- To skip the remaining repeats of the current cycle and to proceed to the next cycle, enable Run-Time Protocol Editing and click **Skip to Next Cycle**. For example, you could use this feature when your samples have clearly crossed the threshold, and you want to skip the remaining amplification repeats to proceed to the melt cycle of your protocol.
- Modifications to the protocol are updated on the iCycler LCD display immediately after they are made.
- This feature is available when the protocol is running or paused, and even while the base is ramping.
- After clicking **Step to Next Cycle** all the steps of the current repeat will be performed before continuing to the next cycle.

C. Add Repeats to Current Cycle

- To add 10 repeats to the current cycle, enable Run-Time Protocol Editing and click **Add Repeats to Current Cycle**. This button may be clicked multiple times, but the total number of repeats is limited to 600.
- Modifications to the protocol are updated on the iCycler LCD display immediately after they are made.
- For example, it may be necessary to add more repeats to an experiment amplifying low copies of DNA in order for all samples to cross threshold. You could click **Add Repeats to Current Cycle once** during an amplification cycle with 30 repeats to increase the number of repeats to 40.
- This feature is available when the protocol is running or paused, and even while the base is ramping.

6.1.2 Show Protocol Graph

When the **Show Protocol Graph** button is selected, the bottom half of the **Thermal Cycler** screen displays thermal information about the currently running protocol.

- The **Programmed Setpoint Temperature** and the current **Sample Temperature** are displayed at the top left. If a gradient step is being carried out, the range of temperatures is displayed.
- A graph of the sample temperature is shown in the graphical display at the bottom of the screen. If a gradient step is being carried out, a red trace will indicate the highest sample temperatures and a blue trace will indicated the lowest sample temperatures.
- **Current Run State**: This field displays the status of the instrument; either Running, Idle or Paused.
- **Current step operation**: This field displays the current operation of the instrument; either Ramping or Dwell.

6.1.3 Show Plate Setup Grid

When the **Show Plate Setup Grid** button is selected, the bottom half of the **Thermal Cycler** screen displays the plate layout and fluorophore selection of the running experiment. Note that only the sample types of the loaded wells are displayed. However, you may view standard concentrations and identifiers on the **View Quantities/Identifiers** tab in either the **Library** or **Workshop** modules while a protocol is running.

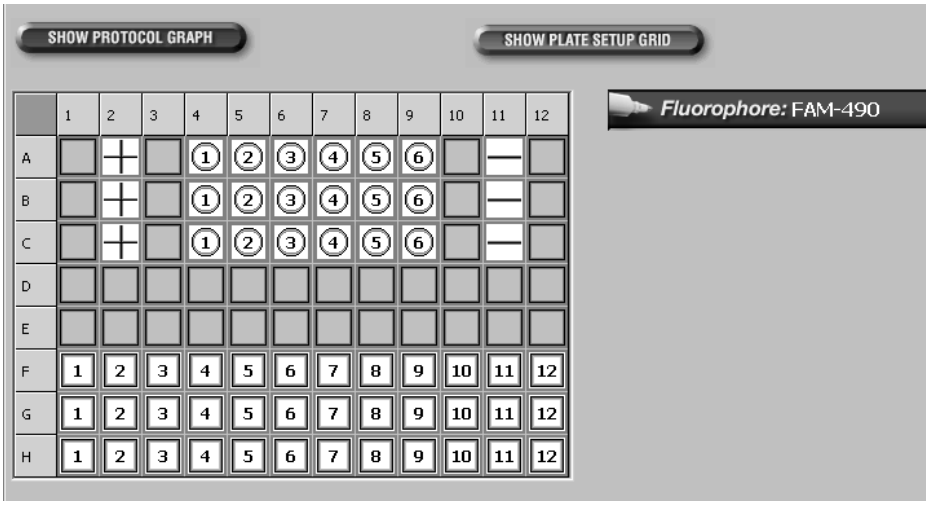


Fig. 6.3. Run Time Central window, Show Plate Setup Grid view.

6.1.4 Pause / Stop

The **Pause / Stop** button allows you to pause a thermal cycling protocol. If you click **Pause / Stop** when the protocol is at a dwell, the iCycler will hold the setpoint temperature and stop counting down the dwell time. If you click **Pause / Stop** button when the protocol is ramping the temperature, the iCycler will continue ramping until it reaches the next setpoint temperature, then pause at that step and stop counting down the dwell time. When you pause the thermal cycler beyond the specified dwell time, the set point temperature is held and the pause appears on the graphical display as a lengthening of that step along the time axis. After the run is complete, you may view the validation report from the iCycler base unit to observe any pauses or terminations of a particular protocol (see iCycler Instruction Manual, Section 4.3).

Clicking **Pause / Stop** pauses the protocol and brings up two new buttons on the screen:

- **End Protocol**: Click this button to terminate the experiment at its current step. Any data collected up to this point are saved and may still be analyzed.
- **Continue Running Protocol:** Click this button to resume running the thermal cycling protocol. The protocol will run to completion unless stopped again.

6.2 Imaging Services

The main use of **Imaging Services** tab is to adjust the masks and to collect persistent well factors. However, you may also use it to capture an image of an experimental plate to check the response of a probe or to assess the completion of a reaction. With a plate image and properly aligned masks, you may obtain fluorescence readings for each of the 96 individual wells.

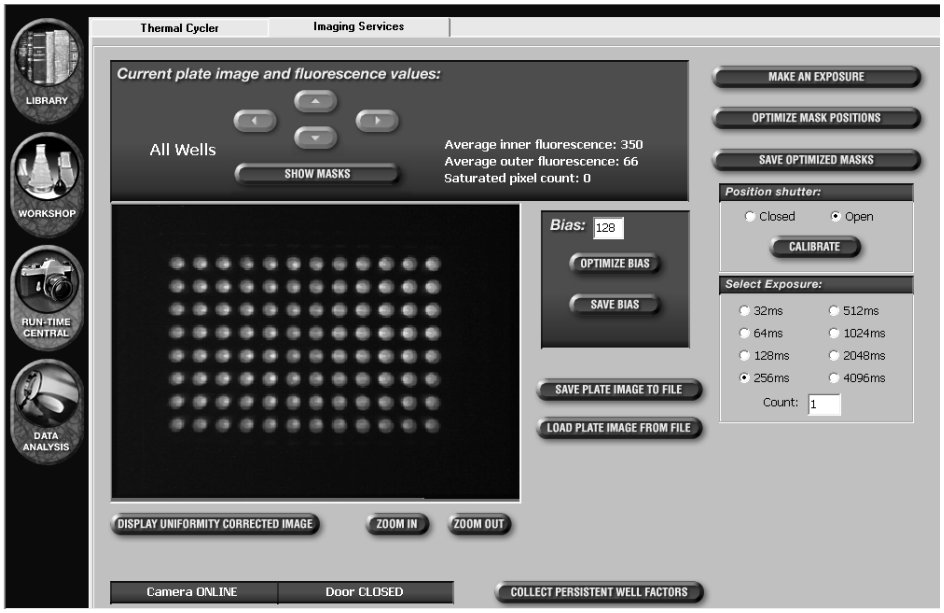


Fig. 6.4. Imaging Services Window.

6.2.1 Description

- The large rectangular display located below the **Current plate image and fluorescence values**: heading is the plate image viewer. The plate image viewer displays a test pattern until an actual image is taken or loaded.
- Immediately above the plate image viewer is the **Show Masks / Hide Masks** button. Click this button to toggle the display of the green masks outline on and off. The masks are used to define the location of each individual well. When the masks firsts appear, all wells are selected, and the text “All Wells” appears to the left of the Show Masks / Hide Masks button. With the masks displayed, clicking on any one square will select that individual well, and that well’s ID will be displayed to the left of the Show Masks / Hide Masks button. Click anywhere inside the plate image, outside of the masks to select all wells once again.
- The **arrow keys** found above the **Show Masks / Hide Masks** button are used to manually position the masks. If an individual well is selected, the arrow keys will reposition only the selected well.
- Also shown above the plate image are the **Average inner fluorescence**, **Average outer fluorescence**, and the average **Saturated pixel count** readings for all 96 wells of the plate. When you select a single well, the surrounding mask becomes blue and the inner and outer readings and saturation of just that particular well are displayed. Inner fluorescence refers to the fluorescence reading taken from the innermost area of each well. Outer fluorescence refers to the fluorescence reading taken from the outer area bordering each well. The saturated pixel count refers to the number of saturated pixels. Saturated pixels are indicated in pink.
- The **Make an Exposure** button appears in the upper right corner of the display. Pressing this button initiates an exposure. The resulting image is displayed in the plate image viewer.

- Below the **Make an Exposure** button is the **Optimize Mask Positions** button. Pressing this button realigns the masks, using well A1 as a reference point. See section 6.2.2 for detailed information about aligning the masks.
- Below the **Optimize Mask Positions** button is the **Save Optimized Masks** button. Pressing this button saves any modifications made to the mask alignment. Note that you must save optimized masks prior to running an experiment for the new mask alignment to be applied during data collection. A dialog box will prompt you to save any changes made to the mask if you leave the **Imaging Services** tab before saving the newly optimized mask positions.
- Found below the **Save Optimized Masks** button is the **Position Shutter** box. This box contains the **Closed** and **Open** radio buttons, as well as the **Calibrate** button. When the Closed radio button is selected, the camera's excitation shutter is closed, blocking light to the samples and CCD detector. When the camera is neither taking exposures nor collecting data, the shutter is in the closed position. When the Open radio button is selected, the camera's excitation shutter is opened, allowing light into the camera and readying the camera to make an exposure. Note that whenever the software is restarted, neither the Closed nor Open radio button is selected, as the software is uncertain about the status of the camera shutter. Pressing **Calibrate** will reset the excitation shutter to the closed position. In general, it is a good idea to hit the Calibrate button after turning the camera off and back on.
- Below the **Position Shutter** box is the Select Exposure box. The **Select Exposure** box contains a radio button list of different exposure times, as well as a **Count** field. Exposure times are listed in terms of milliseconds, and they vary by a factor of two. In general, shorter exposure times yield dimmer images, whereas longer exposure times yield brighter images. In addition to exposure times, you may also specify the number of counts, or the number of exposures to be collected and averaged before being displayed, within the Count field. The count field defaults to one.

  Note: The selected exposure time affects only exposures made via the **Make an Exposure** button in the **Imaging Services** tab. The MyiQ software automatically selects the best exposure time and number of counts for data collection steps during real-time experiments.
- To the right of the plate image viewer is the **Bias** box, which contains the **Bias** field, as well as the **Optimize Bias** and **Save Bias** buttons. Bias is a numerical value controlling the black and white offset of exposures made on the MyiQ. Readjusting the bias every time the exposure time is changed may improve image quality, since the optimal bias varies at different exposure times. You may manually input a bias value, although we recommend you use the **Optimize Bias** button to let the software find the best bias value for the selected exposure time. The bias is set as soon as the bias field is updated, and you may make a new exposure with an optimized bias setting. After optimizing the bias, you may click the **Save Bias** button to save the current bias setting.

Note: The selected bias setting affects only exposures made via the **Make an Exposure** button in the **Imaging Services** tab. The MyiQ software automatically optimizes the bias at the start of every real-time experiment.

- Below the **Bias** box are the **Save Plate Image to File** and **Load Plate Image from File** buttons. These buttons are used to either save the current image to disk or load a saved image from disk, respectively. Plate image files are created and saved with the extension .ISI, and they are accessible only in the **Imaging Services** tab of the **Run-Time Central** module.
- Below the plate image viewer is the **Display Uniformity Corrected Image / Remove Uniformity Corrections** button. Pressing this button toggles image uniformity correction on and off. You may use this feature to apply the software-based uniformity corrections to plate images displayed in the **Imaging Services** plate image viewer.
- Also found below the plate image viewer, are the **Zoom In** and **Zoom Out** buttons. These buttons are used to either enlarge or shrink the current image, respectively.
- The current status of the MyiQ camera and the iCycler lid (door) are indicated in the bottom left side of the window. You may not run an experiment or make exposures in Imaging Services unless the camera is online and the reaction module door is closed.
- The **Collect Persistent Well Factors** button is in the bottom right part of the window. Press this button to initiate the collection of persistent well factors, and to update the PersistentWF.ini file. In order to collect persistent well factors, you must prepare and insert an external well factor plate suitable for your experimental plate. See Section 6.2.5 for details on **Collecting Persistent Well Factors**.

6.2.2 Adjusting the Masks

The masks are software templates stored in the mask96.ini file that map the positions of each of the 96 wells onto the CCD camera. The masks must be adjusted upon installation of the MyiQ system, and typically do not require readjustment unless the system is moved or jarred, the camera is removed and remounted, or unless new MyiQ software is installed. It is good practice, however, to occasionally check the positions of the masks.

Each individual mask consists of a pair of concentric squares. The inner square should be centered on the well so that all fluorescent signals from the well fall within it. The outer square surrounds the inner one, and all signal collected within the region defined by the outer square, except for the signal from the region defined by the inner square, is considered background. A data point for each well is calculated by first taking the difference between the inner and outer readings (well signal minus background) at a particular moment in time. The software can automatically position each individual mask in its optimal position. There are also manual methods for adjusting the position of masks. The process of adjusting the masks consists of:

- Optimizing the bias of the detector
- Capturing an image
- Adjusting the mask
- Saving the mask file

Procedure: You must capture an image of a 96-well plate with some fluorophore in each well. It does not matter which fluorophore is used, as long as all wells fluoresce enough to be seen in **Imaging Services**. The same volume and concentration of solution should be present in every well. Using External Well Factor solution at 1x concentration is the most convenient.

1. Dilute 600 µl of 10X external well factor solution in 5.4 ml of ddH_2O and pipet 50 µl of 1X solution into each well of a 96-well plate. Cover the plate with a piece of optically clear sealing tape, spin it briefly to bring all solution to the bottom of the wells and place the plate into the iCycler.
2. Open the MyiQ software and open the **Imaging Services** tab in the **Run-Time Central** module.
3. Click **Optimize Bias** to initiate the process of detector optimization. When the correct bias is reached, the new setting will be shown in the Bias box and the **Save Bias** button will become active. Click **Save Bias** so that the next time bias optimization is initiated, it will begin with this saved setting, making the optimization process faster.
4. Click **Calibrate** to home the excitation shutter to the **Closed** position.
5. Open the excitation shutter by clicking the **Open** radio button.
6. Click **Make an Exposure**. An image of the plate will be displayed. Examine the image for saturated pixels, which are indicated in pink. If you observe saturated pixels, reduce the exposure time, re-optimize the bias and collect another exposure. Continue reducing exposure time until no saturation is detected. If your first image does not show any saturation, increase the exposure time until saturated pixels are detected, then reduce exposure time to the longest time that does not result in saturated pixels.
7. Show the mask, by pointing the mouse to the center of well A1 (the top-left most well), and left click while holding down the Shift key. Alternatively, click on the **Show Masks button**, then use the arrow buttons to move the masks such that the top-left most box is approximately centered over well A1.
8. Click **Optimize Mask Positions** and the software will automatically resize and optimize the mask positions. An alert is displayed when mask alignment is completed or if it fails. If automatic mask alignment fails, repeat steps 7 and 8, aligning well A1 as closely as possible before clicking **Optimize Mask Positions**.
9. When the masks are properly set, click **Save Optimized Masks**. The new positions will be automatically written to the mask96.ini file. The new mask will be loaded and applied for all real-time experiments.

Note: To move an individual mask, first click on the well, turning the mask from green to blue. Now the arrow keys will affect the position of the blue mask only. When a single well is active, its individual identity, present inner and outer readings, and number of saturated pixels are displayed above the image. To restore simultaneous movement to all masks, click anywhere in the image outside the masks. The blue mask will become green, the well information will disappear and the arrows will affect all masks again.

6.2.3 Checking Mask Alignment

It is good practice to periodically inspect the alignment of the masks. To check the alignment of the masks, make an exposure of an external well factor plate or any other uniform plate that fluoresces in all 96 wells. Click **Show Masks** to display the masks and visually inspect the masks to confirm that each well's image is found exactly within the inner square of the corresponding mask position. If the masks are still lined up correctly, you may exit **Imaging Services** and continue using the current mask. Otherwise, realign the masks as described in Section 6.2.2.

6.2.4 Image File

You may save plate images to a file by clicking **Save Plate Image to File** and recall stored ones by clicking **Load Plate Image from File**. With either choice, a standard Windows Save or Open dialog box opens up to choose the destination or source files, respectively. Plate image files are saved with extension .ISI, and may only be viewed in the **Imaging Services** tab of the MyiQ software.

Note: Although ISI files may only be accessed through the **Imaging Services** screen in the MyiQ software, it is possible to make screen shots of your plate images. While viewing the desired plate image file in Imaging Services, press the **Print Screen** key, and then paste the screen shot into the desired application.

6.2.5 Collecting Persistent Well Factors

Well Factor Source: Persistent Well Factors

The persistent well factor approach must be employed whenever there are varying concentrations of the fluorophore in the individual wells of the plate, and for any other experiments for which well factors may not be collected from the experimental plate. Persistent well factors are collected from an external well factor plate containing External Well Factor Solution (Catalog #170-8794) at 1x concentration in all 96 wells (see Section 6.2.5.1). Persistent well factors must be created using the same reaction vessel type, e.g. plate and tape, as used in the experiment. Persistent well factors may be generated, saved to the PersistentWF.ini file and then applied to future experiments performed on the same MyiQ system. In general, persistent well factors are good for about one month, but should be collected again anytime something pertinent to the optical system is changed, such as the optical filters or the camera lamp.

6.2.5.1 Preparing the External Well Factor Plate

An external well factor plate is used to collect new persistent well factors, and may also be used to align the mask (see Section 6.2.2). Use the Bio-Rad External Well Factor Solution (Catalog # 170-8794) supplied as a 10x concentrate.

1. Dilute the 10x solution 1 part to 9 with ddH_2O. Prepare enough of the 1x well factor solution to fill all 96 wells. The external well factor plate must contain the same volume as the experimental plate in each well, and must be prepared in the same type of plate/tubes with the same type of sealing mechanism. For example, if the experimental plate contains 50 µl sample per well and is a Bio-Rad plate sealed with Bio-Rad optical sealing tape, the well factor plate should contain 50 µl 1x External Well Factor Solution in all 96 wells of a Bio-Rad plate sealed with Bio-Rad optical sealing tape.
2. Pipet the appropriate volume of 1x well factor solution into all 96 wells. Cover the wells with the same sealing mechanism as the experimental plate, and briefly spin the plate/tubes to bring all the reagents to the bottom of the wells.
3. You may check the well factor plate in Imaging Services (Section 6.2) to confirm that the 1x well factor solution gives a strong, but non-saturated image somewhere in the exposure range of 256-2048 ms.

6.2.5.2 Collecting Persistent Well Factors

Persistent well factors are collected from an external well factor plate, and the persistent well factor data are saved to the persistentWF.ini file, which is located in the C:\Program Files\BioRad\MyiQ\Ini folder by default. Additionally, every persistent well factor data file generated is also saved and archived to the C:\Program Files\Bio-Rad\MyiQ\Ini\PWFBackup folder by default, with the date and time of collection appended to the file name. To collect persistent well factors:

1. Prepare a well factor plate as described above.
2. Place the well factor plate into the reaction module and, in the **Imaging Services** tab of the **Run-Time Central module**, click **Calibrate** to reset the positioning of the excitation shutter (which defaults to the closed position). At this point, check mask alignment, as described in Section 6.2.2.
3. Click **Collect Persistent Well Factors**. The **Run Prep** screen will appear with the plate setup and protocol for persistent well factor collection already loaded. Note that you cannot edit the **Well Factor** protocol or plate setup.
4. Specify the sample volume of the well factor plate in the **Sample Volume** box.
5. Click **Begin Run** and the persistent well factor data collection process will begin.

This protocol will cycle the well factor plate three times between 95°C and 60°C. Then it will hold at 60°C for 90 seconds while optical data are collected and the well factors are calculated. A message box stating that "Persistent Well Factors have been generated" will appear when Persistent Well Factors have been successfully produced.

The new persistent well factor data will be saved to the persistentWF.ini file. The old persistentWF.ini file is overwritten, but can still be viewed in the C:\Program Files\Bio-Rad\MyiQ\Ini\PWFBackup archive folder. Note that you may reload an archived persistent well factor file by renaming it as PersistentWF.ini and moving it to the \Ini folder.

Cycle	Repeats	Step	Dwell Time	Setpoint
1	3	1	00:10	95.0
		2	00:30	60.0
2	2	1	00:45	60.0
3	1	1	00:10	60.0

Fig. 6.5. Persistent well factor protocol.

Fig. 6.6. As Persistent Well Factors are collected.

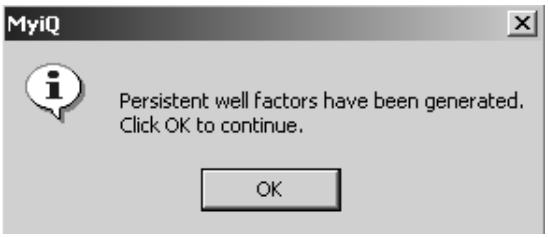


Fig. 6.7. After persistent well factors have been generated.

Section 7
Data Analysis Module

Introduction

The **Data Analysis** Module is where data are both presented and analyzed. When the MyiQ software opens, the Data Analysis icon is in the inactive state and the module is inaccessible. The Data Analysis module opens automatically during the execution of a protocol from **Run-Time Central**, or when opening a stored data file from the **Library**. The Data Analysis module consists of five separate tabs:

- **View/Save Data**
- **PCR Quantification**
- **Standard Curve**
- **Melt Curve**
- **End Point Analysis**

Each of these tabs is discussed in detail below. In each tab, except the **View/Save Data** tab, a **Reports** button is active.

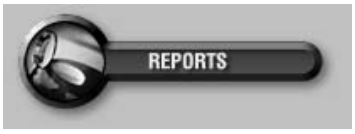


Fig. 7.1. Reports button.

Click this button to open up a customizable report template specific to the current window. Select the type of report, the mode of data presentation, and the destination (printer or file) for the report.

