
MyiQ™ Single-Color Real-Time PCR Detection System

Instruction Manual



Safety Information

Grounding

Always connect the MyiQ optics module to a three-prong, grounded AC outlet using the AC power cord provided with the system. Do not use an adapter to a two-terminal outlet. Always ensure that you set the module power switch to the off position when you connect or disconnect power cords.

Handling

Handle all components of the MyiQ system with care and with clean, dry hands at all times. The optical system contains mirrors and lenses that may shatter if the unit is dropped or struck with great force. If the unit is damaged such that internal components or wires are exposed, contact your local Bio-Rad office immediately. Do not attempt to repair or power on the instrument.

Servicing

The only user-serviceable parts of the MyiQ optics module are the lamp and filters. Call your local Bio-Rad office for all other optics module and thermal cycler related service. When you replace the lamp or filters, open only the outer casing of the MyiQ optics module. The camera lamp may get extremely hot during system operation. Do not attempt to remove the lamp without powering off the instrument and allowing the system to cool for at least 15 minutes. To prevent skin burns and fire hazards, do not attempt to operate the MyiQ system while the camera case is open. Do not open the casing of the MyiQ optics module when the instrument is in use.

Temperature

For normal operation, the maximum ambient temperature should not exceed 40°C. To ensure adequate cooling of the system, maintain a clearance of at least 4 inches around the sides of the MyiQ optics module. 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 optics module in extreme humidity (that is, greater than 90 percent) or where condensation can short internal electrical circuits or fog optical elements.

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 to the instrument not performed by Bio-Rad or an authorized agent.

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Section 1

Introduction to the MyiQ Single-Color Real-Time PCR Detection System

Nucleic acid amplification and detection are among the most valuable techniques used in biological research today. Real-time detection of PCR products is made possible by including in the reaction a fluorescent chemistry that reports an increase in the amount of DNA with a proportional increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequence-specific primers or probes.

Real-time analysis of PCR enables truly quantitative analysis of starting template copy number with accuracy and high sensitivity over a wide dynamic range. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The MyiQ™ Single Color Real Time PCR Detection System builds on the strengths of the iCycler® Thermal Cycler, which provides the optimum performance for PCR.

The MyiQ system features a tungsten halogen lamp as the light source. The filter-based optical design allows the use of optimal wavelengths of light for excitation and emission, resulting in excellent sensitivity and discrimination between multiple fluorophores. The CCD detector captures a simultaneous image of all 96 wells. This results in a comprehensive data set illustrating the kinetic behavior of the data during each cycle.

Simultaneous image collection insures that well-to-well data may reliably be compared. The MyiQ system reports data on the PCR in progress in real time, allowing immediate feedback on reaction success. All of these features demonstrate that the MyiQ system hardware was built to promote reliability and flexibility.

The MyiQ software includes features that make software easy and useful. The software is designed for convenience – offering speedy setup and analytical results. Many functions are presented graphically for ease of use. Tips on usage are available as your mouse rolls over the buttons. In addition, the Online Help Manual is available at all times simply by pressing the F1 key. The MyiQ software automatically analyzes the collected data at the touch of a button, yet leaves room for additional optimization of results based on your analysis preferences.

1.1 Setting up the MyiQ System Hardware

The MyiQ system should be installed on a clean, dry, and level surface. Identify an appropriate work area for the installation process prior to unpacking any system components. The entire installation process should take approximately 15 minutes to complete.

1.1.1 System Checklist

MyiQ Optics Module (170-9744), 1 box

- Power cord
- MyiQ optics module
- MyiQ software installation disk (170-9755)
- Amplification tech notes CD (version 1.0)

iCycler Chassis (170-8701), 1 box

- Power cord
- iCycler chassis
- iCycler thermal cycler operating instructions

Optical Kit (170-9752), 1 box

- Optical reaction block
- Modified sliding rear cover for iCycler thermal cycler
- Serial cable
- USB cable
- Filter extraction tool
- Optical tape applicator tools (3)
- Optics support bracket
- Support bracket screws
- Hex driver
- Hex screws

- Spare part: halogen lamp
- External well factor solution (packaged separately, store at -20°C)

Contact your local Bio-Rad office if any system components are missing or damaged.