7.1 View/Save Data Tab

This window presents a view of the plate setup in four possible modes, each one displaying different information about the wells of the plate. The block temperatures are displayed to the right of the plate setup if a gradient Real-Time data collection step was programmed. If data was collected at more than one step then the information in this view is from the step chosen in the PCR Quantification tab (see Section 7.2.5).

It is in this window that post-run editing of the plate setup is carried out. When post-run editing, the sample type, identifier and standard quantity of any well can be altered. The units of measure and the fluorophore name for the entire plate may also be changed. Saving a data file is also initiated in this window.

Fig. 7.2. View/Save Data Window.

7.1.1 Viewing the Plate Setup

In each of the four views, the well identifier and sample type are listed. If a well has been removed from the analysis through the **Select Wells** box, which is accessible from the **PCR Quantification** tab, the identifier is overwritten with the word EXCLUDED. To toggle between the four different views, click on one of the four radio buttons, which are displayed across the top of the plate layout:

- **Ct**: This view is available for amplification experiments analyzed in PCR Baseline Subtracted or PCR Baseline Subtracted Curve Fit mode. It lists the threshold cycle for each well.
- **Concentrations**: This view is available for amplification experiments analyzed in PCR Baseline Subtracted or PCR Baseline Subtracted Curve Fit mode only if sufficient standards are defined to generate a standard curve via the PCR Standard Curve tab. Once the PCR Standard Curve tab has been opened, you may select this option in the View/Save Data tab. This view lists the calculated concentration of every unknown on the experimental plate together with the defined standard quantities.
- **Standard Quantities**: This is the default view. The starting quantity defined for each standard is shown.

- **End Point**: This view is available for amplification experiments analyzed in the **End Point Analysis** tab after negative and/or positive controls have been defined. Once an analysis has been performed in the End Point Analysis tab, you may select this option in the **View/Save Data** tab. This view lists positive controls as "Positive", and negative controls as "Negative". Unknowns called as positives are labeled "+", and unknowns called as negatives are labelled "-". Samples which cannot be called as positives or negatives are labelled "?".

7.1.2 Post-Run Editing

After an experiment is completed, you may edit the following attributes of the plate setup:

- Sample type of each well
- Identifier of each well
- Replicate number of each well
- Standard concentration of each well
- Units of measure for the entire plate
- Name of fluorophore for the entire plate

As the modifications are made, the data are reanalyzed with the new conditions. If the data file is subsequently saved, it is saved with the newly modified definitions, but the original definitions are also saved along with the data file. The changes persist until the wells are edited again or until **Restore Original Definitions** is clicked.

To edit a well description

1. Click on the well in the grid display. The **Edit Well** box will open up at the bottom of the screen, and the current definition of the well will be displayed.

Fig. 7.3. Post-run editing.

2. Choose the new sample type from the pull down menu.
3. Enter a new replicate number if desired.
4. If the sample type has been changed to a standard, enter a starting concentration for that standard. If the sample type remains a standard, enter a new starting concentration if desired.
5. Enter a new sample identifier if desired.
6. If the sample is a member of a replicate group, the changes can be applied to all members of the current replicate group by clicking the **Yes** check box in the column labeled **Apply to Replicates**?

7. To effect the edited changes and reanalyze the data file, click **Apply Changes to this Well**.
8. Edit any other wells you desire in the manner described above. Click **Hide** to make the **Edit Well** box disappear.
9. When all edits have been completed, save the data set again. The original definitions of all wells will be saved along with the edits. Click **Save ODM File**. The **View and Save Analysis Settings** window will open. Click **Save ODM File** in the new window, and a standard Save dialog box will open. Specify the destination directory, enter a new name, and click **Save**, or just click **Save** to overwrite the existing file. You may still restore the original definitions of all wells if you overwrite the existing file.

7.1.3 Saving ODM Files

When a data file is saved, all data analysis parameters, including the baseline cycles, the threshold, the fraction of data included in the analysis, and the type of digital filtering are saved along with post-run edits, melting curve peak edits, the raw data, and the name of the plate setup and protocol files used in the data analysis. The next time the file is opened, the data will be presented with the same settings for all analysis parameters. If any wells are excluded from the analysis when the data file is saved, they will still be excluded from the analysis the next time the file is opened. All wells can be restored to the analysis at any time by using the **Select Wells** feature and the original data are always preserved each time a file is saved.

In addition, there is also a convenient auto save feature that will automatically save a data file with all changes made to the analysis parameters anytime the software is closed or another data file is opened.

- To review data analysis settings and manually save a data file:
  1. Click **Save ODM file** from the **View/Save Data** tab. The **View and Save Analysis Settings** window will open.

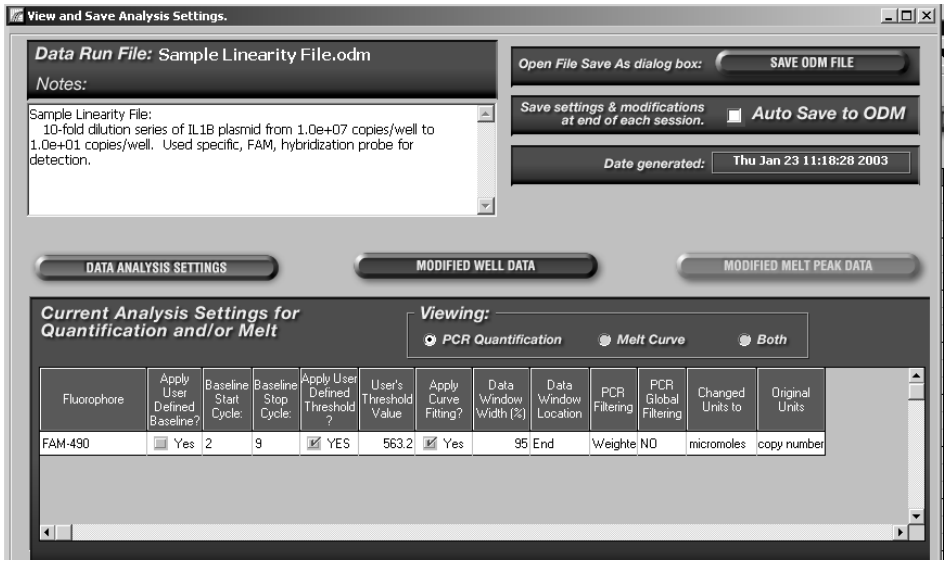


Fig. 7.4. View and Save Data Analysis Settings window.

2. In this window, you can review the data analysis parameters that will be saved by clicking **Data Analysis Settings**. A spreadsheet will open at the bottom of the window, displaying the present settings for all amplification data analysis parameters. The next time the file is opened, all these settings will be applied again automatically.

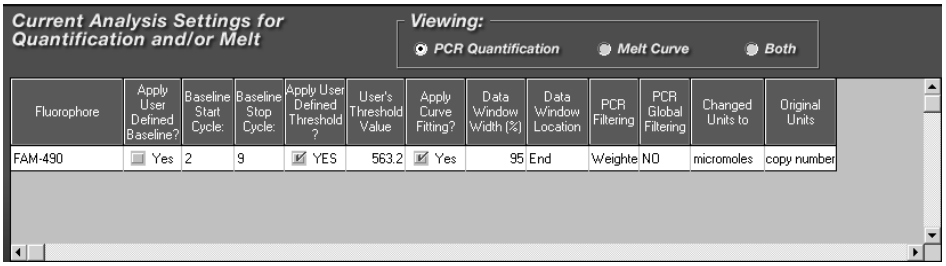


Fig. 7.5. Data Analysis Settings spreadsheet.

3. If any post-run edits were made to one or more wells, that information is also stored to the data file. Click **Modified Well Data** to open a different spreadsheet at the bottom of the window. The current definitions, as well as the original definitions, are listed for each modified well.

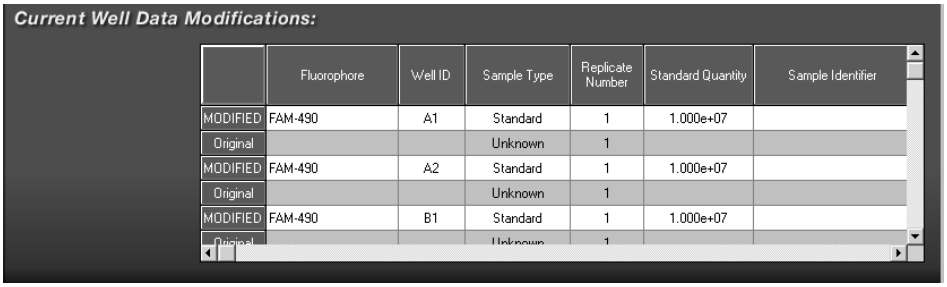


Fig. 7.6. Modified Well Data Spreadsheet.

4. If edits were made to any melting curve peaks, those changes are recorded and saved with the original settings. View them by clicking **Modified Melt Peak Data**.

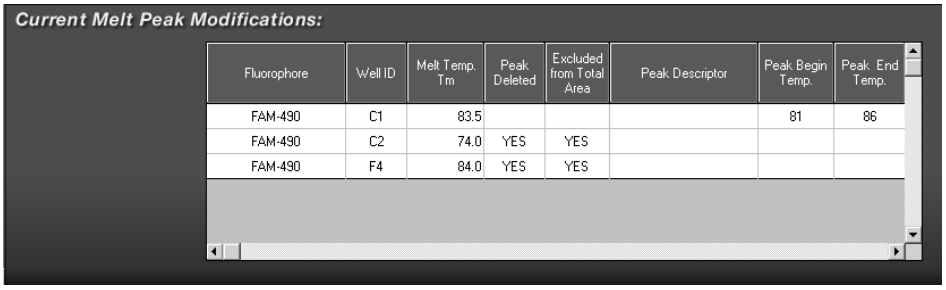


Fig. 7.7. Modified melt Peak Data spreadsheet.

5. Click **Save ODM File**.

6. A standard Save dialog box will open. Name the file, specify a destination directory, and click **Save**, or just click **Save** to overwrite the existing data file.

- To use the Auto save feature:
    1. Click **Save ODM file** from the **View/Save Data** tab. The **View and Save Analysis Settings** window will open.
    2. Click the **Auto Save to ODM** check box to make the software automatically save the data file and all analysis parameters each time you close the software or load a new data file.

Fig. 7.8. Auto Save.

If this box is not checked, each time the software is closed or a new data file is opened without saving the currently displayed data file, a warning will be displayed.

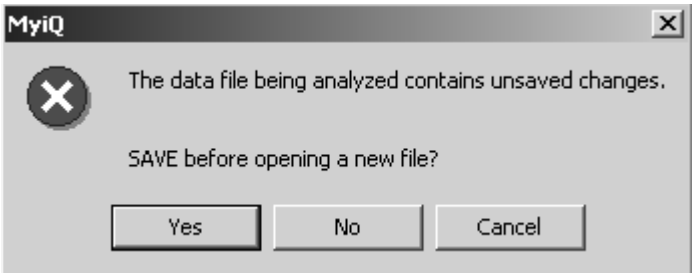


Fig. 7.9. Save data warning.

To save the current file and its analysis parameters, click **Yes** and a standard save dialog box will open. Choose a file name and click **Save**. Otherwise, click **No** in this window, the software will close or the desired data file will open, and all changes to the current file will be lost.

7.2 PCR Quantification Tab

At the beginning of the second repeat of the real-time data collection step, the **Data Analysis** module will become active, and the **PCR Quantification** tab will open, displaying the PCR amplification plot with data from the first repeat of the data collection step. The PCR amplification plot will be updated at the beginning of each repeat of the data collection step and will lag by one cycle. For example, at the beginning of the fourth repeat of the data collection step, data from repeat 3 are added to the plot. As data are collected, they are displayed on the PCR amplification plot in Background Subtracted mode. Furthermore, we recommend that you do not multi-task on the computer hosting the MyiQ during data collection steps.

Data analysis may begin upon the opening of a saved data set (see Section 4.4) or it may begin at the completion of an experiment. Only one data file may be open at a time. The PCR Quantification tab may only be opened for data files containing amplification data. For experiments lacking amplification data, such as Melt Curve-only or End Point-only experiments, the PCR Quantification tab will be labelled N/A and will remain unavailable.

7.2.1 Amplification Plot

The Amplification plot displays the relative fluorescence values for each well at every cycle. Each trace represents a single well, and at each cycle a single data point is plotted, which is the calculated mean of all data collected for that well during the particular cycle. As the analysis mode is changed from **Background Subtracted** to **PCR Base Line Subtracted** or **PCR Base Line Subtracted Curve Fit**, the plot is modified. It is possible to zoom in on the plot, and as you do, the labels on the graph axes are maintained.

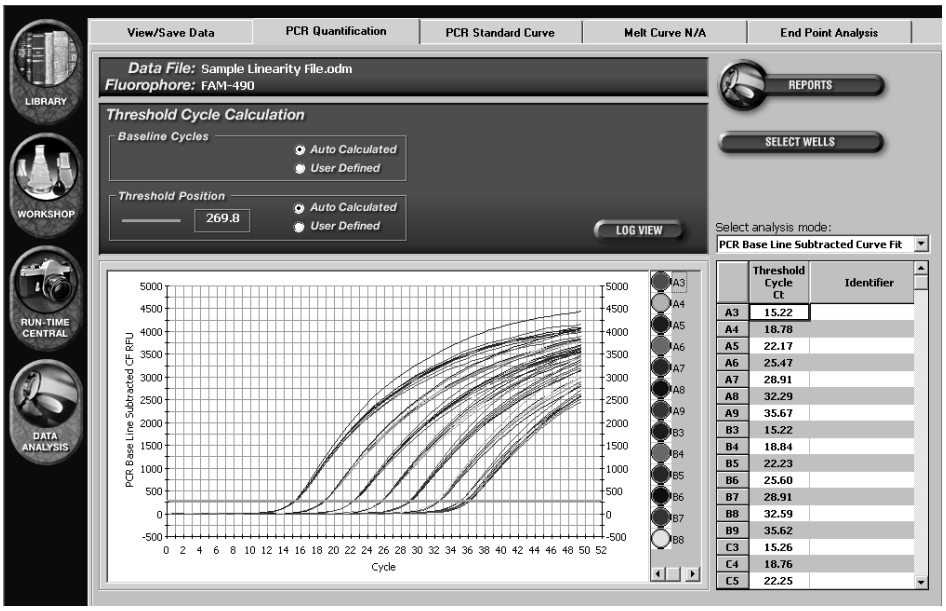


Fig. 7.10. PCR Amplification Plot.

- To zoom in on the plot, hold down the **Shift**, and then click and drag the mouse over the area of the plot you wish to zoom in on.
- To zoom out in small controlled steps, press **Control-Z** repeatedly.
- To zoom out at once, type **R** or choose **Restore Graph** from the context menu (right click on the plot to access the context menu).
- You can choose to limit the display to just one or more traces by clicking on the desired paint pots to the right of the amplification plot, holding down the **Control key** to make multiple selections at a time. To show all traces again, type **S** or choose **Show All Traces** from the context menu.
- You can toggle between the Semi-logarithmic view and normal view using the Log View/Normal View button. The text on this button is for the alternate view and does not represent the view currently displayed.

Fig. 7.11. The Log View Button.

7.2.2 Amplification Plot Context Menu

There are many data analysis or data display parameters that can be accessed by a right mouse click on the PCR amplification plot. This will open up a context menu.

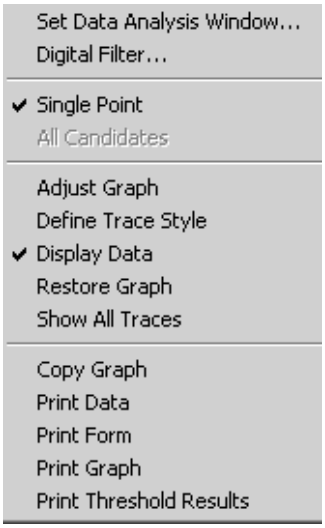


Fig. 7.12. PCR Quantification Plot Context menu.

A. Set Data Analysis Window…

By default, the PCR Base Line subtracted plots are constructed using the mean of the readings taken over the last 95% of each cycle. The number of data points collected depends on the exposure time and the dwell time at the cycle. You can change the percentage of data points used (the window) and you can choose to use data from the beginning of the cycle, the end of the cycle, or anywhere in between as follows:

1. Click the right mouse button on the **PCR Quantification** plot and choose **Set Data Analysis Window…** from the context menu. The **Set Data Analysis Window** will appear.
2. If you want data from the **Beginning of Cycle** or the **End of Cycle**, click the appropriate radio button and use the up and down arrows or the keyboard to input the percentage of data points to be used. Then click **OK** to reanalyze the experiment.
3. To center the analysis around data collected somewhere else in the cycle, first click the **Pick Window from Plot** radio button. Note that you may not select the Pick Window from Plot option while in PCR Base Line Subtracted Curve Fit mode. The instructions will be displayed in the window.

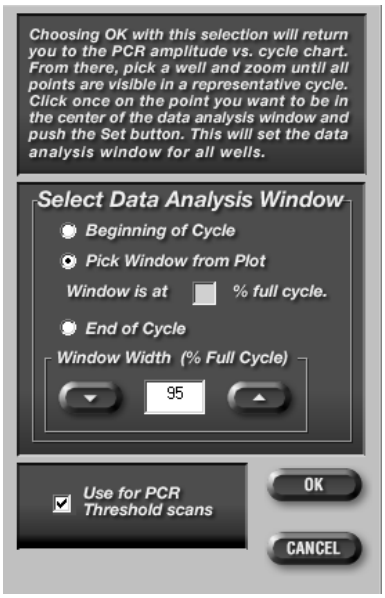


Fig. 7.13. Set Data Analysis window.

4. Use the up and down arrows or the keyboard to input the **Window Width (% Full Cycle)** for data analysis. If you choose 20, for example, then the analysis will include 10% of the data points on either side of the center point you will select in step 8.

5. Click **OK** to return to the PCR Quantification Plot.

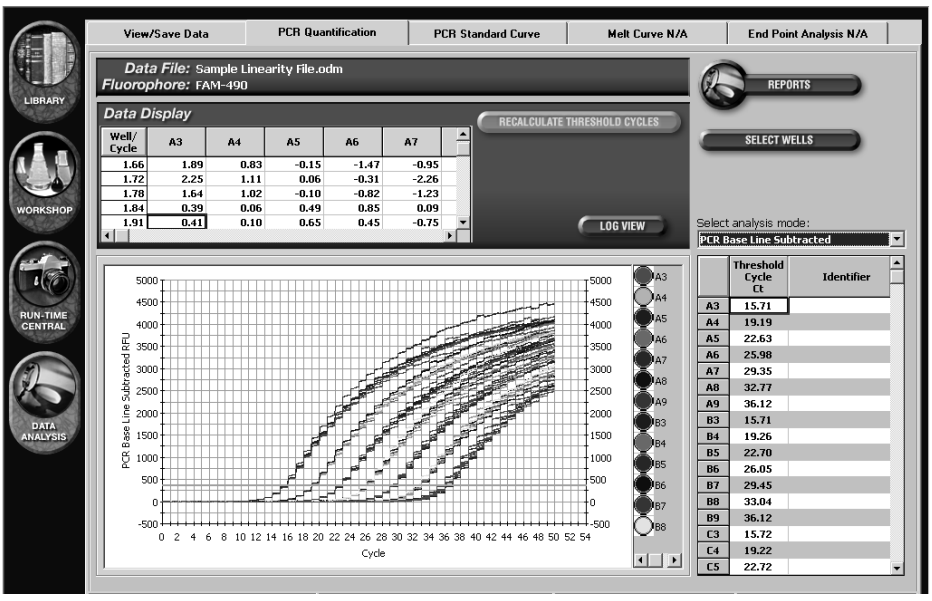


Fig. 7.14. All Candidates View.

6. The PCR Quantification plot will be displayed in the **All Candidates** mode, showing every single data point for each well. (In the default view of the plot, the individual data points are not actually shown. A smooth trace that passes through all the individual points is shown. It is easier in the subsequent steps if the data points are represented by some symbol. You can select a symbol by first choosing **Define Trace Style** option from the same context menu (see Section F). A spreadsheet will open showing wells and individual data points.

7. Choose a single well by clicking on its paint pot to the right of the plot. While holding down the Shift key, drag the mouse across a section of the plot to zoom in on. You may zoom in more than once. (Figure 7.15)

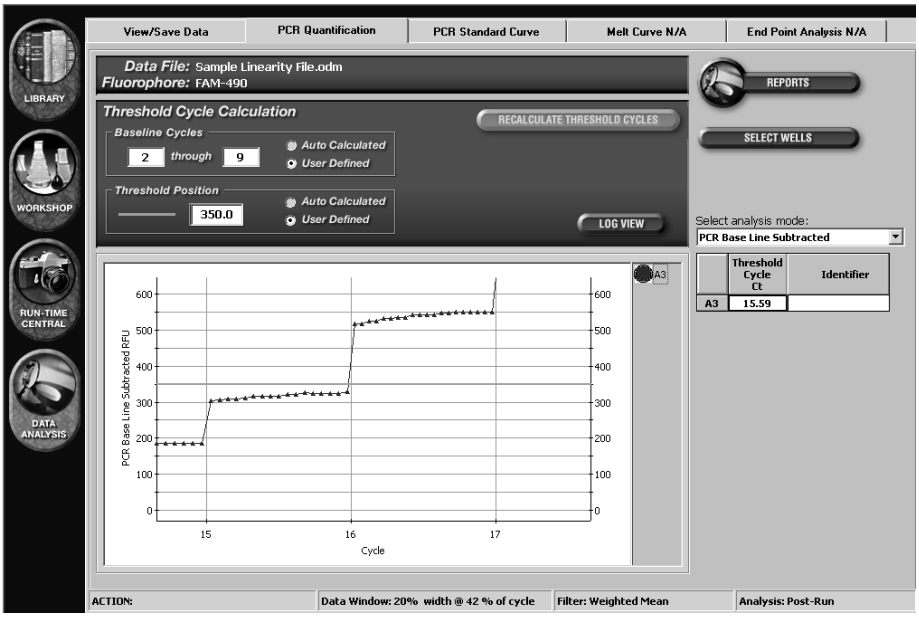


Fig. 7.15. Zoomed in view.

8. Click on the data point you want to center the data window upon. As you place the pointer on a data point, its reading and cycle number will be highlighted in the Data Display box.