Accessories

In addition, the following accessories are required to complete the installation process:

- Scissors
- Optical-quality sealing film or tube caps
- #2 Phillips screwdriver
- Calibrated micropipets
- Optical-quality PCR plates or tubes
- Aerosol barrier pipet tips

1.1.2 Installing the Optics Module on the iCycler Chassis

Handle the MyiQ optics module and iCycler thermal cycler with care and with clean, dry hands during unpacking and assembly. Do not handle the MyiQ system components with wet hands.

1. Remove the existing rear cover from the iCycler chassis by sliding the cover towards the front of the iCycler base.
2. Install the modified sliding rear cover provided with the optical reaction block, ensuring that the notch is oriented towards the rear of the iCycler thermal cycler.
3. Push the sliding rear cover on top of the chassis as far back as possible.
4. Rotate the green latches on the optical reaction block up towards the open lid.
5. Lift the optical reaction block by the handle and install it onto the chassis. Lower the front portion of the reaction block so that it engages with the chassis before the rear portion. The rear of the block lid should fit over the front lip of the sliding rear cover.
6. Secure the optical reaction block in place by rotating the green latches downward.
7. Close the optical lid.

1.1.3 Installing the Support Bracket

A support bracket with roller is provided for the MyiQ optics module. It is mounted to the rear of the iCycler thermal cycler.

Align the optics module support bracket with the two holes on the rear of the iCycler thermal cycler.

Using a #2 Phillips screwdriver, adjust the height of the bracket with two of the appropriate screws provided with the system accessories. Both of the screws should be approximately in the center of the slots on the bracket.

1.1.4 Installing the MyiQ Optics Module

1. Remove the plastic sheath and protective label from the optics module and place the optical module on a flat surface, taking care not to touch the inside of the nose portion of the module.
2. Remove the protective label from the optical lid.
3. Slide the optics module onto the U-bracket.
4. Secure the optics module to the U-bracket using the two long, thin hex screws and the hex driver provided.
5. After the optics module is secure, confirm that the optics module and lid assembly can be opened and closed readily.

If the lid is difficult to open, lower the support bracket slightly before tightening the bracket mounting screws.

If the lid is difficult to close, try raising the support bracket slightly before tightening the rear screws.

1.1.5 Connecting Power and Communication Cables to the MyiQ System

Before connecting any communication or power cables to the system, confirm that the power switch for the iCycler thermal cycler and the MyiQ optics module are in the OFF position.

1. Close the optical reaction block by sliding the lid forward and pressing down on the lid handle. On the right side of the optics module are three connectors.

2. Using the cables provided in the MyiQ Optical Kit, establish power and communication with the computer as follows:
 - **Recessed 3-pin power connector:** Connect the supplied power cord between the optics module and a grounded power outlet. This connection provides power only to the optical module; a separate power cord must be connected to the iCycler thermal cycler.
 - **Serial connector:** A serial connector is located at the rear of the iCycler thermal cycler. Connect the serial cable to the rear of the iCycler chassis and to the serial port on the side of the MyiQ optics module. This connection enables communication between the MyiQ software and the iCycler thermal cycler.
 - **USB port connector:** Connect the supplied USB cable between the MyiQ optics module and a USB 2.0 high-speed enabled port on the computer. Data are transferred to the computer via this cable. This single connection directs the operation of both the MyiQ optics module and the iCycler thermal cycler by the MyiQ Optical System Software. At the right rear corner of the optical reaction block is a single connector.
 - **Positive docking connector:** This self-aligning connector is secured into place when the MyiQ optics module is installed on the iCycler chassis. This connection senses when the lid handle is lifted.

1.2 Installing the MyiQ Software

Locate the software installation disk provided with the MyiQ system. This installation disk is compatible with computers running the Windows XP and/or Windows 2000 operating systems.