9. Click **Set** at the bottom of the window to initiate recalculation. In this example, the PCR Base Line Subtracted plot will be recalculated using 20% of the data points collected near the middle of each cycle.

10. Use the context menu, by right clicking on the PCR quantification plot, to **Show All Traces**.

B. Digital Filter… There are two intra-cycle data filtering options available by choosing **Digital Filter…** from the context menu: a **Weighted Mean** and a **Rolling Boxcar**. The default filter is the weighted mean and it is the only one available during data acquisition.

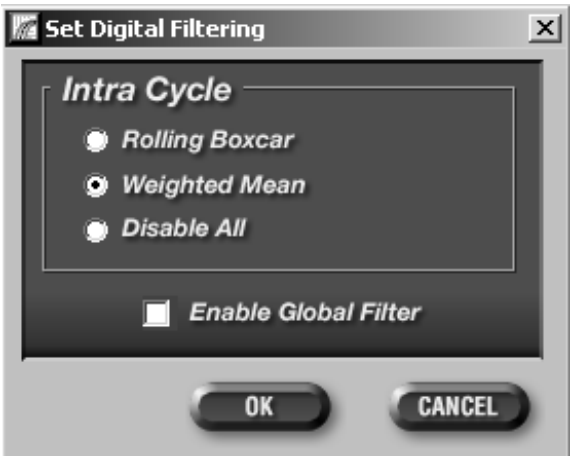


Fig. 7.16. Set Digital Filters.

The weighted mean is determined by the equation

$O_i = (R_i + c * M)/(1 + c)$

where:

O_i is the filtered value for data point i

R_i is the unfiltered value for data point i

c is a weight factor with default value of 2

M is the arithmetic mean of all data points for the well within the given cycle.

The rolling boxcar filter is the arithmetic mean of data readings i - (w-1) to i where w is the filter width. For example if you want to calculate the 20th data point (i=20) and the width is 4 (w=4), you take the mean of data points 17 through 20. Then data point 21 is the mean of data points 18-21, data point 22 is the mean of data points 19-22, etc.

The above filters are applied only within a cycle. A global filter that smoothes data from cycle to cycle is also available by clicking **Enable Global Filter**. The global filter operates on the trace for a given well using all cycles together in a single pass. Global filtering should normally be reserved for data that appear significantly noisy, with very jagged traces, and should not be applied routinely.

C. Single Point. This is the default mode of data presentation in which all data collected at a particular step are averaged and the average is plotted. For example, if four data points are collected during the third repeat of an amplification cycle, the mean of those four data points is plotted at cycle 3. The alternative to viewing the data in **Single Point** mode is to view the data in **All Candidates** mode.

D. All Candidates. In this mode, every single data point that is collected at each cycle is plotted. Normally this feature is used only in conjunction with the Set Data Analysis Window, but is sometimes very informative about the behavior of the PCR reaction and probe hybridization. The automated data analysis features cannot be used when the data are displayed in **All Candidates** mode. The alternative to viewing the data in All Candidates mode is to view the data in **Single Point** mode.

E. Adjust Graph. The amplification plot may be rescaled or changed from linear to log or vice versa, by this selection from the context menu. A new window will open.

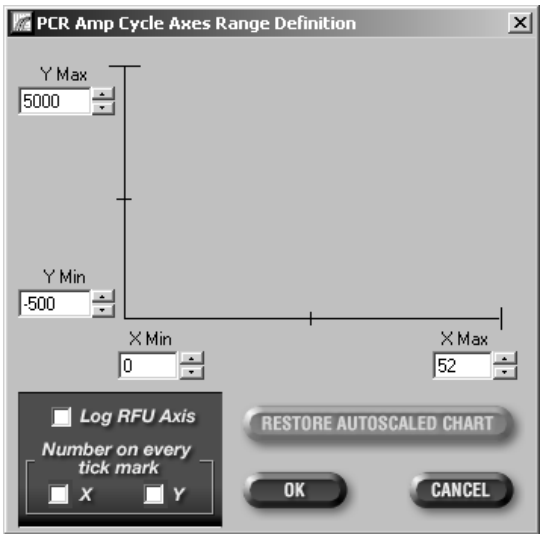


Fig. 7.17. Adjust Graph.

- The maximum and minimum values for both axes may be entered directly by first double clicking on and deleting the existing settings. Alternatively, the up and down arrows can be used to set the maximum and minimum values for both axes.
- Change to a semi-logarithmic display by clicking **Log RFU Axis**. Deselect this check box to revert to a linear plot.
- You may also specify whether to label every tick mark on either the X or Y axis of the plot with a number by clicking the appropriate **Number on every tick mark** check box.
- The plot settings may be reverted to the default by clicking **Restore Autoscaled Chart.**

F. Define Trace Style. This feature allows one to customize the display. Trace color and the symbol (if any) used to represent the data points may be changed on a well-by-well basis or in groups of sample types. All changes may be previewed before they are applied.

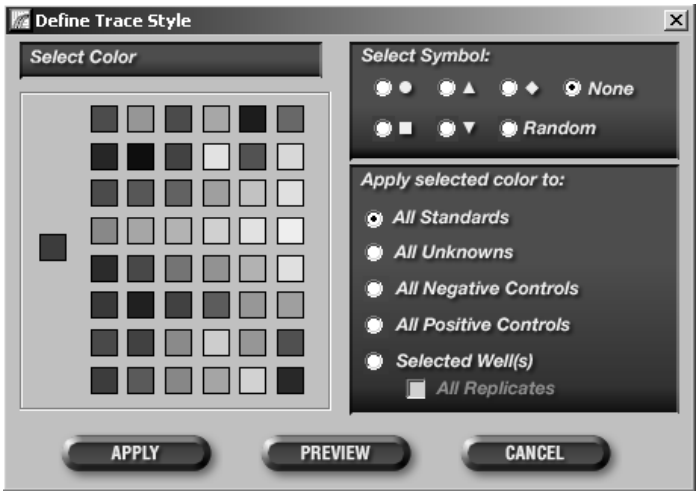


Fig. 7.18. Define Trace Style.

To modify a trace style:

1. Right mouse click on the Amplification plot and then select **Define Trace Style** from the context menu.
2. Choose the color for the trace.
3. Choose the symbol type.
4. Choose the type of traces to be modified (e.g., All Standards). If you want to choose on a well-by-well basis, click **Select Well(s)** and the display will change to show a representative grid.

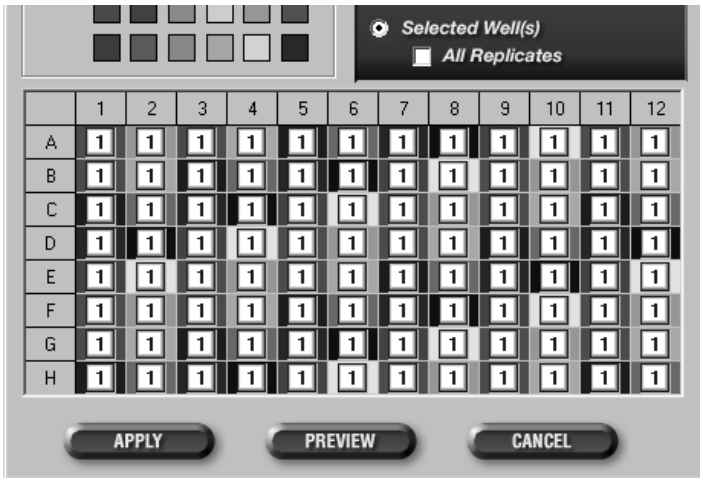


Fig. 7.19. Well selection for Define Trace Style.

5. Click on the wells to be changed in the grid. Click a column or row heading to change the entire column or row. The edge of each well in the grid will be outlined with the selected trace color. You may also click the **All Replicates** check box to apply these settings to replicates of wells that you click on.

6. Click **Preview** to see the changes and, if they are satisfactory, click **Apply**. It is not necessary to first click Preview, but after Apply is clicked, there is no way to restore the default settings without closing and reopening the file.

G. Display Data. To view the individual readings for each well, click the right mouse button over the plot and choose **Display Data** from the context menu. A small spreadsheet will appear above the amplification plot. Across the top are the well numbers, and down the sides are the cycle numbers. Use the scroll bars to navigate the data. In single point mode, each cycle is represented by one point that is the mean of all data points collected during that cycle. In All Candidates mode, each individual data point is displayed.

Note: These data may be copied to the clipboard for direct import into text and spreadsheet programs by clicking the top left corner of the spreadsheet and then typing **Control C**.

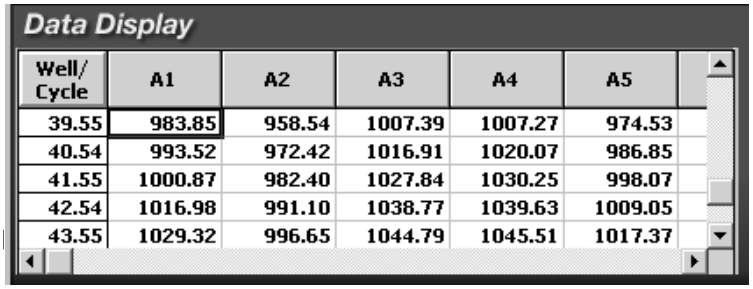


Fig. 7.20. Display Data.

H. Restore Graph. Use this to redraw the graph after zooming. The graph may also be restored by pressing the **R key**.

I. Show All Traces. Select this option to show all traces after singling out just one or more traces in the Amplification plot display. The traces may also be restored by pressing the **S key**.

J. Copy Graph. This will copy the Amplification plot to the clipboard for import into other programs.

K. Print Data. This option is only available when the **Display Data** spreadsheet has been opened from the context menu. Selecting this option prints the Display Data spreadsheet.

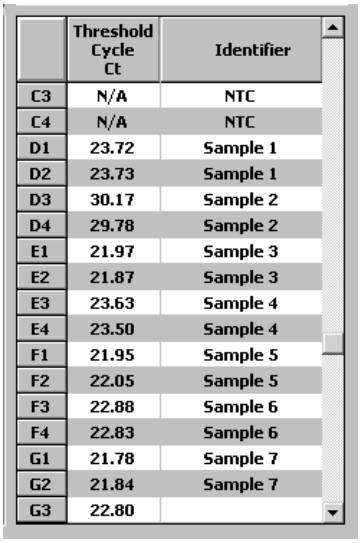
L. Print Form. This prints the entire page.

M. Print Graph. This prints only the graph.

N. Print Threshold Results. This prints the threshold cycles for each well as they appear to the right of the graph when the data are analyzed in **PCR Baseline Subtracted** or **PCR Baseline Subtracted Curve Fit** mode.

7.2.3 PCR Quantification Data Display

After the data are brought to **PCR Baseline Subtracted** or **PCR Baseline Subtracted Curve Fit** Mode, threshold cycles are determined and presented in the threshold cycle display box along with any identifier entered in the plate setup.



	Threshold Cycle Ct	Identifier
C3	N/A	NTC
C4	N/A	NTC
D1	23.72	Sample 1
D2	23.73	Sample 1
D3	30.17	Sample 2
D4	29.78	Sample 2
E1	21.97	Sample 3
E2	21.87	Sample 3
E3	23.63	Sample 4
E4	23.50	Sample 4
F1	21.95	Sample 5
F2	22.05	Sample 5
F3	22.88	Sample 6
F4	22.83	Sample 6
G1	21.78	Sample 7
G2	21.84	Sample 7
G3	22.80	

Fig. 7.21. PCR Quantification Threshold Cycle data display.

7.2.4 Select Analysis Mode

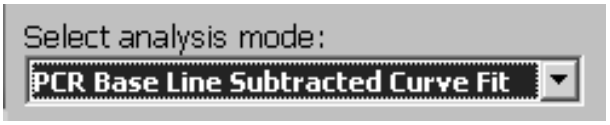


Fig. 7.22. Select Analysis mode.

- **Background Subtracted**. The background subtracted data are the relative fluorescence of each fluorophore after normalizing for exposure time and applying well factors. No further analysis is possible on background subtracted data.

- **PCR Baseline Subtracted**. In order to determine threshold cycles, construct standard curves and determine the concentration of unknown samples, the data must be PCR baseline subtracted. The PCR baseline subtracted trace is determined by fitting the best straight line through the recorded fluorescence of each well during the baseline cycles. The best fit data are then subtracted from the background subtracted data at each cycle to generate the PCR baseline subtracted trace. In the automated analysis mode, the optimal baseline cycles for each well are determined individually. In manual mode, the user selects one set of baseline cycles to be applied globally to every trace.

- **PCR Baseline Subtracted Curve Fit**. The PCR Baseline Subtracted data are fit to a smoothed curve using a balanced flank, centroid-finding digital filter. The curve fit process is performed in such a way that threshold crossing (Ct) is left invariant for all traces.

7.2.5 Select Data Set

If more than one data collection step was specified in the protocol file, this selection box will appear in the **PCR Quantification** window. By default, the step specified as the REAL-TIME step is the first one analyzed. To change to any other step(s), choose the desired step from the pull down menu.

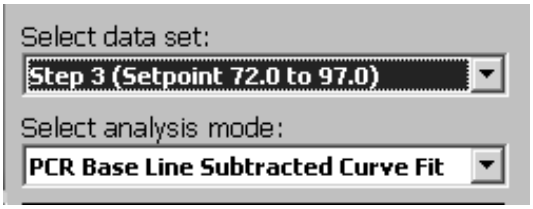


Fig. 7.23. Select Data Set.

7.2.6 Select Wells

Any subset of monitored wells may be included or excluded from the data analysis through the **Select Wells for Analysis** window. The original data are always preserved and excluded wells may be added back to the analysis at any time. However, removing wells from the analysis will affect calculation of the threshold and, hence, can affect standard curve calculation and quantification of unknowns.

Fig. 7.24. Select wells.

To select the wells included in the data analysis:

1. In the **PCR Quantificatio**n window, click **Select Wells** and the **Select Wells for Analysis** window will open.

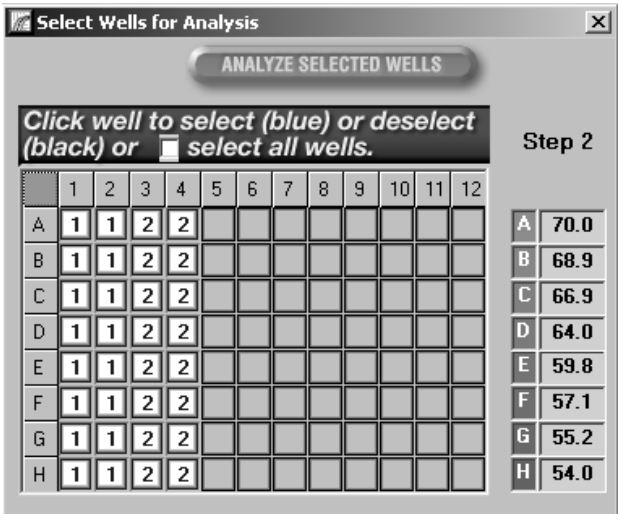


Fig. 7.25. Select Wells display.

2. To eliminate a row or column, click the appropriate letter or number heading, respectively. To eliminate an individual well, click on the well. Wells included in the analysis are shown in blue and wells excluded from the analysis are shown in black.
3. After selecting the desired wells, click **Analyze Selected Wells**. It will take a few moments for the data to be removed and for recalculations to be completed.
4. To add a well back to the analysis, reverse this procedure. You can add back wells one at a time, or an entire row or column at once. Alternatively, you may click the **Select all wells** box to restore all data to the analysis.

Note: Data are not permanently removed by this procedure. They are eliminated only from the present analysis, and you may always restore them to the analysis via the **Select Wells** button.

7.2.7 Threshold Cycle Calculation

Once the data are analyzed in **PCR Baseline Subtracted** or **PCR Baseline Subtracted Curve Fit** mode, the Threshold Cycle Calculation box appears.

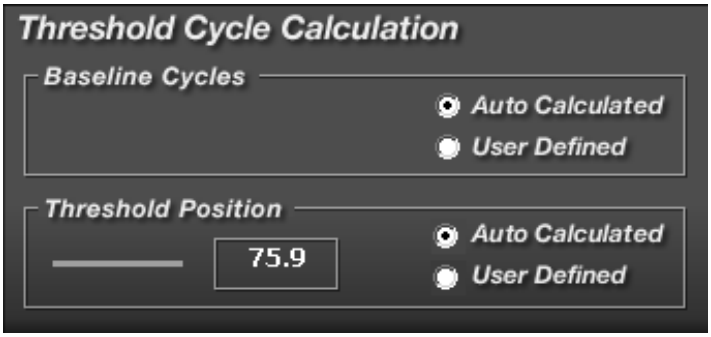


Fig. 7.26. Threshold Cycle Calculation.

By default, the baseline cycles and the threshold are automatically calculated when in **PCR Baseline Subtracted** or **PCR Baseline Subtracted Curve Fit** mode. The baseline cycles are determined individually for each trace, so that, for example, the trace of well A1 may have baseline cycles of 2–10, while the trace for well H12 may have baseline cycles of 4–24. The automatic threshold calculation is done in one of two ways. If there are standards defined on the experimental plate, the threshold is adjusted to attain the highest possible **Correlation Coefficient** value, for the **PCR Standard Curve**. If there are no standards on the plate, or not enough standards are valid, the threshold is determined by calculating the second derivative of each trace and looking for the point of maximum curvature. While the baseline cycles are determined individually for each trace, the threshold is a global value, and must be the same for all traces.

In the automatic calculation, the last 95% of the data collected at each cycle are included in the analysis, and the weighted mean digital filter is applied. Any or all automated analysis features may be manually overridden. It is allowable to combine some automated analysis with manually selected analysis parameters, for example, automatic determination of baseline cycles may be combined with manual specification of the threshold cycle.

The amount of data included in the analysis can be modified by choosing **Set Data Analysis Window…**, and the type of digital filtering can be modified by choosing **Digital Filter…** from the context menu on the PCR Amplification plot.

- **Manual definition of baseline cycles**. To override the automatic calculation of individual baseline cycles, click **User Defined** near the baseline cycles display. Two new baseline cycle fields will appear; one for the beginning and one for the end. User defined baseline cycles default to cycles 2-10. Enter new values into either or both fields and then click **Recalculate Threshold Cycles**. Note that when manually defining the threshold values, all traces are analyzed with the same baseline cycles.

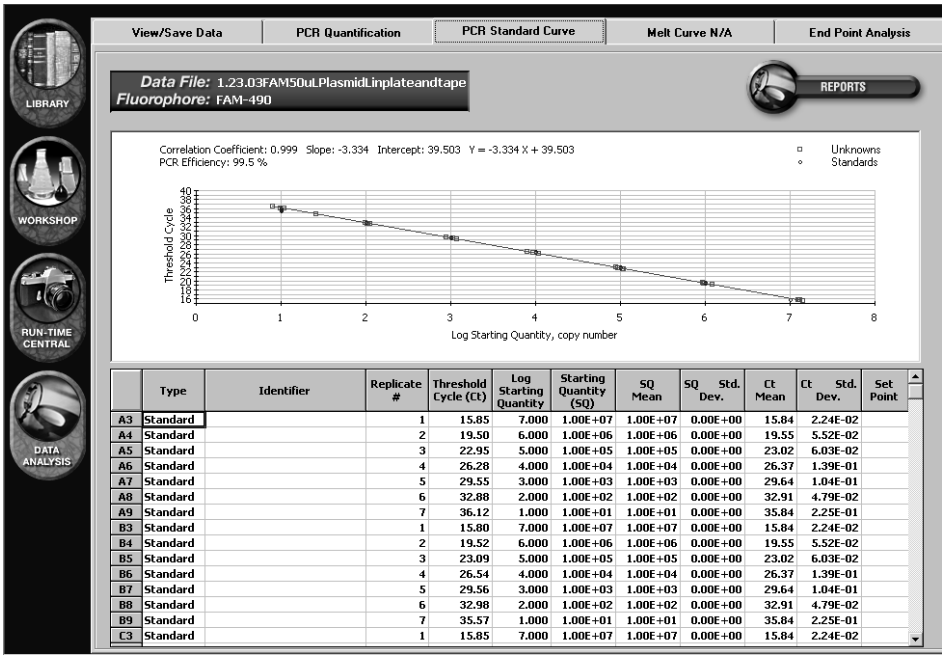
The purpose of the baseline cycle calculation is to characterize and correct for drift in the background fluorescence over the course of the experiment. Data are generally improved by extending the baseline cycles to include as many cycles as possible before any of the traces begin to rise above background.

- **Manual definition of threshold**. To manually define the threshold, click **User Defined** near the displayed threshold position. The last user-defined threshold will be recalled. If no user-defined threshold has ever been specified, then a default value of 10 times the mean standard deviation of all traces over the baseline cycles will be used. The new threshold may be specified in two ways: enter it directly into the Threshold Position field or drag the orange threshold bar with the mouse and move it to the desired location. When the new threshold is set, click **Recalculate Threshold Cycles**.

The threshold value is a specific RFU value that serves as a reference for comparing your amplifications. The cycle at which each sample crosses the threshold value is known as the threshold cycle, or Ct. The threshold should not be set outside the region of exponential amplification – generally before the the slope of the PCR Amplification plot begins to decline. This is best observed in the logarithmic view as the region of the plot that appears to be a straight line. Observing the data in logarithmic view can also make it easier to identify anomalous wells or outliers.

7.3 Standard Curve Window

Once PCR Baseline Subtraction or PCR Baseline Subtraction Curve Fit has been carried out, the standard curve is generated. To view it, select the **PCR Standard Curve** tab. Note that you must have valid standards defined on your plate setup file to generate a standard curve. If you did prepare a standard curve, but forgot to define the standards in the plate setup, see Section 7.1.2 for details on Post-Run Editing.