1. Insert the MyiQ Optical System Software installation CD in a CD-ROM drive.
2. If the installation program does not begin automatically, click Run in the Start menu and then type X:\MyiQ\Setup, where X is the drive letter of the CD-ROM drive. For example, if the CD-ROM is the E drive, type E:\MyiQ\Setup.
3. Follow all screen prompts to finalize the installation. Certain configurations of Windows 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 for the Program Files/Bio-Rad folder or the Program Files/Bio-Rad/MyiQ folder so that you can save protocol, plate setup, and data files.

If, after you change the protection on either of these folders, you still cannot write to the folders beneath the Program Files/Bio-Rad/MyiQ folder, check the properties of each folder. Specifically, in the Properties window Securities tab, ensure that the check box which allows inheritable permissions to propagate to that folder has been selected.

1.2.1 Calibrating the Instrument

Before using the MyiQ system for the first time, four procedures must be performed on the new instrument: Camera Driver Installation, Mask Alignment, Background Calibration, and Persistent Well Factor Collection.

1. Power on the iCycler thermal cycler and the MyiQ optics module.
2. Follow all screen prompts to finalize the camera driver installation.
3. Allow the instrument optics to warm up for 10 minutes prior to performing the Mask Alignment, Background Calibration or Persistent Well Factor Collection procedures.
4. Open the MyiQ system software by double-clicking on the shortcut icon located on the Windows desktop, or by selecting the MyiQ program icon from the Bio-Rad folder in the Windows Start menu.

1.2.2 Overview of the MyiQ Software

The MyiQ software is divided into four sections, called 'modules'. Icons representing each of the modules are always shown on the left side of the screen. The active or selected module has bright green background, whereas unselected modules have a dark green background. Each module is subdivided into windows which perform a specific function for that module.

The four modules in the MyiQ software are the:



Workshop Module: This module is used to select a Plate and Protocol and Run an experiment. It is also where experimental files are selected and opened for Data Analysis. The Workshop Module consists of a Setup and Plate Summary window. The Protocol, Plate, Run Set and Data File tabs which can be used to select, open, edit or create files. In Setup the selected Protocol and Plate Setup can be Run or the selected data file can be opened for analysis in Data Analysis.



Run-Time Central Module: This module is used to initiate and monitor experimental runs. It is accessed from the Workshop Module, once the Protocol and Plate Setup have been chosen, by clicking Run or Run End Point.



Data Analysis Module: This module contains a suite of tools enabling you to conduct thorough and varied analyses of your experimental data. Within this module are screens for Quantitative, Melt Curve/Peak, End Point, and Gene Expression analyses. The Edit Plate screen permits post-run editing of the experimental plate setup, allowing you to correct erroneous sample type assignments. The Data Analysis module is opened automatically when you open a saved data file from the Workshop.



Calibration Module: In order to extract the best data from your real time PCR experiment, the MyiQ system must be calibrated. These simple and easily-performed calibration routines are accessed in the Calibration module. There are calibration routines for Mask Alignment, Background Calibration, and Persistent Well Factor Collection.

Section 2

Quick Guides to Running Experiments on the MyiQ System

2.1 Calibration Quick Guide

You calibrate the MyiQ Camera using the MyiQ software Calibration Module.

2.1.1 Calibration Overview

You must perform the following steps in order to calibrate the MyiQ Camera:

1. Mask Alignment
2. Background Calibration
3. Persistent Well Factor Generation

What You Will Need

You need the following to calibrate the MyiQ camera:

1. iCycler iQ External Well Factor Solution
2. 2 x 96-well PCR plate or preferred reaction vessel
3. Optical quality sealing tape or preferred sealing method

Calibration Preparation

You must prepare two plates: the external well factor plate and the background calibration plate.