	Type	Identifier	Replicate #	Threshold Cycle (Ct)	Log Starting Quantity	Starting Quantity (SQ)	SQ Mean	SQ Std. Dev.	Ct Mean	Ct Std. Dev.	Set Point
A3	Standard		1	15.85	7.000	1.00E+07	1.00E+07	0.00E+00	15.84	2.24E-02	
A4	Standard		2	19.50	6.000	1.00E+06	1.00E+06	0.00E+00	19.55	5.52E-02	
A5	Standard		3	22.95	5.000	1.00E+05	1.00E+05	0.00E+00	23.02	6.03E-02	
A6	Standard		4	26.28	4.000	1.00E+04	1.00E+04	0.00E+00	26.37	1.39E-01	
A7	Standard		5	29.55	3.000	1.00E+03	1.00E+03	0.00E+00	29.64	1.04E-01	
A8	Standard		6	32.88	2.000	1.00E+02	1.00E+02	0.00E+00	32.91	4.79E-02	
A9	Standard		7	36.12	1.000	1.00E+01	1.00E+01	0.00E+00	35.84	2.25E-01	
B3	Standard		1	15.80	7.000	1.00E+07	1.00E+07	0.00E+00	15.84	2.24E-02	
B4	Standard		2	19.52	6.000	1.00E+06	1.00E+06	0.00E+00	19.55	5.52E-02	
B5	Standard		3	23.09	5.000	1.00E+05	1.00E+05	0.00E+00	23.02	6.03E-02	
B6	Standard		4	26.54	4.000	1.00E+04	1.00E+04	0.00E+00	26.37	1.39E-01	
B7	Standard		5	29.56	3.000	1.00E+03	1.00E+03	0.00E+00	29.64	1.04E-01	
B8	Standard		6	32.98	2.000	1.00E+02	1.00E+02	0.00E+00	32.91	4.79E-02	
B9	Standard		7	35.57	1.000	1.00E+01	1.00E+01	0.00E+00	35.84	2.25E-01	
C3	Standard		1	15.85	7.000	1.00E+07	1.00E+07	0.00E+00	15.84	2.24E-02	

Fig. 7.27. Standard Curve Window.

- At the top of the plot, the correlation coefficient, the slope, intercept, and equation of the best-fit line, as well as the calculated PCR efficiency are displayed. At the bottom of the window is a spreadsheet that lists for each well the sample type, identifier, threshold cycle, calculated or input starting concentration, and statistics (means and standard deviations) for threshold cycles (Ct) and calculated starting quantities (SQ). The Set point column reports the temperature of data collection when a temperature gradient is included in the protocol.
- The data in the standard curve spreadsheet may be copied to the clipboard for export to another program by clicking in the top left corner and pressing **Control-C**.
- You may zoom in on the standard curve plot by holding Shift and dragging the mouse across the desired region. To zoom out in controlled steps, press **Control-Z** repeatedly. Alternatively, right mouse click on the plot and choose **Restore Graph** or simply press **R** to restore the graph to default settings.
- A right mouse click on the standard curve plot will bring up a context menu with the following options:

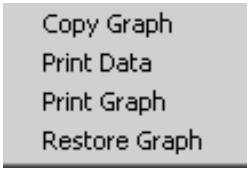


Fig. 7.28. Standard Curve Context menu.

Copy Graph will copy the graph to the clipboard.
Print Data will print the spreadsheet data.
Print Graph will print only the graph.
Restore Graph is only active after zooming in. This will zoom the plot out again.

7.4 Melt Curve Window

Melting curve data are displayed in this window. To open the **Melt Curve** window, the current data file must have included a melt curve analysis step. If the current data file does not include a melt curve step, the **Melt Curve** tab will remain unavailable and tagged **N/A**.

7.4.1 Melting Curve Plot

The melting curve plot has two presentations. The first plot is of the temperature versus fluorescence. These are the data as acquired during execution of the melting curve protocol.

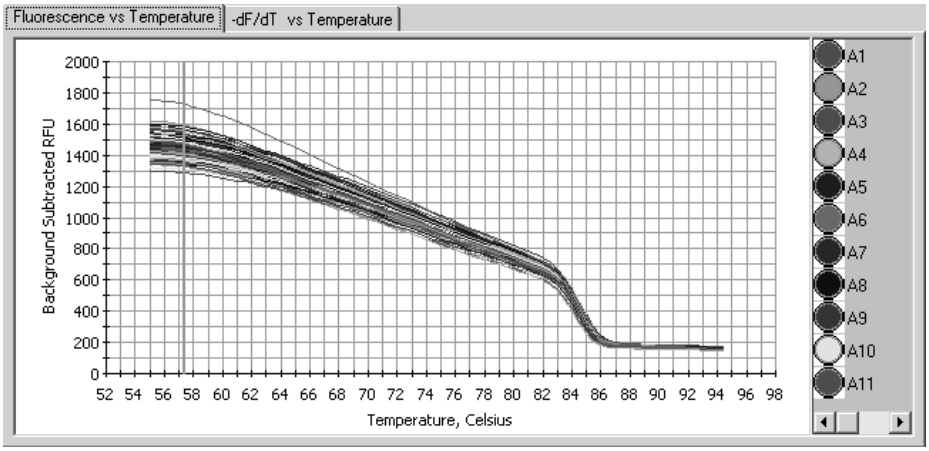


Fig. 7.29. Fluorescence vs Temperature Plot.

The second plot is of the negative first derivative of the temperature versus fluorescence (-dF/dT) plotted against temperature. It is from this plot that melting peak temperatures are derived.

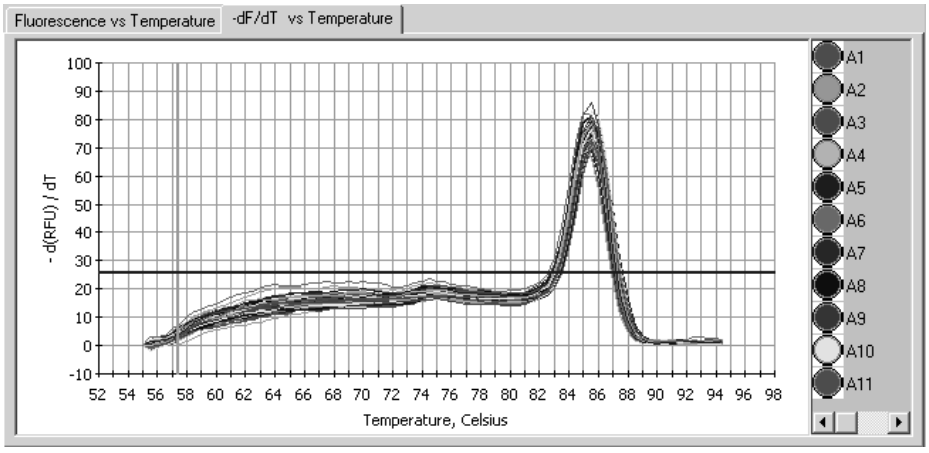


Fig. 7.30. First derivative plot.

- It is possible to display the traces of just one well by clicking on its paint pot on the right side of the plot. To display several wells, hold down the **Control** key as you click the paint pots.
- To show all the traces after singling out just one or more traces, press the **S** key.
- To zoom in on the plot, hold down **Shift** and drag with the mouse.
- To zoom out at once, choose **Restore Graph** from the context menu or press the **R** key. To zoom out in controlled steps, press **Control-Z** repeatedly.

7.4.2 Melting Curve Plot Context Menu

A right mouse click on either of the melting curve plots brings up a context menu.

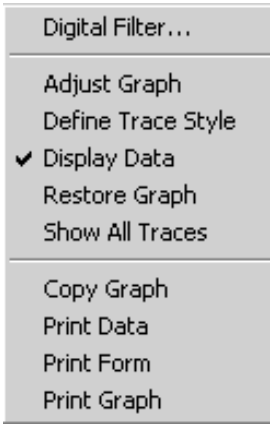


Fig. 7.31. Melting Curve Plot Context menu.

A. Digital Filter. The melt curve data are digitally filtered by default. The type, if any, of filtering may be changed through this entry. The digital filtering is described in the discussion on the **PCR Quantification** window (see Section 7.2.2).

B. Adjust Graph. This feature allows you to change the way that the plot is presented. It is described in the discussion on the PCR Quantification window (see Section 7.2.2).

C. Define Trace Style. This allows you to customize the traces on the melting curve plots. It is described in the discussion on the PCR Quantification window (see Section 7.2.2.).

D. Display Data. This selection will open a **Data Display** spreadsheet that presents, for each well, the RFU collected at each temperature. The data may be copied to the clipboard for import into other programs by clicking in the top left corner and then pressing **Control-C**.

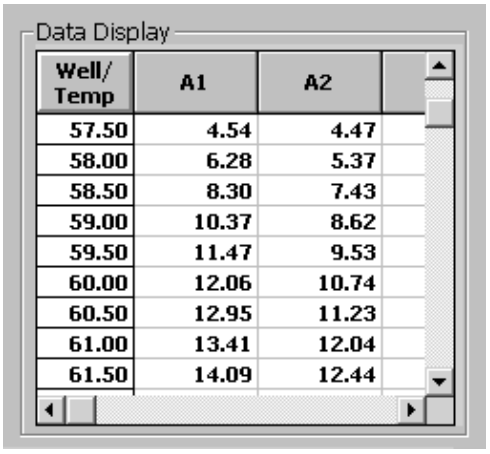


Fig. 7.32. Melting Curve Plot Data Display.

E. Restore Graph. Use this to redraw the graph after zooming. The graph may also be restored in steps by pressing **Control-Z**, or all at once by pressing the **R** key.

F. Show All Traces. Select this to restore any trace that was removed from the Melt Curve plot. The traces may also be restored by pressing the **S** key.

G. Copy Graph. This will copy the displayed Melt Curve plot to the clipboard for import into other programs.

H. Print Data. This option is only available once the **Data Display** spreadsheet has been opened from the context menu. Selecting this option will print the Data Display spreadsheet.

I. Print Form. This prints the entire page.

J. Print Graph. This prints only the graph.

7.4.3 Melting Curve Data Spreadsheet

The software will automatically identify and characterize all peaks above the blue Peak bar and then display the results in a spreadsheet. Note that you must click **Apply Changes to Melt Peaks** after moving the Peak bar in order to update the changes.

- For each well, the peaks are listed in order of decreasing melting temperature.

- Each peak is assigned a Peak ID that begins with the well number followed by peak number. For example, if there are six peaks found for trace A1, the peaks are labeled A1.1 through A1.6.

	Identifier	Peak ID	Melt Temp.	Peak Descriptor	Begin Temp.	End Temp.
A1		A1.1	85.5		81.0	90.0
A2		A2.1	85.5		81.0	91.0
A3		A3.1	86.0		81.0	90.0
A4		A4.1	86.0		81.0	90.0
A5		A5.1	86.0		81.5	91.5
A6		A6.1	86.0		79.5	90.5
A7		A7.1	86.0		80.5	90.5
A8		A8.1	86.0		81.0	92.0
A9		A9.1	85.5		81.5	90.0
A10		A10.1	85.5		80.0	90.0

Fig. 7.33. Melting Curve Plot Spreadsheet.

- For each peak, the beginning and ending temperatures are listed along with the melting temperature, which is defined as the highest point in the melting peak. The peak beginning and ending temperatures may be adjusted manually as described in section 7.4.6 below. There is also a field for the entry of a descriptive name for each peak. The melt peak data may be copied to the clipboard for import into another program by clicking in the top left corner and pressing **Control-C**.

7.4.4 Select Wells

Any subset of monitored wells may be included or excluded from the data analysis through the **Select Wells for Analysis** window. The original data are always preserved, and excluded wells may always be added back to the analysis at any time. This feature has been described in previously in the discussion of the **PCR Quantification** window (See Section 7.2.6).

7.4.5 Open/Save Settings

The melting curve data are saved automatically at the end of the protocol to the specified ODM file. Each time the data are saved, all modifications to the analysis, including adjustments to melting temperatures and peak deletions, are be saved to the file, and the next time the file is opened, all modifications are applied to the data again. However, the original data are always preserved and may always be restored by clicking **Undo All Melt Peak Changes**.

To save a data file, start by clicking **Open/Save Settings**. In the **View and Save Analysis Savings Window**, review changes to the analysis by clicking **Modified Melt Peak Data**, then click **Save ODM File**. At the Save dialog box, enter a new filename if desired and then click **Save**. This procedure is presented in detail in the discussion of the PCR Quantification Window (See Section 7.1.3).

7.4.6 Peak Bar

Only peaks that are above the Peak bar, represented by the blue horizontal line displayed across the graph, are included in the analysis. The position of the peak bar can be manually adjusted with the cursor.

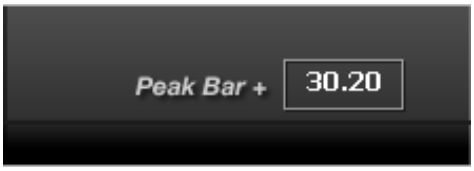


Fig. 7.34. Peak Bar.

1. To adjust the peak bar, click and drag the bar to a new position. The new peak height will be displayed in the **Peak Bar** field, and the **Apply Changes to Melt Peaks** button will become active.
2. Click **Apply Changes to Melt Peaks** to update the spreadsheet.
3. The melt peak spreadsheet will be updated, and the melt peaks will be renumbered to reflect the changes.

7.4.7 Temperature Bar

The orange temperature bar is a convenient way to identify the temperature at any point. Click and drag the vertical orange bar with the cursor to the desired point and the temperature will be displayed in the **Temperature Bar** box. This bar is also used to edit melting peak begin and end temperatures.

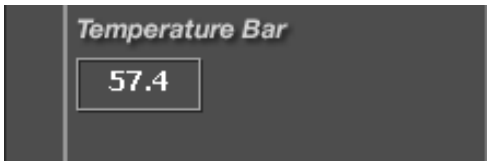


Fig. 7.35. Temperature Bar.

7.4.8 Edit Melt Peak Begin/End Temps

You can override the automatic assignment of the beginning or ending temperature for any peak as follows:

1. Check the **Edit Melt Peak Begin/End Temps** box.
2. You may click on the paint pot of the trace to be edited to get a better view.
3. Click in the Begin Temp. or End Temp. column, depending on which value you wish to edit.
4. Drag the orange temperature bar to the new Begin Temp or End Temp. As you drag the bar, the temperature field is updated.

Note: When editing melt peak temperatures, the Begin Temp must always be lower than the End Temp. In addition, you may not edit the Begin and End Temps in such a way that they completely overlap an adjacent peak for the same well. You may, however, delete the adjacent peak and then apply the desired changes made to the Begin Temp and/or End Temp.

5. Click **Apply Changes to Melt Peaks**.

6. To restore all traces, right mouse click on the melting curve plot and choose **Show All Traces** from the context menu.
7. The original beginning and ending temperatures may be restored by clicking **Undo All Melt Peak Changes**.

7.4.9 Delete Peaks

Peaks may be removed from the spreadsheet and the analysis in the following manner:

1. Check the **Delete Melt Peaks** box.
2. Within the melt peak spreadsheet, click on any cell in the row of the peak you want to delete. The peak will be removed from the spreadsheet.

Note: You may not delete the last melt peak for any particular well. If there is only one melt peak reported for a certain well, you may not delete this peak.

3. Click **Apply Changes to Melt Peaks** to effect the removal of the peaks. The remaining melt peaks will be renumbered. The deleted peaks can be later restored to the analysis by clicking **Undo All Melt Peak Changes**.

Section 8
End Point Analysis

The **End Point Analysis** module provides a convenient method of analyzing final RFU, or Relative Fluorescence Unit, values. End Point analysis can be performed in two ways: by selecting the **End Point Analysis** tab for an existing data file, or by clicking **End Point Analysis Run** to initiate the collection of End Point data from a sample plate.

8.1 End Point Analysis of Existing Data Files

Within the **Data Analysis** module, any MyiQ data file with PCR Quantification data can be viewed in the **End Point Analysis** tab. However, the corresponding PCR Quantification data must be in either the **PCR Base Line Subtracted** view or the **PCR Base Line Subtracted Curve Fit** view before the **End Point Analysis** tab is enabled. Furthermore, you may choose to include only certain wells under either tab, since well selection is synchronized between the **PCR Quantification** tab and the **End Point Analysis** tab.

8.2 Performing an End Point Analysis Run (also called End Point Only Run)

End Point Analysis Runs require a current or "valid" Persistent Well Factor file (see Section 6.2.5). During an **End Point Analysis** run, two repeats of a 30-second step at 60°C will be performed.

To initiate the collection of End Point data from a sample plate:

1. Insert the plate into the reaction module.
2. Create or open a plate setup file from either the **View Plate Setup** tab or the **Edit Plate Setup** tab. Note that any positive or negative controls defined in the loaded plate setup will be exported to the **End Point Analysis** table after the run. However, you may always define all your controls post-run from the **End Point Analysis** tab and/or the **View/Save Data** tab.
3. Click on the **End Point Analysis Run** button, and the **Run Prep** tab will appear.

Fig. 8.1. End Point Analysis Run Button.

4. Specify the sample volume of your experimental plate in the **Sample Volume** box.

Fig. 8.2 End Point Begin Run Button

5. Click **Begin Run**, give the data file a name, and the End Point Analysis run will begin. Upon the completion of End Point data collection, the End Point Analysis tab will appear, displaying the results.

8.3 Analyzing Endpoint Data

End Point data are displayed immediately following an **End Point Analysis Run**. Although any file with amplification data may be analyzed post-run in both the **PCR Quantification** and **End Point Analysis** tabs, an **End Point Analysis Run** may only be analyzed in the **End Point Analysis** tab.

The **End Point Analysis** tab is comprised of several sections; each one described below.

8.3.1 Methods

Below the file and fluorophore information is the **Method** box. The **Method** box allows you to select the method of assigning positive and negative values to your unknowns. The **Method** box consists of the following three choices:

- **Positives**: Select this method to use positive controls to define/call unknown samples. Samples are considered positive if they are greater in RFU value than the positive control average minus the tolerance.

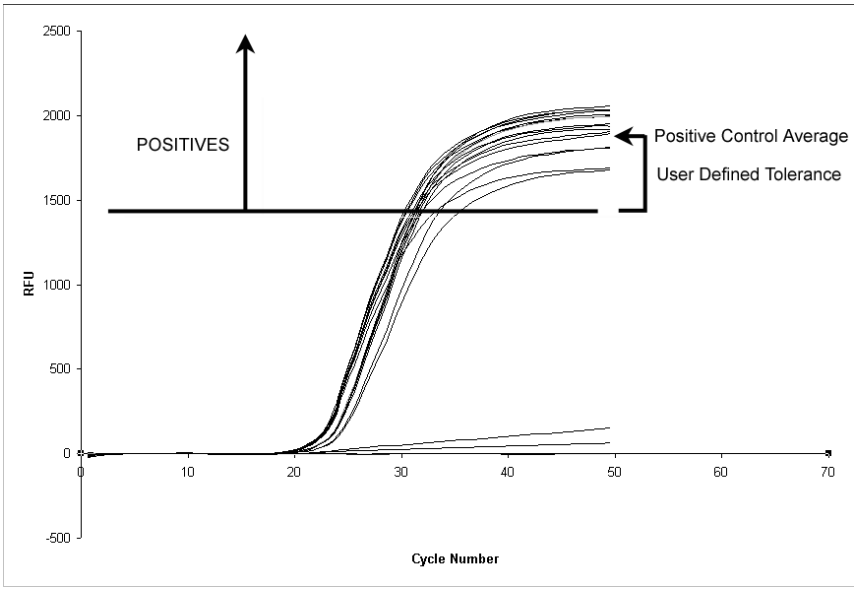


Fig. 8.3. Positive Method.

- **Non-Negatives**: Select this method to use negative controls to define/call unknown samples. Samples are considered positive if they are greater in RFU value than the negative control average plus the tolerance.

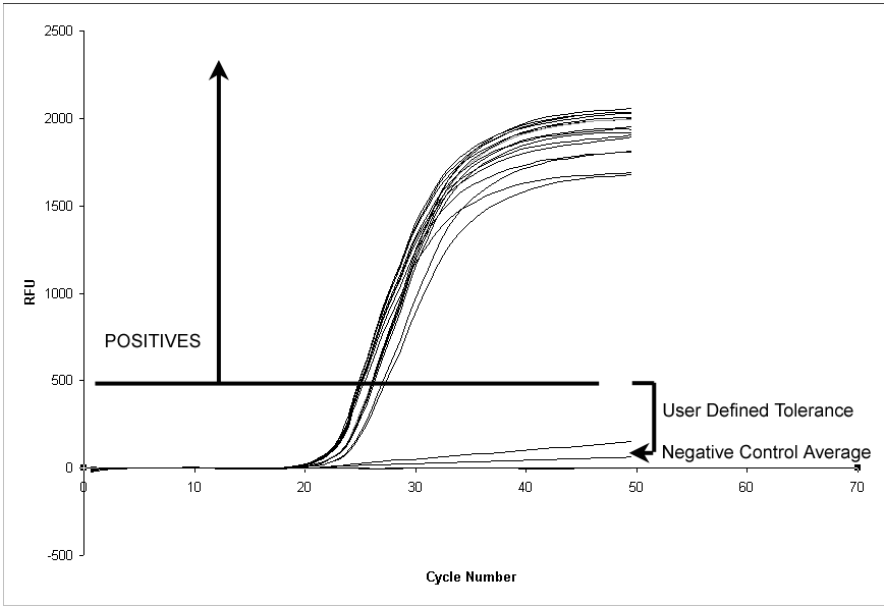


Fig. 8.4. Non-Negative Method.

- **Positives & Negatives**: Select this method to use positive and negative controls to define/call unknown samples. Samples are considered positive if they are greater in RFU value than the positive control average minus the tolerance, and are considered negative if their RFU value is less than the negative control average plus the tolerance.