Preparation of an External Well Factor Plate

(For Mask and Persistent Well Factor calibrations)

To prepare an external well factor plate:

1. Dilute the 10x External Well Factor Solution to 1x with water.
2. In Plate 1 pipet equal volumes of the 1x external well factor solution into ALL wells.
3. Seal the plate using the sealing method you will use in your experiment.

Preparation of an Background Calibration Plate

Prepare a background calibration plate by sealing Plate 2 using the sealing method you will use in your experiment.

2.1.2 Mask Alignment

Before you align the mask, complete the following steps:

1. See Calibration Overview about preparing the external well factor plate.
2. Place the external well factor plate in the iCycler.
3. Select the Mask tab in the Calibration module.

To align the mask:

1. Click Home.
2. Click the Filter Position 2 option button.
3. Click Take an Exposure.
4. Click Show Mask.
5. If any pink pixels are present in the mask, reduce the exposure time and retake an exposure. Keep reducing the exposure time and retaking the exposure until no pink pixels are present.
6. Click Optimize Mask.
7. Click Save Mask.

This completes Mask Alignment calibration. Next, perform Background Calibration.

2.1.3 Background Calibration

Before you perform background calibration, complete the following steps:

1. See Calibration Overview about preparing the background calibration plate.
2. Ensure that the mask has been aligned.
3. Place the Background calibration plate in the iCycler instrument.
4. Select the Background tab in the Calibration module.

To perform background calibration:

1. Select a well seal.
2. Select a vessel type.
3. Click Collect Background Factors.
4. When MyiQ completes the Background Calibration run, a dialog box appears with the message, "Background Calibration Run Complete."
5. Click OK to exit.

This completes background calibration. Next, perform Persistent Well Factor calibration.

2.1.4 Persistent Well Factors Calibration

Before you perform a Persistent Well Factor Calibration run, complete the following steps:

1. See Calibration Overview about preparing the external well factor plate.
2. Ensure that the mask has been aligned and that Background Calibration has been performed.
3. Place the external well factor plate in the iCycler.
4. Select the Well Factors tab in the Calibration module.

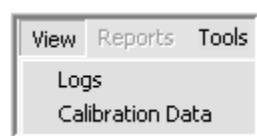
To perform a Persistent Well Factor Calibration run:

1. Select a sample volume.
2. Select a well seal.
3. Select a vessel type.
4. Click Collect Persistent Well Factors.
5. When MyiQ completes the Persistent Well Factor Calibration run, a dialog box appears with the message, "Persistent Well Factor Calibration Run Complete".

6. Click OK to exit.

This completes Persistent Well Factor calibration.

2.1.5 Viewing Calibration Files



Calibration files can be viewed by selecting Calibration Data from the View menu.

The background factors file is in the Background Factors folder in the MyiQ Program Folder.

Persistent Well Factors are in the Well Factors folder in the MyiQ Program Folder.

2.2 Protocol Quick Guide

Protocols can be:

1. Selected,
2. Created, and
3. Edited from the Protocol Viewer of the Workshop module.

2.2.1 Selecting a Protocol

To select a protocol.

1. Locate the folder by using the browser below the Protocol button.
2. Click the protocol name. The selected protocol appears in the Selected Protocol window.

Note: The MyiQ software has a number of sample protocol files that you can use.

2.2.2 Editing or Creating a Protocol

- Edit a selected protocol by clicking Edit in the Selected Protocol window.
- Create a protocol from a protocol template by clicking Create New in the Selected Protocol window.

Note: Clicking Edit or Create New in the Selected Protocol window opens the Protocol Editor.

Edit the protocol change the appropriate fields in the spreadsheet by performing one or more of the following five tasks:

1. Edit the Dwell Time and Setpoint temperature.

Click in the Dwell Time or Setpoint cell, and then enter the Dwell Time or Setpoint temperature. To enter 10 seconds, type 0 followed by : then 10 (that is, as the time appears in the spreadsheet). Alternatively 10 seconds can be entered as 0.10.

2. Acquire data.

Click in the Data Acquisition column, and then click Real-Time at the step you want to collect real-time data. Click Melt Curve if data from a melt curve is required.