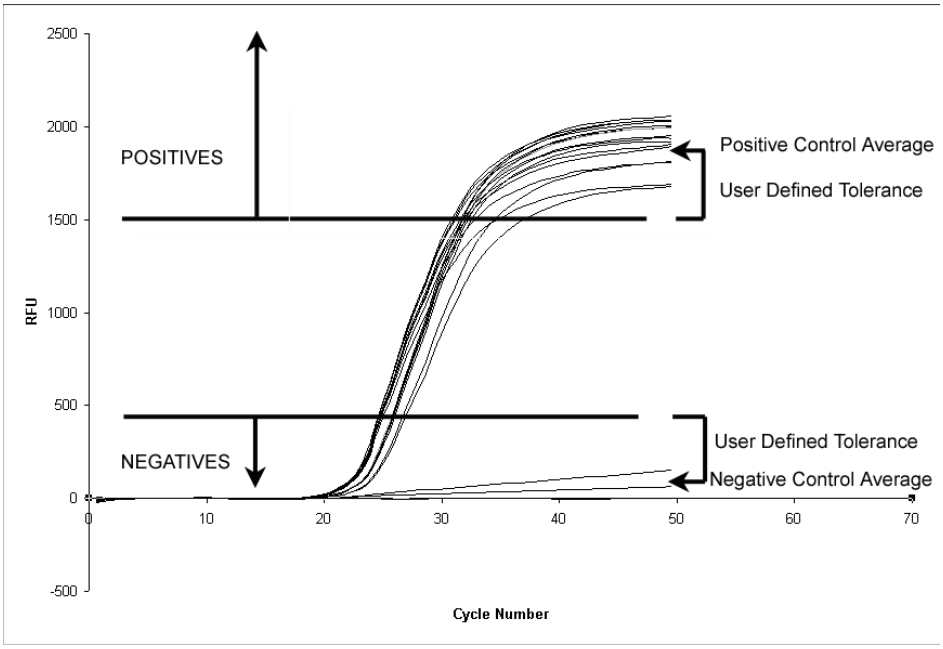


Fig. 8.5. Positives & Negatives Method

8.3.2 End Point Tolerance

End Point Tolerance defines the margins for sorting unknowns as positives or negatives. How the tolerance variable and the type of tolerance are applied depends on which **Method** is selected. The **End Point Tolerance** box consists of the two choices:

- **RFUs**: This is the default Tolerance choice and should be selected if you would like to use an absolute RFU value for the tolerance value. The minimum RFU tolerance value is 2, whereas the maximum is the absolute value of the highest RFU minus the lowest RFU value. The default RFU tolerance value is 10% of the total RFU range (see below).

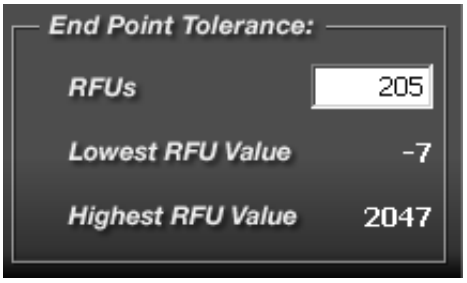


Fig. 8.6. End Point Tolerance using RFU values.

- **Percent of Range**: This Tolerance choice is available as a context sensitive menu and is accessed by right clicking on the **End Point Tolerance** box. Select this setting to use a percentage of the RFU range for the tolerance value. The minimum percent of range is 1%, whereas the maximum percent of range is 99%. The default percent of range tolerance is 10%.

The definition of the **RFU range** is dependent on the **Method** chosen.

For the **Positive** Method - the range is the Positive control Average minus the Lowest RFU value.

For the **Non-Negative** Method - the range is the Highest RFU value minus the Negative control average.

For the **Positives & Negatives** Method - the range is the Positive control average minus the Negative control average

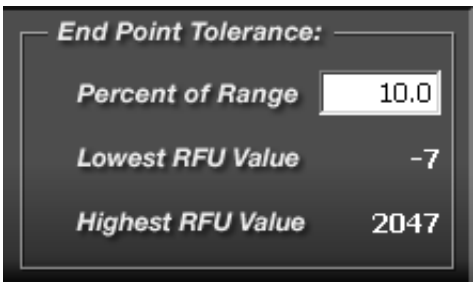


Fig. 8.7. End Point Tolerance using Percentage of Range values.

Below the **Method** box, are the **End Cycles to Average** and **Number of Ranks** text boxes as well as the **Sort Data by Call** check box.

8.3.3 End Cycles to Average

- **End cycles to Average** is the number of cycles from the last cycle, that will be used to calculate an average End Point RFU value. The end cycles to average field defaults to 2 for End Point Only runs, and 5 for non-End Point Only runs. In order for non-End Point Only data to be analyzed in the **End Point Analysis** tab, at least 6 repeats at data acquisition must be performed.

8.3.4 Number of Ranks

- The **Number of Ranks** allows assignment of samples into distinct groups based on their RFU values. The absolute range (Highest RFU minus Lowest RFU) is divided by the number of ranks selected. The default rank value is 10 and the minumum number of ranks is 3.

  Colored rank boxes, displayed below **Number of Ranks** and **Sort Data by Call**, symbolize the number and order of ranks in the End Point Analysis. To the right of the colored rank boxes are five color-gradation buttons that allow a change in the color scheme of the rank boxes, once the data are analyzed.

8.3.5 Sort Data by Call

This function is used to sort End Point Samples into Positive and Negative Calls (as well as No Calls wells which do not fall into the previous two categories). Sort Data by Call is essentially the Positives & Negatives method (see Section 8.3.1) but also includes sorting and color coding the Positive and Negative Calls. The Ranking function is diabled in this mode.

8.4 Results

The Results box lists the following information:

- **Source of Data**: Displays the analysis mode of the source data. For End Point Only Data this is **Background Subtracted**. For Endpoint Analysis of PCR Quantification runs the source of data must be either **PCR Base Line Subtracted** or **PCR Base Line Subtracted Curve Fit**. You may alter the analysis mode of the source data in the **PCR Quantification** tab of the Data Analysis module.
- **Current method**: Displays the currently selected **Method** for assigning positive and negative values to unknowns.
- **Tolerance**: Displays the currently selected mode of **Tolerance** calculation. Immediately beneath the displayed mode is the Tolerance value. If RFUs are chosen then the **Set Tolerance** in RFUs is given. If **Percentage of Range** is chosen then the Calculated Tolerance in RFUs is displayed.
- **Range**: The value displayed here is dependant on the Method chosen.

  If the **Positives** method is chosen then the **Range** is the Positive Controls Average RFU minus the Lowest RFU.

  If the **Non-Negatives** Method is chosen then the **Range** is the Highest RFU minus the Negative Controls Average RFU.

If the **Postives & Negatives** method is chosen then the **Range** is the Postive Controls Average RFU minus the Negative Controls Average RFU.

- **+ Controls Average RFUs**: Displays the average RFU of all positive controls. No value is shown if there are no positive controls defined.
- **+ Control - Tolerance**: In the **Postives** or **Positives & Negatives** Methods, samples with values equal to or higher than this amount will be called as Positive. No value is shown if there are no positive controls defined.
- **- Controls Average RFUs**: Displays the average RFU of all negative controls. No value is shown if there are no negative controls defined.
- **- Control +Tolerance**: In the **Negatives** Method, samples with values equal to or higher than this amount will be called as Positive. In the **Positives & Negatives** Method, samples with values less than this amount will be called Negatives. No value is shown if there are no negative controls defined.

8.5 End Point Analysis Spreadsheet

The End Point Analysis Spreadsheet contains the necessary data to perform End Point Analysis, and also displays the values assigned to unknowns after the **Analyze** button is pressed. The table headings include the following:

- **Well**: Lists the **well ID** of each sample. You may click on the **Well** column header to sort the table by wells. Note that you may also include or exclude certain wells from **End Point Analysis** via the **Select Wells for Analysis** window, which can be toggled via the **Select Wells** button.
- **Sample Type**: Lists the sample type of every well, as defined in the data's plate setup. You may click on the **Sample Type** column header to sort the table by sample type.
- **End RFUs**: Lists the absolute RFU averages for each well, as calculated from the end cycles, which are specified in the **End Cycles to Average** text box. You may click on the **End RFUs** column header to sort the table by end RFUs.
- **Define Controls**: Lists any positive or negative controls defined in the original plate setup file. You may also add new controls or edit existing controls in this column by clicking on the drop down menu on the right side. The options are (+) Positive, (-) Negative or blank. Alternatively, you may type the letter 'p' or the plus sign (+) to select a positive control, and you may also type the letter 'n' or the minus sign (-) to select a negative control. The controls specified in this column are used in the End Point Analysis calculations that assign positive or negative values to the unknowns.
- **Unknowns Call**: If a well is not defined as a positive control or a negative control in the Define Controls column, it is considered an unknown for End Point Analysis. This column displays the value assigned to each unknown after the **Analyze** button has been pressed. Unknowns Call may be (+) positive, (-) negative or blank. You may click on the **Unknowns Call** column header to sort the table by Unknowns Call.
- **Unknowns Ranking**: Lists the rank into which each unknown falls. An unknowns ranking depends on the total number of ranks and the End RFUs value. You may click on the **Unknowns Ranking** column header to sort the table by Unknowns Ranking.

- **Identifier**: Lists the identifier for every well, as defined in the data file's plate setup.

8.6 Analyzing the Data

Clicking the **Analyze** button performs the final calculations used to determine unknown sample calls. The **Analyze** button is in the active state (Analyze button green) before analyzing data and in the inactive state (Analyze button gray) after an analysis. The Analyze button will remain in the active state even if Method and Tolerance parameters are incorrect. An error message will be displayed if one or more of the following conditions is present:

- If the **Positives** method is selected and there isn't at least one positive control defined in the End Point Analysis spreadsheet
- If the **Non-Negatives** method is selected and there isn't at least one negative control defined in the End Point Analysis Spreadsheet
- If the **Positives & Negatives** method is selected and there isn't at least one positive control and at least one negative control defined in the End Point Analysis spreadsheet
- If no controls are defined
- If the range is less than one (i.e. the negative control RFU average is larger than the positive control RFU average).

End Point Analysis cannot be performed if any of these error conditions is present when the **Analyze** button is clicked.

After you are satisfied with your End Point Analysis, you may choose to view, save, or print a customized End Point Analysis report. To access End Point Analysis reports, click on the **Reports** button while the **End Point Analysis** tab is displayed.

Section 9
Care and Maintenance

9.1 Cleaning the Unit

Take care not to spill liquids onto or into the iCycler or the MyiQ optical module. Clean up of the iCycler reaction module may be done using a lint-free cloth or paper towel. The outer casings of the iCycler and the MyiQ optical modules may be cleaned using a soft, lint-free cloth and water.

Warning: Do not attempt to clean any of the mirrors or lenses found inside the MyiQ optical module, as they are extremely sensitive and fragile. Following the recommended guidelines for operation, the camera optics should not require cleaning. In any case, contact Bio-Rad Technical Services if you suspect a problem with the optical system. Do not attempt to disassemble or clean the optical system yourself.

9.2 Replacing the Lamp

When replacing the lamp, you must only use lamps supplied by Bio-Rad. Bio-Rad lamps are subject to additional tests and standards geared specifically toward Real-Time data collection. Lamps from alternative sources, which may appear to be similar, may not deliver the same optical quality, performance, and lifetime as those supplied by Bio-Rad. If the camera is to remain continually on, we recommend the lamp to be replaced every 6 months.

Note: When a lamp is overdue for replacement, it may flicker sporadically, causing a wavering of the data in all wells simultaneously, as shown in Figure 9.1. Installing a new lamp will alleviate this problem (see Figure 9.2).

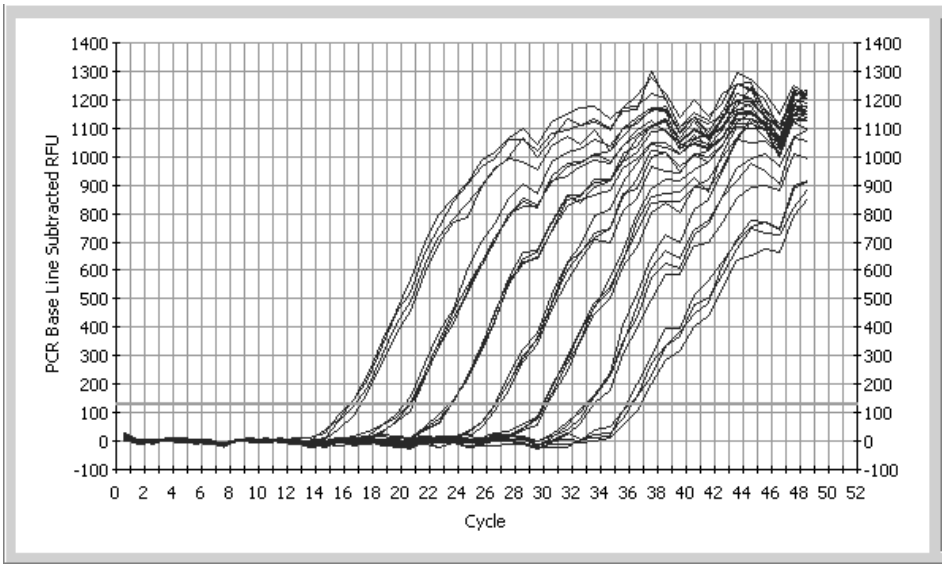


Fig. 9.1. Data Collected with a lamp in need of replacement.

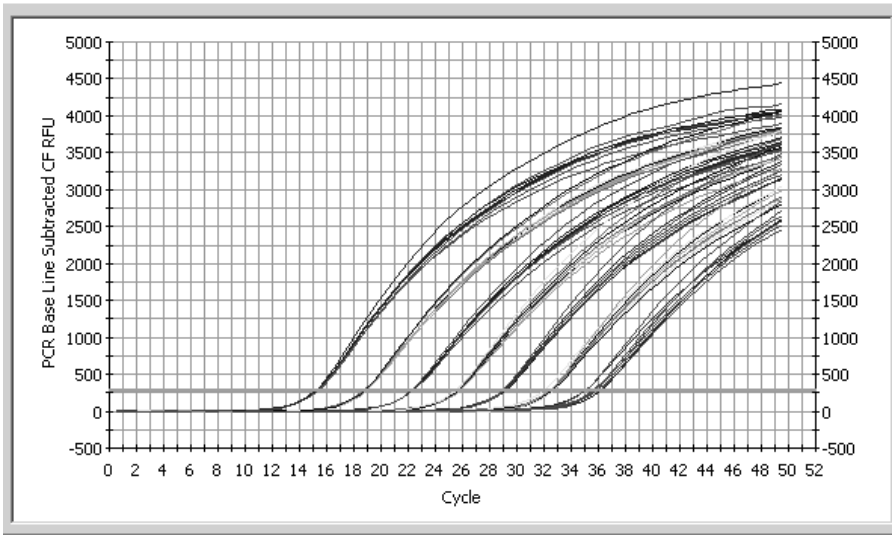


Fig. 9.2. Good data collected after replacing the lamp.

Caution: Take care when changing the lamp, as it is hot. Allow at least 15 minutes after turning off the MyiQ Camera module before removing the lamp.

The lamp is located on the right side of the Optical Module immediately behind the right latch (Figure 9.3). To replace the lamp:

1. Turn off the power supply to the optical module.
2. Disconnect the power cord from the Optical Module power supply.
3. Unlock the left and right latches.
4. Slide the cover toward the rear to expose the lamp compartment.
5. Push up on the lamp spring clip to release the lamp from the bracket.
6. Lift the lamp out of the socket.
7. Installing the new lamp is the reverse process. Hold the new lamp by the outer reflector. Do not touch the bulb of the new lamp, as oil from your fingers may degrade the performance and lifetime of the bulb. Be sure the spring clip is down before inserting the lamp into the socket. Push the lamp firmly into the bracket, then close the case and lock the latches.

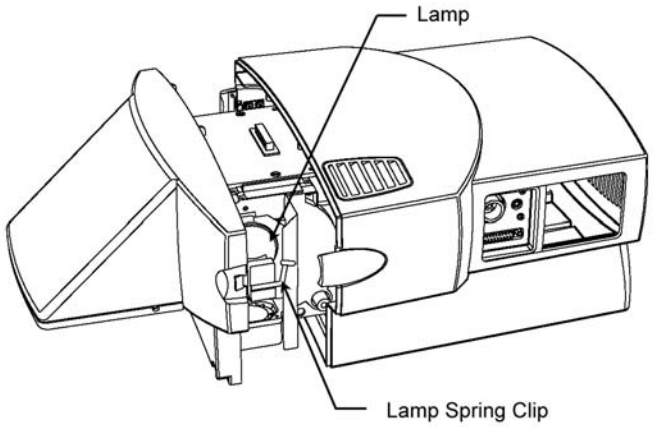


Fig. 9.3. Replacing the Lamp.

Appendix A
Specifications

Input Power	100–250 VAC ± 10% 50–60 Hz 250 VA max., 95W typical
Environmental	
Operating temperature	20–28°C
Storage temperature	20–45°C
Humidity	20–80%, noncondensing
Descriptive, Optical Module	
Size	width 12.2” (31. cm) height 13.5” (34.3 cm) depth 21.5” (54.6 cm)
Weight	15 lbs (6.8 kg) net max
Power Supply Size	width 3.75” (9.5 cm) height 2.56” (6.5 cm) depth 7.4” (18.8 cm)
Power Supply Weight	2 lbs (0.9 kg) net max
Data communications	bidirectional PC parallel port
Regulatory Compliance	TUV CE

In regions where fluctuating power is a problem, Bio-Rad recommends the use of Uninterruptable Power Supplies to protect the iCycler base and the MyiQ Optical system. The manufacturer should be APC or equivalent. The base requires a UPS with 1000 VA; this should be dedicated to iCycler thermal cycler. The computer requires a 650 VA UPS and the MyiQ Optical Module requires a 250 VA UPS.

Appendix B
Minimum Computer Specifications

A computer with the following system specifications is required for running the MyiQ optical module.

- Any IBM-compatible computer with a 500 MHz or faster Pentium processor
- Operating system with Windows 2000, Windows XP, and Windows NT US version (with Service Pack 3 or higher)
- Microsoft Internet Explorer (v5.0 or higher)
- 256 Mb RAM minimum, 512 Mb recommended
- 6 GB Harddrive minimum
- CD ROM drive
- 1.44 MB capacity 3.5” floppy disk drive
- Mouse
- 57.6 Kbps Serial port
- Bi-directional Parallel port (EPP)
- 1024 x 768 minimum screen resolution with true color mode (24 or 32 bits)
- IEEE 1284 Parallel Port Cable (m-m)

The following are also recommended:

- USB port for printer
- A USB compatible color printer
- A modem with an Internet connection (suggested for downloading new releases)
- An uninterrupted power supply (UPS). At least 1000 VA for the base iCycler, 650 VA for the computer and 250 VA for the MyiQ module.
- Use of shielded (rather than unshielded) cables

Appendix C
Warranty

The MyiQ Real Time PCR Detection System is warranted against defects in materials and workmanship. If any defects should occur during the warranty period, Bio-Rad will replace the defective parts without charge. However, the following defects are specifically excluded:

1. Defects caused by improper operation.
2. Repair or modifications done on the MyiQ Real-Time PCR Detection System by anyone other than Bio-Rad Laboratories.
3. Using tubes, plates, or sealing materials not specified by Bio-Rad Laboratories for use with the MyiQ Real-Time PCR Detection System.
4. Deliberate or accidental misuse.
5. Damage caused by disaster.
6. Damage due to use of improper solvent or sample.

The warranty does not apply to fuses.

For inquiry or request for repair service, contact Bio-Rad Laboratories after confirming the model and serial number of your instrument.

For Technical Service call your local Bio-Rad office, or in the U.S. call 1-800-4BIORAD (1-800-424-6723).

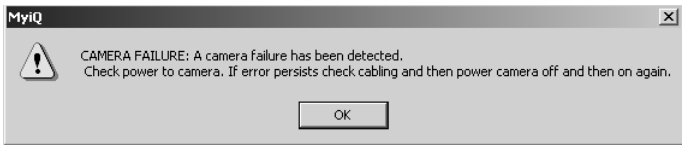
Perkin-Elmer does not guarantee the performance of this instrument.

Appendix D
Product Information

Catalog Number	Product Description
170-8720	**iCycler with 96 x 0.2 ml Reaction Module**, includes iCycler base with the 96 x 0.2 ml iCycler Reaction Module, In-Sample temperature Probe (0.2 ml tube size), Comprehensive Instruction Manual, Quick Reference Card, PCR tubes, and power cord.
170-9740	**MyiQ Single-Color Real-Time PCR Detection System**, includes MyiQ Optical Upgrade for 96-well reaction module, optical power supply, MyiQ filter set, software interface CD-ROM, optical quality 96-well PCR plates, communication cables, power cord, instructions.
170-8740	**iCycler Multi-Color Real-Time PCR Detection System,** includes four-color iCycler Optical upgrade for 96-well reaction module, optical power supply, basic filter set, iCycler iQ validation solution, software interface CD-ROM, optical quality 96-well PCR plates, communication cables, power cord, instructions.
223-9441	**96-well 200 µl Thin Wall PCR Plates**, 25 per box
223-9443	**96-well 200 µl PCR Plate Caps**, for 223-9441, 300 per box
223-9444	**Optical Quality Sealing Tapes**, optimized for use with 223-9441, 100 sheets
170-8794	**iCycler iQ External Well Factor Solution**
170-8780	**Fluorescein Calibration Dye**
170-8756	**Replacement Halogen Lamp**

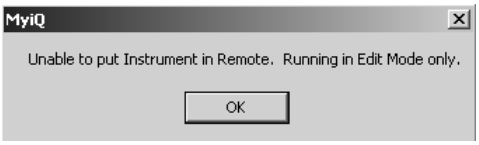
Appendix E
Error Messages and Alerts

E.1 Software Startup



Cause: The detector is not responding to the computer's parallel port when the software is launched.

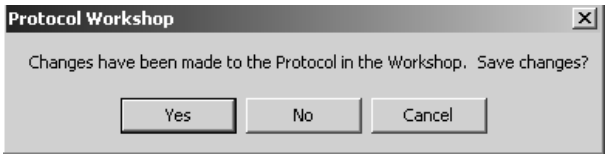
Solution: Power up the detector or check the parallel port connection.



Cause: When the MyiQ software is launched, no iCycler base unit is recognized via the serial port. In this mode, you can still edit protocols and plate setups and conduct data analysis

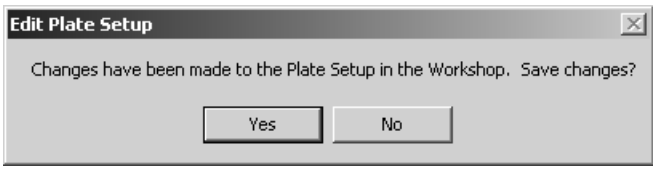
Solution: No action is necessary, unless this message is displayed when an iCycler base unit is connected to the computer running the software. In that case, be sure that the base unit is powered up and check the serial cable connection.

E.2 Workshop and Library: Protocol and Plate Setup



Cause: The currently opened thermal protocol has been edited in some way, but the changes were not saved before you chose to open another protocol or close the application.

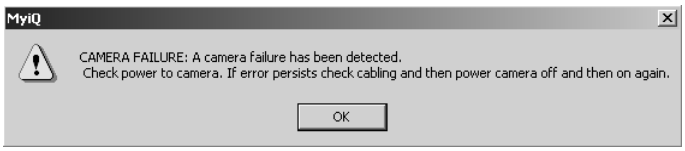
Solution: Only one protocol may be active at once, so you must choose to abandon edits or save them before you may open another protocol file. If you close the application without saving the protocol first, all changes will be lost.



Cause: The currently opened plate setup has been edited in some way, but the changes were not saved before you chose to exit the software or to open another plate setup file.

Solution: Only one plate setup may be active at once, so you must choose to abandon edits or save them before you may open another plate setup file. If you are closing the software, all changes to the plate setup will be lost if you do not save them.

E.3 Run Time Central

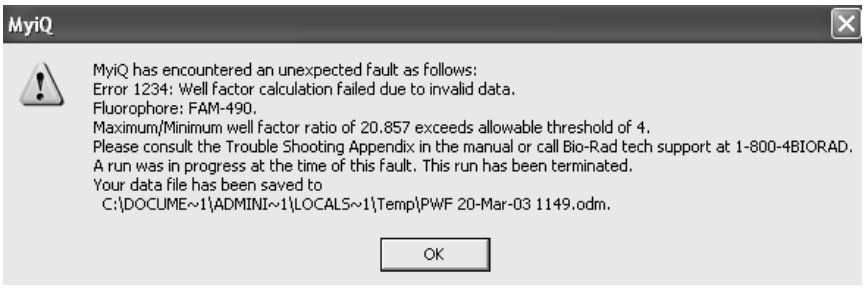


Cause: The software is has lost signal from the MyiQ optical module.

Solution: Be sure that the power supply is on and that the parallel cable is securely attached to the detector and the computer. If camera failure is detected during an experiment, the software will pause the protocol until the camera power is restored and you click **Continue Running Protocol**.

Cause: The lid has been opened while an experiment is in progress, the lid is not closed completely, or the miniature phone plug connector has become unplugged.

Solution: Close the lid securely. If the lid is opened during an experiment, the software will pause the protocol until the camera lid is closed and you click **Continue Running Protocol**. If this does not solve the problem, confirm that the miniature phone plug connector is connected as described in section 1.1 of this manual.

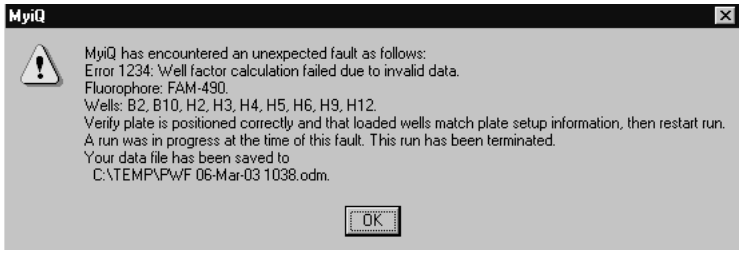


Cause: Invalid well factor data was collected. Either negative well factor values were recorded, or the highest to lowest well factor ratio exceeds 4.0. Thus, at least one well contains relatively low levels of fluorescence or deviates significantly from the rest of the wells on the plate you are attempting to collect well factors from.

Solution: If you are collecting persistent well factor data, check to make sure that you have inserted an external well factor source plate, with all wells loaded, as described in Section 6.2.5. Make sure your plate contains no bubbles and that the liquid sits at the bottom of each loaded well (centrifuge your plate in a plate spinner).

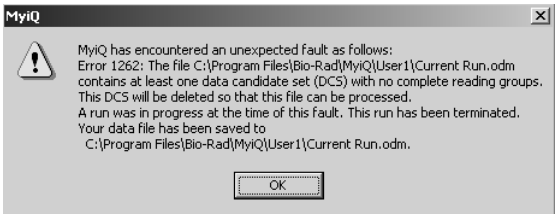
If you still receive this error after checking your plate, view an image of the empty block in Imaging Services at 4096ms and note any large "hot spots" of fluorescence. Clean these portions of the reaction block with 70% alcohol and water to remove the spots, and then try collecting well factors again. If these bright spots still persist, contact Bio-Rad Technical Support.

If there are no significant "hot spots" observed in the empty block and you still receive this error, view an image of the external well factor plate or experimental plate in Imaging Services and note any of the selected wells that differ significantly from the rest. Remake your plate and try collecting well factors again. Contact Bio-Rad Technical Support if the problem persists.



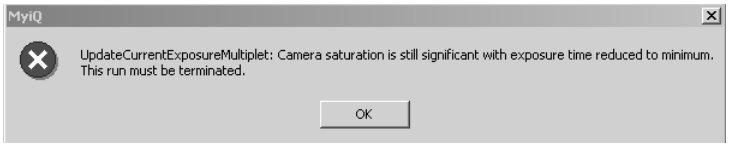
Cause: The plate from which you are attempting to collect well factors was not inserted into the reaction module, or is too dim to collect valid well factors.

Solution: In any case confirm that you have loaded the appropriate plate into the reaction module. If you are collecting well factors from the experimental plate, confirm that you have added an appropriate amount of probe labeled with a suitable fluorophore (visible with the 480/40x – 540/50m filter set), or switch to using persistent well factors instead. If this error occurs while collecting persistent well factors, create a new external well factor plate and try collecting persistent well factors again.



Cause: The data collection step of the protocol was not sufficiently long to collect at least one complete reading group. A complete reading group consists of a set of exposures taken during the same cycle.

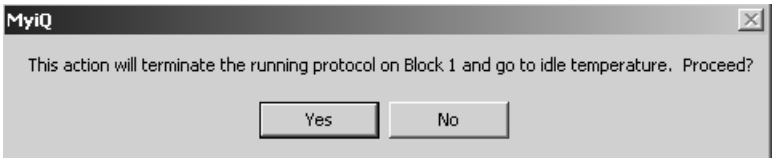
Solution: Edit the thermal protocol file and extend the dwell times of the affected data collection step. If this happens early during execution of the amplification part of the protocol, you can edit the protocol and restart the experiment. If it happens late during the amplification, after the traces have begun to rise above the baseline, you may have sufficient data for complete analysis. However, you should edit the protocol before using it again.



Cause: One or more wells on the experimental plate are so bright that the detector cannot collect a non-saturated image.

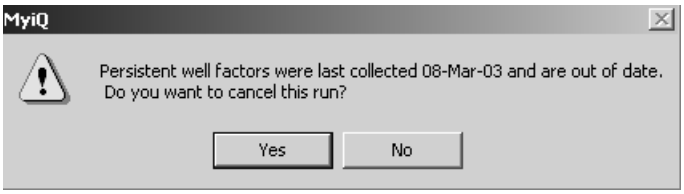
Solution: If this occurs at the beginning of the protocol, try reducing the concentration of fluorophore present. If it occurs near the end of the run, you may have sufficient data to complete the analysis; save the data file.

This message will also appear if either the excitation or the emission filter is missing. Ensure that the filters are correctly installed (see Appendix K).



Cause: This box is displayed when you attempt to terminate a protocol before it has ended.

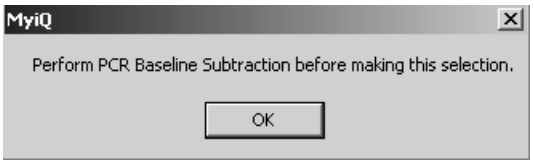
Solution: If you wish to end the protocol click Yes, otherwise click No and the protocol will resume.



Cause: The Current PersistentWF.ini file, which is by default found in the C:\ProgramFiles\Bio-Rad\MyiQ\Ini, is more than one month old. Outdated persistent well factors may lead to poorly normalized data.

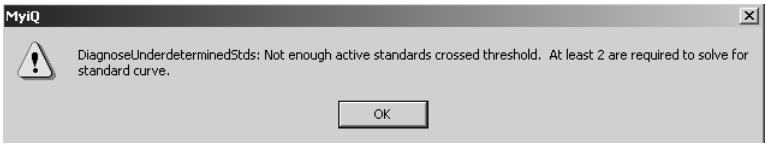
Solution: Collect new Persistent Well Factors as described in Section 6.2.5.

E.4 Data Analysis



Cause: You have tried to perform some other data analysis before PCR Base Line subtraction. Until PCR Base Line subtraction is completed, no other analysis options may be carried out.

Solution. Choose PCR Base Line Subtracted mode or PCR Base Line Subtracted Curve Fit mode from the PCR Quantification tab.



Cause: Not enough standards crossed threshold to calculate a standard curve.

Solution: Try changing the data analysis parameters, especially the threshold setting. If the problem persists, it indicates failure of the standards to amplify and the experiment will have to be repeated.

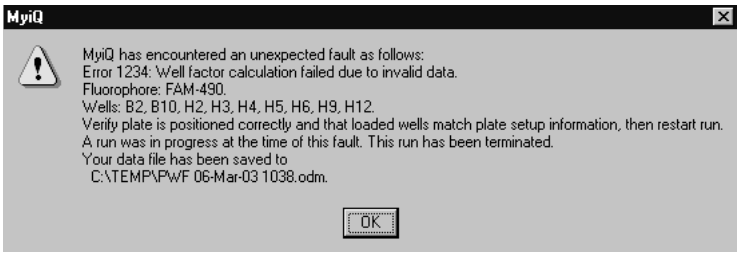
E.5 Imaging Services

Cause: Invalid well factor data was collected. Either negative well factor values were recorded, or the highest to lowest well factor ratio exceeds 4.0. Thus, at least one well contains relatively low levels of fluorescence or deviates significantly from the rest of the wells on the plate you are attempting to collect well factors from.

Solution: If you are collecting persistent well factor data, check to make sure that you have inserted an external well factor source plate, with all wells loaded, as described in Section 6.2.5. Make sure your plate contains no bubbles and that the liquid sits at the bottom of each loaded well (centrifuge your plate in a plate spinner).

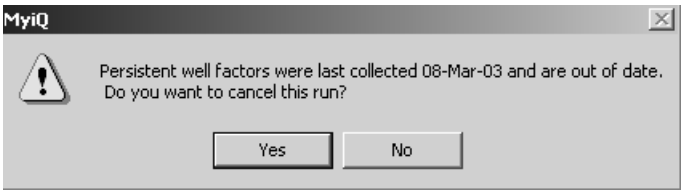
If you still receive this error after checking your plate, view an image of the empty block in Imaging Services at 4096ms and note any large "hot spots" of fluorescence. Clean these portions of the reaction block with 70% alcohol and water to remove the spots and then try collecting well factors again. If these bright spots still persist, contact Bio-Rad Technical Support.

If there are no significant "hot spots" observed in the empty block and you still receive this error, view an image of the external well factor plate or experimental plate in Imaging Services and note any of the selected wells that differ significantly from the rest. Remake your plate and try collecting well factors again. Contact Bio-Rad Technical Support if the problem persists.



Cause: The plate from which you are attempting to collect well factors was not inserted into the reaction module, or is too dim to collect valid well factors.

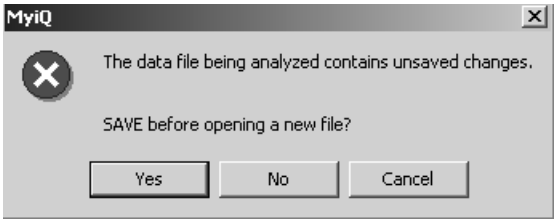
Solution: In any case confirm that you have loaded the appropriate plate into the reaction module. If you are collecting well factors from the experimental plate, confirm that you have added an appropriate amount of probe labeled with a suitable fluorophore (visible with the 480/40x – 540/50m filter set), or switch to using persistent well factors instead. If this error occurs while collecting persistent well factors, create a new external well factor plate and try collecting persistent well factors again.



Cause: The Current PersistentWF.ini file, which is by default found in the C:\ProgramFiles\Bio-Rad\MyiQ\Ini, is more than one month old. Outdated persistent well factors may lead to poorly normalized data.

Solution: Collect new Persistent Well Factors as described in Section 6.2.5.

E.6 Exiting Software



Cause: The Save data dialog box opens automatically if you try to exit the software or open a new data file without saving modifications made to the current data set (for example, you may have altered the baseline cycles settings or the analysis mode).

Solution: Save the data files before exiting the software or opening new data files. Alternatively, you may click the cancel button and choose not to resave the current data file.

Cause: The software is trying to restore the iCycler base to local control (i.e. so you can program regular PCR protocols with the front panel) before closing down, but the base is not responding.

Solution: If the base has been powered off, this message is normal, click **OK** and the software will close normally. If the base is still on, displaying the text Remote Host Mode, exit the software. If the base unit continues to indicate that it is in remote host mode, power the base off and back on. If the message still persists, call your local Bio-Rad office or Technical Service.

Appendix F
Hardware Error Messages

The following are error codes produced by the sddcamdll.dll file. Error codes present a message box with the title “SddCamDll.” The table below lists the error codes, their probable cause, and possible solutions.

Message	Possible Cause(s)	Possible Solution(s)
`Can’t allocate image buffer (low memory)`	When the dll starts, an image buffer is allocated in memory. This message appears if not enough memory is available.	1. Close other applications. 2. Reboot the computer. Note: Sometimes applications may cause “memory leaks”, slowly depleting available RAM. Rebooting resets the memory.
`No bi-directional port at nnnh.`	The port address selected does not point to a valid parallel port. If the system was working previously, the port settings have been changed in the BIOS or under Settings.	1. Reset BIOS values. 2. Check under Settings to see if the port address was changed for LPT1.
`Failed 1st Readback` `Failed 2nd Readback`	The camera has failed to respond to a test command. and this usually occurs if the camera is not powered up, if the cable is unplugged, or if there is a bad cable.	1. Check the camera to be sure the power is on. 2. Check the cable connection. 3. Replace the cable.
`Timeout waiting for IMGRDY bit`	The camera failed to respond after an exposure command was sent successfully.	Turn the camera off, then on to reset it.

The following are error codes produced during initialization, which is when most relevant messages appear.

Message	Possible Cause(s)	Possible Solution(s)
`Set CamPort error: -1`	Couldn't find the 'Well X' and/or 'Well Y' values in the sdd.ini file (Algorithm section). The sdd.ini file was not found or has become corrupted.	Reinstall the sdd.ini file.
`Set CamPort error: -1,-2`	Couldn't verify the existence of the selected parallel port. The port settings may have been damaged in the BIOS or under Settings.	Select new BIOS settings
`Set CamPort error: -3,-4`	Bi-directional test failed for the selected parallel port. The port settings may have been damaged in the BIOS or under Settings.	Select new BIOS settings.
`Set CamPort error: 5,-6`	The parallel port is okay but the camera is not responding. A cable may have come loose.	1. Check that the camera is turned on. 2. Check all cable connections.
`Set CamPort error: -7`	1. The PortBase value is missing from the sdd.ini file, or 2. The sdd.ini file could not be found, or 3. The mask96.ini file is invalid or could not be found.	Check the sdd.ini file and the mask96.ini file in c:\programfiles\Bio-Rad\iCycler\ini.

Appendix G
Additional Troubleshooting

Problem	Cause	Solution
No image observed upon making exposures in Imaging Services	The excitation shutter is closed	Select the "Open" option for Position Shutter in Imaging Services and make another exposure.
	The lamp has failed	Slide the camera casing back to see if the lamp has failed. If the lamp has failed, replace it as described in Section 9.2.
	The correct optical filters are not installed properly	See Appendix K
RFU values remain at zero throughout the course of an entire experiment	The sample plate does not contain a detectable fluorophore	See Appendix K and repeat the experiment with a detectable fluorophore
	The reactions are not amplifying	Optimize the reactions
	The lamp has failed	Slide the camera casing back to see if the lamp has failed. If the lamp has failed, replace it as described in Section 9.2.
	The correct optical filters are not installed properly	See Appendix K
PCR amplification or Melt Curve plots exhibit a wavering of the data from all wells simultaneously	The lamp is overdue for replacement and is flickering	See Section 9.2

Appendix H
Description of MyiQ Data Processing

1. Fluorescent data may be collected for real-time analysis or for post-run analysis. Data are simultaneously collected from all 96 wells during the entire dwell time of the steps defined for analysis; the number of individual readings on each well depends on the exposure time and the dwell time. With a 45-second dwell time and a 512 ms exposure time, as many as 35 data points may be collected at each cycle for every well.

2. Every time data are collected, two separate readings are taken for each of the 96 wells: the inner reading and the outer reading. The inner reading is the average of all the pixels in the area defined by the mask as the inner well region. The outer reading is the average of all the pixels in the region immediately surrounding the inner well region and represents background. The net reading for a well is the difference between the inner and outer reading for that well. The area of each well inner and outer is defined by the mask file. It is the net readings that are used in preparing the PCR Amp Cycle plot.

3. Data collection begins at an exposure time of 2048 ms by default. The pixels in the camera are monitored for saturation at each cycle, and if a significant number of pixels are saturated, then the MyiQ Detector will automatically reduce exposure time in half. The next time saturated pixels are detected, the exposure time will be reduced by half again, and this will continue each time the detector measures significant saturation. Eventually all data are normalized to the 2048-ms exposure time; that is, data collected at 1024 ms are doubled and data collected at 512 ms are quadrupled.

4. At the beginning of the second repeat of the Real-Time data collection step, the PCR Amp Cycle plot is automatically displayed showing the first data points.

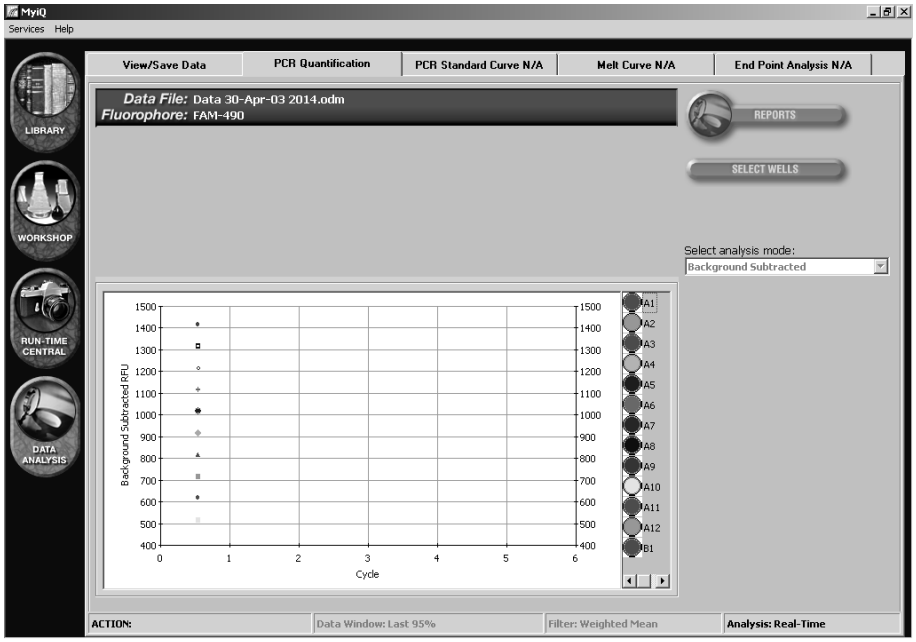


Fig H.1. The PCR Amp Cycle Plot.

There are three views of the data available: Background Subtracted and PCR Base Line Subtracted, PCR Base Line Subtracted Curve Fit.

- The Background Subtracted data displayed in the PCR Amp Cycle plot are the mean of the last three net readings in the cycle. The Background Subtracted data are also normalized to a constant exposure time.
- The PCR Base Line Subtracted data displayed in the PCR Amp Cycle plot are calculated by first fitting a straight line through the Background Subtracted data of the specified baseline cycles. This line is extended beyond the last baseline cycle for each subsequent cycle. Next, the calculated RFU value of the straight line is subtracted from the actual background subtracted reading (inner-outer) at each cycle. The result is then plotted against the cycle number.
- The PCR Base Line Subtracted Curve Fit data displayed in the Amp Cycle plot are calculated by fitting to a smooth curve using a balanced flank, centroid-finding digital filter. The curve fit process is performed in such a way that threshold crossing (Ct) is left invariant for all traces.

5. From the PCR Base Line Subtracted plot, the software will calculate the threshold fluorescence for the experiment and then determine the threshold cycle for each well. The threshold is the level of fluorescence that is considered to be significantly above the background level of fluorescence measured in the early cycles of the amplification.
6. A graph is constructed by plotting the log of the beginning concentration of template in each standard well against that well's threshold cycle. A least squares linear regression is fit to these data to produce a standard curve. Next the beginning concentration of each well defined as an unknown is calculated by mapping the threshold cycle of that well to the standard curve. If the unknown was amplified in replicate samples, then a standard deviation is also calculated for the beginning concentration.

   Example: Consider the following simplified data set for wells A1 and H12. The data presented in this table represent the mean of the last reading at the indicated cycle.

		A1		H12	
Cycle #	**Exposure Time**	**Inner**	**Outer**	**Inner**	**Outer**
1	2048ms	350	118	400	150
2	2048ms	360	129	405	154
5	2048ms	355	122	400	147
10	2048ms	360	125	405	150
15	2048ms	365	125	408	154
20	2048ms	412	135	460	165
25	1024ms	315	69	350	88

A. The Background Subtracted graph is constructed by plotting the net (inner-outer) reading for each well at each cycle. The net difference at cycle 25 was doubled to normalize the exposure time to 2048 ms.

Cycle #	A1	H12
1	232	250
2	231	251
5	233	253
10	235	252
15	240	254
20	277	295
25	492	524

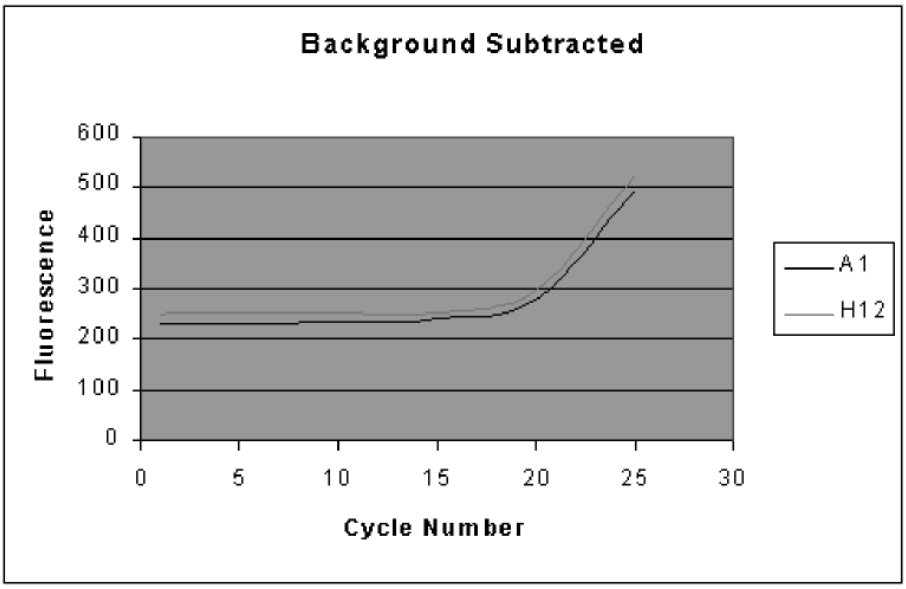


Fig.H.2. Background Subtracted Plot.

B. The first step in constructing the PCR Baseline Subtracted graph is to calculate a straight line fit through the net readings for the specified baseline cycles (in our example from cycles 2 through 10, inclusive).

The equation of the straight line that best describes the data collected between cycles 2 and 10 in well A1 is

Fluorescence = Cycle * (0.490) + 230.2

The equation of the straight line that best describes the data collected between cycles 2 and 10 in well H12 is

Fluorescence = Cycle * (0.092) + 251.5

C. Now the difference between the fluorescence predicted by the equation of the line fit and the actual background subtracted fluorescence is plotted to produce the PCR Base Line Subtracted plot.

	Background subtracted		Predicted		Final	
Cycle #	**A1**	**H12**	**A1**	**H12**	**A1**	**H12**
1	232	250	230.7	251.6	1.3	-1.6
2	231	251	231.2	251.7	-0.2	-0.7
5	233	253	232.7	251.9	0.3	1.1
10	235	252	235.1	252.4	-0.1	-0.4
15	240	254	237.6	252.9	2.4	1.1
20	277	295	240.0	253.3	37.0	1.7
25	493	525	242.5	253.8	250.5	271.2

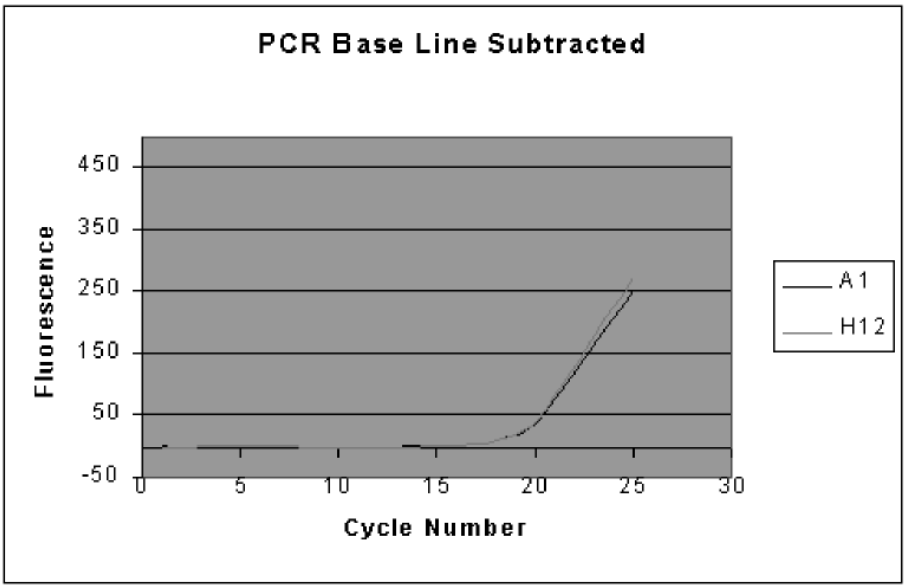


Fig. H.3. PCR Base Line Subtracted Plot.

D. The next operation by the software is to identify the threshold. The first step is to calculate the standard deviation of the readings between cycles 2 and 10, inclusive, for each well in the PCR Base Line Subtracted plot. Then the mean of all standard deviations is multiplied by the default factor of 10 and this level of fluorescence is considered the default threshold value. Suppose in this example that the average standard deviation of each well was 1.2, then the threshold would be set at 12 by default.

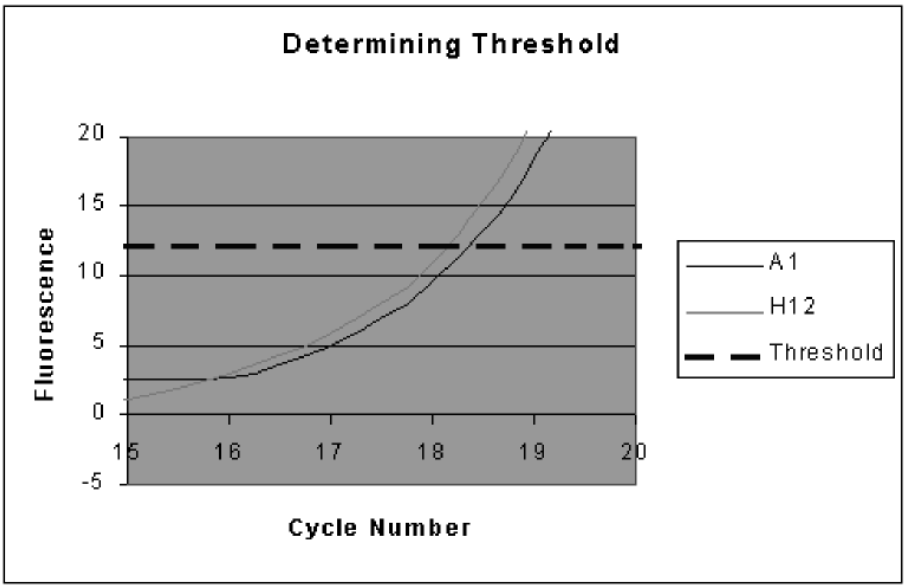


Fig. H.4. Determining Threshold Level.

E. Finally the threshold cycle is determined by dropping a perpendicular line from the trace of a well as it crosses the threshold. In this example, the software would assign a threshold cycle of 18.35 for well A1 and 18.12 for well H12. See Figure H.5.

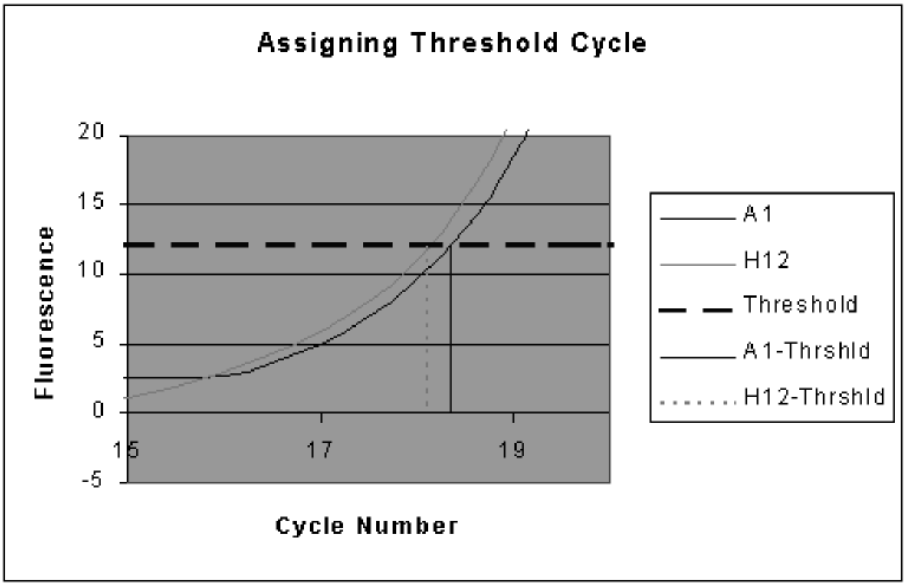


Fig. H.5. Assigning a Threshold Cycle.

7. Understanding the standard curve

The standard curve is made from a known sample. The log of the copy number (x-axis) is plotted versus the threshold cycle (y-axis) to generate this graph.

The equation of the line and the correlation coefficient are displayed on the standard curve graph.

Correlation coefficient:

The correlation coefficient, or **r-value**, is a measure of how well the predicted values, e.g.the standard amounts entered for a sample “fit” with the experimental data. That is, how well the data from the experiment fit to the values assigned to the standards.

The correlation coefficient is a number between 0 and 1. If there is no relationship between the predicted values and the actual values then the correlation coefficient is 0 or very low (the predicted values are no better than random numbers). As the strength of the relationship between the predicted values and actual values increases so does the correlation coefficient. A perfect fit gives a coefficient of 1.0. Thus, the higher the correlation coefficient, the more accurately your experimental data fit the expected values.

Efficiency:

The slope of the standard curve is directly related to the efficiency of your reactions. In turn, the efficiency can give you valuable information about the chemistry of your reaction. The slope of the standard curve is related to the efficiency through the following equation:

Efficiency = $[10^{(-1/\text{slope})}] - 1$.

A slope of -3.322 represents an efficiency of 100%. A higher absolute value of the slope (|s|) will yield an efficiency of less than 100%. A lower |s| will yield an efficiency that is greater than 100%. In either case, the reaction should be optimized.

At 100% efficiency, the template doubles after each cycle during exponential amplification. Several design factors influence efficiency, such as the length of the amplicon, the G/C content of the amplicon, and secondary structure. The dynamics of the reaction itself can also influence efficiency. Variations in the dynamics can result from such sources as the enzymes used in the reaction and non-optimal reagent concentrations.

Appendix I
Uploading New Versions of Firmware

Bio-Rad is committed to continuous improvement in iCycler features. Towards that end, the iCycler firmware will be upgraded to offer new features on a regular basis. We will make a firmware upgrade diskette available at the time of each new release and announce the release on our web site, www.bio-rad.com.

The iCycler is upgraded via serial port connection to a PC Computer. The computer serial port must support a 57600 baud rate. The connection may be made with a standard 9 pin serial cable. The firmware upgrades may be ordered from Bio-Rad using part number 170-8737.

Upgrading the Embedded Firmware. The upgraded version of the firmware and a utility for loading it are on the diskette described above. First insure that the serial cable is connected and that the computer and iCycler are both powered on.

1. Log onto the iCycler with a user name.
2. Create a new folder on the computer hard drive. Name the folder “Upgrade”.
3. Insert the floppy disk into the drive.
4. Open the floppy disk directory and locate the Utilities folder. Open the folder called \Base Unit\Firmware Upgrade and copy the files Upgrade.exe and icycupdt.bin to the newly created Upgrade folder.
5. Open the Upgrade folder on the C drive and double click on Upgrade.exe. The utility will open and assess the current version and the new version of firmware to be down-loaded. If they are the same, there is no need to proceed. If they are different and you want to proceed with the download, confirm this by selecting “yes” and the utility will automatically download the new version of the firmware over the serial port.
6. When the upgrade is complete, a message will be displayed telling you that the upgrade has been successfully completed. You must turn the iCycler off and back on to implement the new version of firmware.

Appendix J
Melt Curve Functionality

Melt curve is a dynamic tool used to measure the melting temperature (T_m) of double stranded DNA molecules. DNA duplexes can be visualized by either incorporation of DNA-binding dyes (e.g.SYBR Green I) or by hybridization with fluorescently labeled probes. In the case of DNA-binding dyes and non-cleavable hybridization probes, fluorescence is brightest when the two strands of DNA are annealed. As the temperature is raised towards the T_m of the duplex, the fluorescence will decrease at a constant rate (constant slope). At the T_m, there is a dramatic reduction in the fluorescence with a noticeable change in slope. The rate of this change is determined by plotting the negative first derivative (-dF/dT) versus temperature. The greatest rate changes yield visible peaks, representing the T_m of the double-stranded DNA complexes.

When Should I Use Melt Curve?

Two major applications for melt curve are: peak identification (number of amplified products) and characterization of molecular beacons. These two applications are typically used as a guide for improving real-time PCR assay development.

1.1 Peak Identification

It is well known that successful real-time PCR amplification is dependent on optimization of many factors. One of the most important of these factors is determining the specificity of the primers chosen for amplification. A particular primer pair may induce primer-dimer formation and/or amplify other non-specific products. In turn, these non-specific products can greatly reduce amplification efficiency as well as diminish the overall dynamic range of a standard curve. Therefore, identification of all products amplified by a particular primer pair would be useful for optimization of real-time PCR assays.

DNA-binding dyes, such as SYBR Green I, are typically used for non-specific visualization of amplified product. SYBR Green I binds to double-stranded DNA and fluoresces 50-100 times brighter than when not bound to DNA. Since these dyes bind to any double-stranded DNA molecule, they cannot distinguish between multiple products amplified in the same reaction. SYBR Green I can be used to monitor real-time amplification with a particular primer pair.

The number of amplified products can then be identified by melt curve analysis, allowing you to check for any non-specific source of fluorescence in the PCR. At temperatures below the T_m of the amplified product, SYBR Green I will bind to the double-stranded DNA and fluoresce brightly. As the T_m is reached, the DNA denatures and releases SYBR Green I, causing a sharp decline in fluorescence. This decrease in fluorescence is plotted as Fluorescence vs. Temperature. Plotting the negative first derivative of this data versus the temperature change (–dF/dT vs. Temperature) results in a melting peak and T_m for each amplified product. The T_m for each peak is dependent on the length of the amplified DNA as well as the G/C content of the sequence. Primer-dimers, which are typically shorter in length, usually melt at a much lower T_m than the intended product and are therefore easy to distinguish. Secondary or non-specific products can be of varying lengths and sequences, and therefore have a large range of possible melting temperatures.

Without melt curve, you can only be sure that the fluorescence observed in the amplification is really the intended product by running the products on a gel.

Identification of primer-dimers and non-specific products by melt curve can help the user understand any abnormalities seen in the amplification plot. For example, the dynamic range of a dilution series will be greatly affected by the presence of primer-dimers. Other non-specific products could also affect the efficiency of the amplification. Redesigning primer sequences to avoid primer-dimer formation and adjusting annealing temperatures to prevent non-specific product amplification are just two ways in which real-time PCR assays can be optimized. After adjustments have been made, amplification and melt curve protocols can be repeated to ensure that primer-dimers and other non-specific products have been minimized or eliminated.

1.2 Characterization of Molecular Beacons

Fluorescently labeled molecular beacon probes are used for specific quantitation of a particular product in real-time PCR assays. Melt curve analysis can assist in characterizing a molecular beacon for real-time PCR amplification by distinguishing the best temperature for data collection. Molecular beacons are labeled with a fluorophore at the 5' end and a quencher molecule at the 3' end. They consist of a probe sequence, complementary to the target DNA and two arm sequences of five or more nucleotides flanking both sides of the probe. The arms are not complementary to the target, but they do complement one another. When the arms anneal, the molecular beacon assumes a hairpin structure, bringing the 5' and 3' ends of the molecule together. This brings the fluorophores and quenchers in close proximity, allowing the fluorescence to be quenched. When a beacon is bound to its DNA target, the 5' and 3' ends are maximally separated, allowing fluorescence detection of DNA.

A molecular beacon and its target are characterized by three different T_m s: the Tm of the probe:target hybrid, the T_m of the self-complementary arms and the T_m of the primer:target hybrid. For a sensitive quantitative experiment, the T_m of the probe:target hybrid should be higher than the T_m of the arms which, in turn, should be higher than the T_m of the primer:target hybrid. These parameters are necessary for successful real-time quantitative PCR assays. At the first step (95°C denaturation), molecular beacons will be completely melted and open and highly fluorescent. As the temperature decreases towards annealing, it is important for the molecular beacons to bind to the target sequences first. Decreasing the temperature further toward annealing should then cause all unbound molecular beacons to close. This will quench the probe fluorescence and reduce background fluorescence. Finally, at the annealing temperature, the primers should anneal to the target to promote DNA polymerization.

The melt curve feature of the software can be used to determine the melting temperatures of the probe:target hybrid and of the self-complementary arms. This is accomplished by running two separate reactions: the molecular beacon alone and the molecular beacon with a single-stranded DNA complement to the probe sequence. The temperature during the melt curve protocol may be reduced from 95°C or increased to 95°C, as the fluorescence of the two reactions is monitored. For molecular beacons alone, fluorescence will be minimal at low temperatures since all arms are closed. The fluorescence will begin to increase with increasing temperature as the molecular beacons begin to open. When the melting temperature of the complementary arms is reached, there is a sharp increase in

fluorescence as all of the molecular beacons open and the fluorophores at the 5' end are no longer quenched.

In reactions with molecular beacons and the single-stranded complements, fluorescence is usually highest at low temperatures. This is the point at which the probes are bound to the targets and the fluorophores are maximally separated from the quenchers. As the temperature increases and the Tm of the probe:target hybrids are reached, there is a marked change in fluorescence as the molecular beacons are released from the target DNA. Melt curve can assist in designing a molecular beacon real-time PCR assay by determining the best temperature at which fluorescent data should be collected.

Both the T_m of the arms and the T_m of the probe:target hybrid are best revealed in the negative first derivative plot (-dF/dT vs. Temperature). Fluorescent data generated during an amplification protocol should be collected at a temperature at which the unbound molecular beacons are closed (below the T_m of the arms) and the probes bound to the DNA targets are maximal. This will ensure the maximum signal-to-noise ratio in the experiment.

Section 2
Interpretation of Sample Data

A. Peak Identification

Amplification with DNA-binding dyes (e.g.SYBR Green I) provides non-specific quantitation of DNA. Therefore, the presence of non-specific products during the amplification could affect the overall dynamic range of the standard curve and possibly decrease the efficiency of the reaction.

In the following example, IL-1b plasmid was amplified with SYBR Green I in a 5-fold dilution series from $1x10^4$ copies/well to 16 copies/well with primers containing a GGG/CCC overlap at their 3' ends. These primers were designed to produce significant primer-dimers that interfere with the amplification of the specific product. As seen in the amplification plot in Figure J.1, the higher concentrations of plasmid ($1x10^4$, $2x10^3$) can be resolved from one another (~2.3 cycles apart). However, the lower dilutions cannot be resolved. Furthermore, it appears that amplification also occurred in the zero template control. Overall, this standard curve produced a correlation coefficient of 0.99 and a slope = -2.728 (~133% efficiency). Since efficiency is >100%, this suggests that more than one product was amplified and being monitored in real-time.

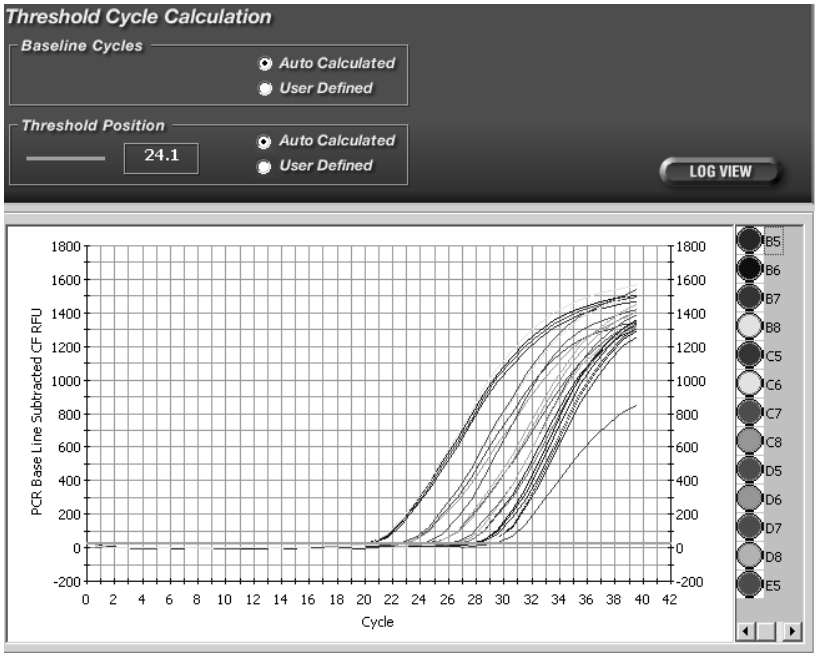


Fig. J.1. Amplification of IL-1b plasmid (5-fold dilution series) with SYBR Green I using primers designed to produce primer-dimers.

Immediately after the amplification, a melt curve was performed to confirm that multiple products were amplified in these reactions. The MyiQ software displays the data collected during melt curve analysis in real-time plotted as Fluorescence vs. Temperature:

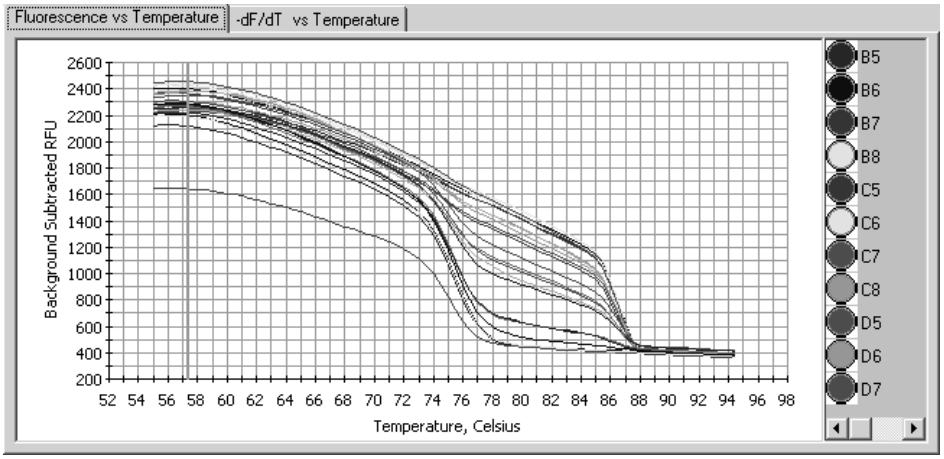


Fig. J.2. Fluorescence vs. temperature plot of data collected during the melt curve run protocol between 55°C–95°C (80 repeats, 0.5 °C increments) following amplification of IL-1b with SYBR Green I.

Figure J.2 reveals melt curve data generated after amplification of wells containing $4x10^2$ copies/well of IL1b plasmid with SYBR Green I (3 replicates). A negative first derivative plot is then generated from this data and presented as the rate of change in fluorescence over temperature or –dF/dT vs. Temperature (Figure J.3). This graph represents the amplified DNA products as distinct melting peaks with specified melting temperatures (T_m):

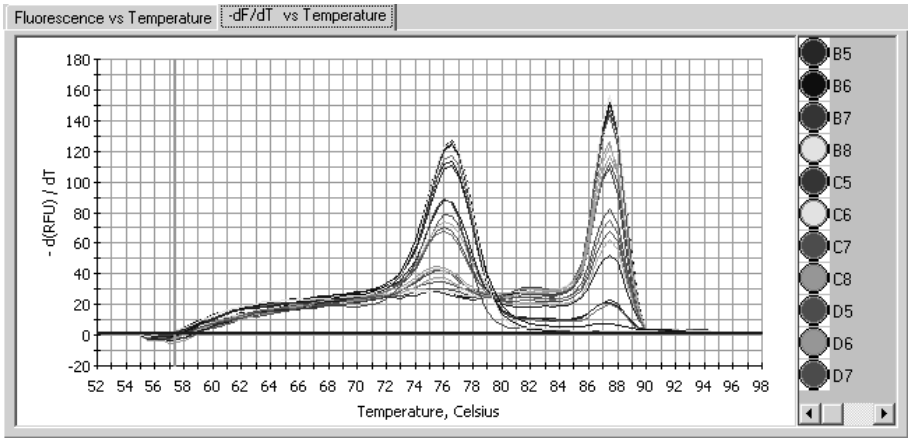


Fig. J.3. Negative first derivative plot of melt curve data collected after amplification of IL-1b plasmid ($4x10^2$ copies/well) with SYBR Green I using primers designed to produce primer-dimers.

Two peaks are identified in the melt curve with melting temperatures of 87.5°C and 76.5°C. Multiple peaks confirm the presence of multiple amplification products including the specific product of interest, plus additional non-specific products. In this example, the additional non-specific peak is due to the presence of primer-dimers since the amplification was performed with primers containing a GGG/CCC overlap at the 3'ends. Primer-dimers typically melt at a lower Tm than the specific amplified product due to their shorter length. Other secondary products will melt at a large range of melting temperatures. This is because Tm of each product is dependent on the length of the product as well as the percentage of G/C in the DNA sequence.

Based on the information obtained from the melt curve, it is apparent that a significant amount of primer-dimers (76.5°C peak) were present during amplification. The melt curve results from the zero-template control wells (Figure J.4) show that all amplification in these wells were due to primer-dimer (single melt curve peak at 76.5°C). Overall, the presence of primer-dimers resulted in competition for reaction reagents and in a reduction of the dynamic range of the standard curve.

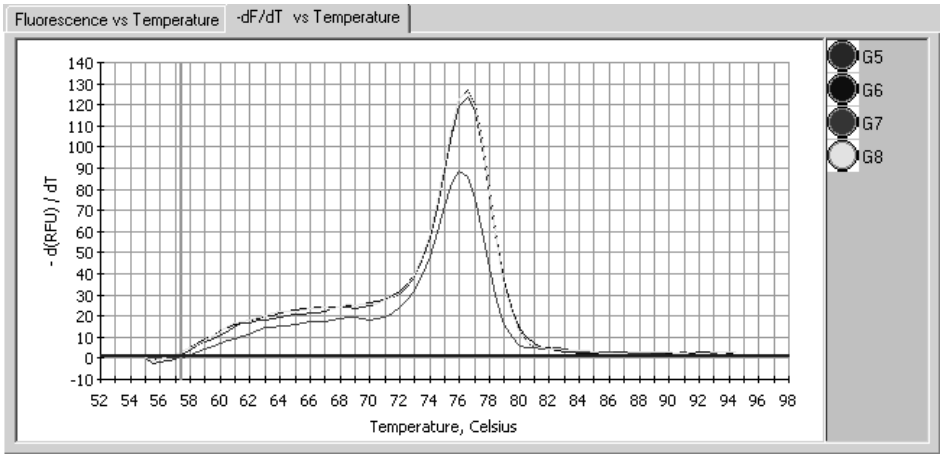


Fig. J.4. Melt curve data collected in the zero template control wells for amplification of IL-1b. These wells contained primers only, yet produced a product peak at 76.5°C, indicating that the amplified product is completely primer-dimer.

Melt curve analysis was used in this example to identify the source of the assay problems. In this particular example, primer-dimer formation was the major contributor to the decrease in dynamic range and efficiency of the reaction. Upon redesigning the primers, a second standard curve was generated as a 10-fold dilution series ($1x10^7$–$1x10^1$ copies/well with a zero template control) using SYBR Green I (Figure J.5).

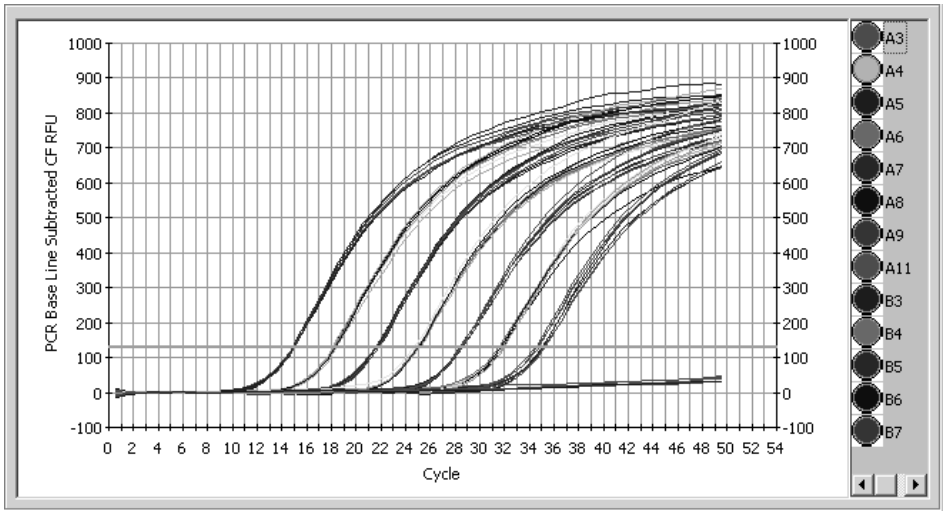


Fig. J.5. 10-fold dilution series of IL-1b plasmid using redesigned primers with SYBR Green I. Concentrations ranged from $1x10^7$ – $1x10^1$ copies/well with a set of replicates as a zero template control.

This standard curve produced a correlation coefficient of 1.000 and a slope = -3.343 (~99% efficiency). Furthermore, no amplification occurred in the zero template control wells. Melt curve analysis of the reactions revealed that all wells (with the exception of the zero template controls) produced a single amplified product at a T_m of 85.5–86.0°C, the T_m of the intended product (Figure J.6).

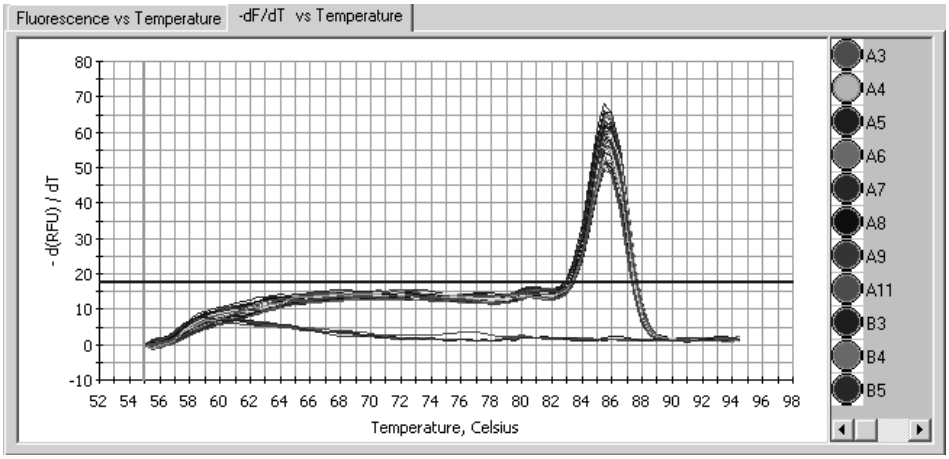


Fig. J.6. Melt curve data collected after IL-1b amplification with redesigned primers. All wells in the standard curve produced a single amplification product. The zero template controls produced no amplified product.

In conclusion, the presence of primer-dimer and other non-specific secondary products severely interferes with the amplification efficiency and the dynamic range of a desired dilution series. By decreasing and/or eliminating non-specific products (redesigning primers, optimizing annealing temperatures etc.), standard curves can be dramatically improved. Furthermore, melt curve analysis is a useful tool for improving assay design.

2.2 Characterization of Molecular Beacons for PCR Quantification

Melt curve analysis can also aid in developing molecular beacon assays for sensitive quantitative real-time PCR. A melt curve can be used to assess the design of a molecular beacon. It can also be used to approximate an annealing temperature for the amplification protocol. In this example, a melt curve was performed on a molecular beacon labeled with FAM and quenched with DABCYL. The beacon was melted alone or in the presence of a single stranded complement that binds to the probe portion of the beacon. The conditions used for the protocol consisted of complete denaturation at 95°C for 1 minute followed by melting at 1°C decrements for 10 seconds from 95°C to 35°C. The fluorescence vs. temperature was plotted in real time during the melt curve run (Figure J.7).

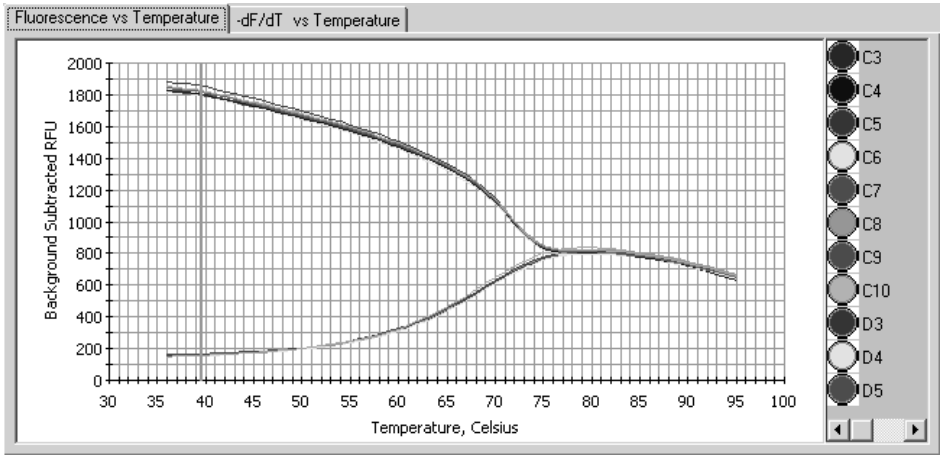


Fig. J.7. Fluorescence vs. Temperature plot of a melt curve protocol for a FAM beacon alone or in the presence of a single stranded complement template.

The negative first derivative plot of fluorescence vs. temperature (**- dF/dT vs. Temperature**) provides information as to whether the beacon was designed correctly. Figure J.8 shows distinct melting peaks for beacon alone and beacon mixed with complement template. The graph indicates that the beacon began to bind to its template around 80°C (an increase in fluorescence or positive peak). By 65°C, all of the available template is bound to beacon probes. The beacon alone trace shows that the highest rate of beacon arms closing beginning at 75°C (a decrease in fluorescence or negative peak). By 53°C, all of the beacon arms have closed. This information indicates that this beacon was designed correctly. The beacon will bind to its template around 80°C before the arms of the unbound beacons close at 75°C. Once the beacons are closed, they are unavailable to bind to template. Therefore, the temperature at which the arms close must be lower than the temperature at which the beacon binds to template.

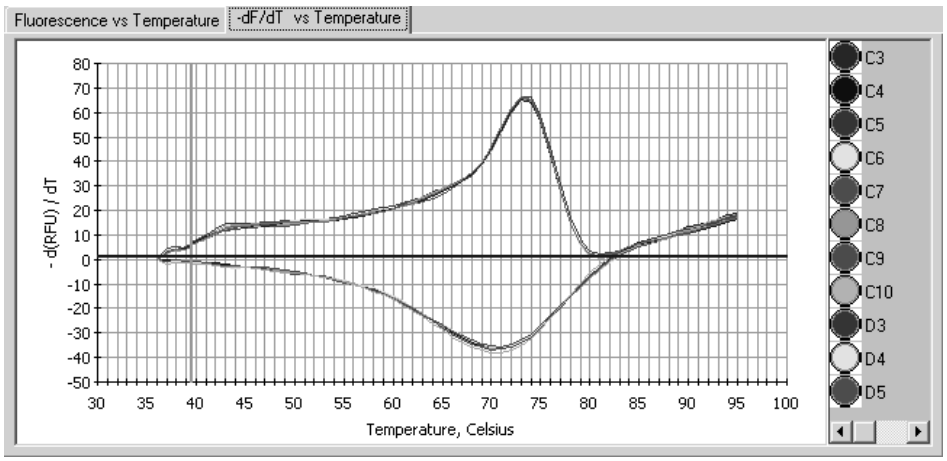


Fig. J.8. Negative first derivative (-dF/dT vs. Temperature) plot of fluorescence vs. temperature of a FAM beacon alone or in the presence of a single stranded complement template. The graph demonstrates the rate of change in fluorescence, with the peaks representing the maximum rate of change.

This plot also can assist the user in choosing the appropriate annealing temperature for amplification. When a molecular beacon probe anneals to its target, the total fluorescence is recorded as amplification data. As amplification products increase, the fluorescence also continually increases. Since the total fluorescence is detected, it is important to choose an annealing temperature where fluorescence of molecular beacon to target is high, and background fluorescence from unbound beacon is low. Based on the data presented in Figure J.8, the best annealing temperature would be between 50–53°C. All available template is bound to probe by 65°C. However, the background fluorescence of molecular beacon alone is still extremely high at this temperature. The arms of the remaining beacons not bound to template will not close completely until 53°C. By choosing an annealing temperature between 50–53°C, the total fluorescence collected for data analysis is primarily from molecular beacon probe binding to template with minimal background fluorescence. Therefore, melt curve analysis is a simple, quick assay for examining molecular beacon design as well as determining the appropriate annealing temperature for amplification.

Appendix K
MyiQ Filter Description and Instructions

The MyiQ is a single color instrument, thus requiring only one filter pair:

Excitation Filter: 480/40x

Emission Filter: 540/50m

Note: The filter designation 480/40x indicates that this filter will allow light from 460 to 500 nM to pass through. The first number, 480, indicates the center of the wavelength of light. The second number, 40, indicates the total breadth of wavelengths of light that can pass through it. X indicates excitation only and M indicates emission only types of filters. Excitation and emission filters are not interchangeable.

The resulting signal is suitable for detecting Fluorescein, FAM, SYBR® Green I, and other fluorophores that excite and emit at similar wavelengths.

The MyiQ is shipped with the specified filters pre-installed, ready for use. Normally, you should not have to reconfigure or replace the optical filters that come with your unit. However, the optical filters are removable and user-serviceable by design.

Note: The "FAM" filters that come pre-installed in the iQ Optical Module are of narrower bandwidth than those pre-installed in the MyiQ optical Module. Bio-Rad Laboratories will not provide support for the use of any optical filters besides the ones that come pre-installed in the MyiQ or obtained from Bio-Rad as a replacement.

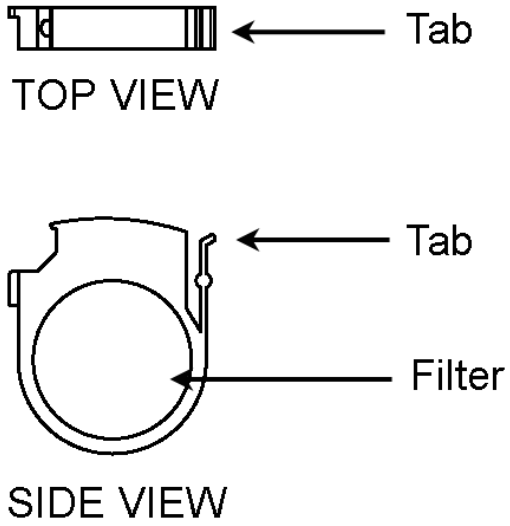


Fig. K.1. Filter in filter holder.

To access the optical filters, proceed as follows:

1. Turn off the power to the Optical Module.
2. Release the two latches on each side of the Optical Module. Slide the housing backwards 2–3” (5–8 cm), exposing a black case, the filter housing. It is not necessary to remove the housing or cables.

3. To access the excitation filter slot, flip open the hatch on the right side of the instrument. The excitation filter is actually set upon an upright wheel that may rotate towards both the front and back of the instrument. If the excitation optical filter is not seen, turn the excitation wheel using the supplied ball end hex driver until you can access the filter. As long as the power to the Optical Module is off, the excitation wheel may be turned freely in either direction. To access the emission filter, remove the plug from the slot at the center of the instrument (see Figure K.2). The emission filter is stationary and always accessible.
4. To remove a filter, grasp it on both sides with the supplied filter removal tool and squeeze the tab in; gently pull the filter up and out.
5. To insert a filter, grasp the filter with the filter removal tool and insert it into a vacant slot. For the excitationfilter, the tab on the filter should be closer to the front of the instrument. For the emission filter, the tab on the filter should be closer to the right of the instrument. Before operating the camera, confirm that neither the excitation filter slot nor the emission filter slot is empty.
6. Close the hatch to the excitation filter slot and replace the plug to the emission filter slot. Close the camera housing by sliding it forward until the latches click. Power the Optical Module back on.
7. Click on the Calibrate button in Run-Tim Central / Imaging Services before collecting images or data.

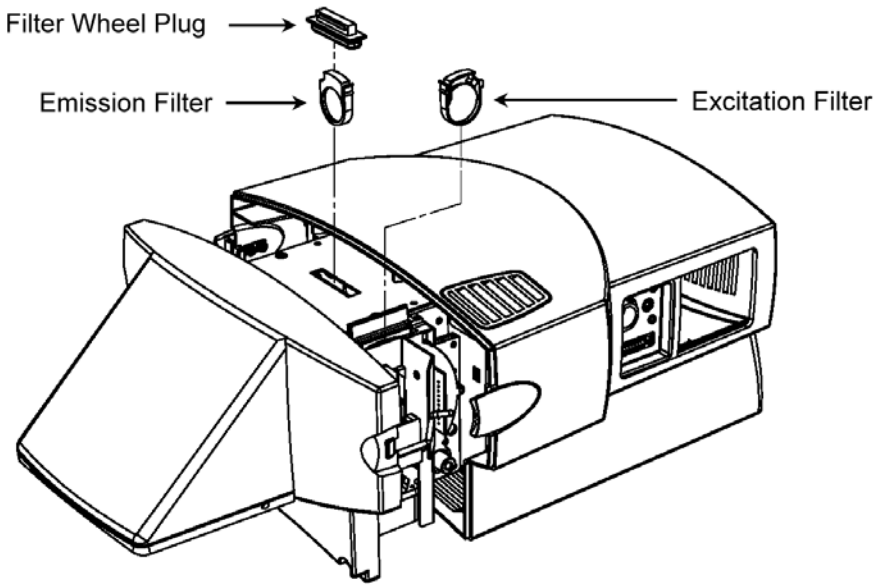


Fig. K2. Installing the filters.

Handle the optical filters with care, as they can crack if dropped. Also, avoid touching the surfaces of the filters, especially with fingers, as this can inpair data quality. Contact Bio-Rad for replacement filters.

Appendix L
MyiQ™ Real-Time PCR Detection System Software Version 1.0 Installation Instructions

This installation disk is for the following operating systems: Windows XP, NT and 2000.

1. Insert the MyiQ installation CD in a CD-ROM drive.
2. If the installation program does not begin automatically, select **Run…** from the Start menu and then type **X:\Setup**, where X is the drive letter of the CD-ROM drive. For example, if the CD-ROM is the E drive, type **E:\Setup**.
3. You must type a number in the serial number field in order for the installation program to continue. It does not matter what number you type in this field.
4. Follow all screen prompts to finalize the installation.

   Administrator-level privileges are required to install the software. If this is a first-time installation of the software, the administrator must also start the application initially.

   Certain configurations of Windows NT, 2000 and XP initialize new folders by assigning Read and Execute permission for the members of the 'Users' group. If you have this type of operating system, and this is a first-time installation, the administrator must change the protection on the Program Files/Bio-Rad folder or the Program Files/Bio-Rad/iCycler folder so that you can save protocol, plate setup and data files. If, after changing the protection on either of these folders, it is still not possible to write to the folders beneath Program Files/Bio-Rad/iCycler, check the Properties of each individual folder to be sure that under the Securities tab, the box is checked that allows inheritable permissions to propagate to that folder.

Bio-Rad Laboratories, Inc.

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France *01 47 95 69 65* ***Germany*** *089 318 84-0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *(852) 2789 3300* ***Hungary*** *36 1 455 8800*
India *(91-124)-2398112/3/4, 5018111, 6450092/93* ***Israel*** *03 951 4127* ***Italy*** *39 02 216091* ***Japan*** *03-5811-6270* ***Korea*** *82-2-3473-4460*
Latin America *305-894-5950* ***Mexico*** *55-52-00-05-20* ***The Netherlands*** *0318-540666* ***New Zealand*** *64 9 415 2280* ***Norway*** *23 38 41 30*
Poland *+ 48 22 331 99 99* ***Portugal*** *351-21-472-7700* ***Russia*** *7 095 721 1404* ***Singapore*** *65-64153188* ***South Africa*** *00 27 11 4428508*
Spain *34 91 590 52 00* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *(886 2) 2578 7189/2578 7241* ***United Kingdom*** *020 8328 2000*

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