Note: Ensure that every thermal protocol has at least one Data Acquisition step.

3. Insert cycles and steps.

Insert a cycle by clicking in the Insert column within the cycle row. Cycles have a blue background. MyiQ inserts the new cycle below the current cycle.

Insert a step by clicking in the Insert column within a step row. Steps have a white background. MyiQ inserts the new step below the current cycle.

Note: You can use the Options cell to customize whether the step or cycle is inserted before or after the current cycle/step, as well as how many steps MyiQ will insert when you insert a cycle.

4. Delete cycles and steps.

Delete a cycle by clicking in the Delete column within a cycle row. Cycles are indicated with a blue background.

Delete a step by clicking in the Delete column within a step row. Steps are indicated with a white background.

5. Save the protocol.

Click Save & Exit Protocol Editing. Type the name of the protocol in the Save As dialog box, and then click Save.

Note: You can only exit the Protocol Editor by clicking **Save & Exit Protocol Editing** or **Cancel & Edit Protocol Editing**.

2.3 Plate Setup Quick Guide

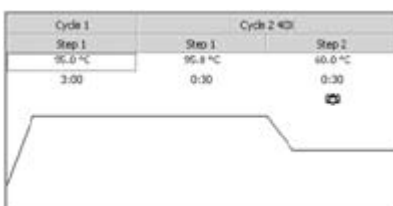
To set up a plate:

1. From the Workshop module:
 - a. Click Create New in the plate setup display window to enter the Plate Setup Editor.
or
 - b. Click Plate. Open the desired plate setup file in the appropriate directory. Double click the file name to go directly to the Plate Setup Editor.
or
 - c. Click Plate. Open the desired plate file in the appropriate directory. Click the file name to open the plate setup in the bottom right pane of the Workshop window. Click Edit to open the plate setup in the Plate Setup Editor.
or
 - d. Click Data File. Open the desired data file in the appropriate directory. Click the file name to open the plate setup associated with the data file in the bottom right pane of the Workshop window. Click Edit to open the plate setup in the Plate Setup Editor.
2. Enter or edit any notes about the plate setup in the Notes box.
3. Enter or edit the sample volume, seal type, and vessel type.
4. Enter or edit a name for the experiment.
5. Click Select/Add Fluorophores and select or edit the fluorophores to be used on the plate. Associate each fluorophore with a unique color.
6. Click a sample type icon.
7. Select the type of replicate loading desired.
8. Click a fluorophore.
9. Click or drag across the plate to define wells with the selected fluorophore and sample type.
10. Continue defining the remaining wells that will contain the first fluorophore by changing to the other sample type icons.
11. To delete a previously defined well, click the Delete All icon, and then click the well.
12. To delete the selected fluorophore from a previously defined well, click the Delete Fluorophore icon, and then click the well.
13. If there are standards in the first dye layer, click Dilution Series and calculate the concentrations of the standards, or type the concentrations in the spreadsheet manually.
14. Click Save & Exit Plate Setup. Give the plate setup file a new name or overwrite the current version of the file. The software adds the extension .pts automatically.

2.4 Running a Real-Time Experiment on the MyiQ

To set up a real-time PCR experiment:

1. Select, Edit or Create a Protocol.



2. Select, Edit or Create a Plate Setup.



3. Click Run.



4. Run the Selected Protocol and Plate Setup.



After you click Run in the Workshop module, MyiQ opens the Initiate Run tab within the Run-Time Central module.

2.4.1 Beginning a Run

To begin a run:

1. Check that the desired Protocol and Plate Setup are displayed in the bottom half of the Initiate Run screen.
2. Select the type of well factors to use by selecting one of the following two check boxes:



3. Click Begin Run.
4. Name the file in the Save Optical Data File dialog box.
5. Click OK.

2.4.2 During a Run

When MyiQ begins the run, MyiQ opens the Monitor Run window. You can see the progress of the run in this window.

At the end of the run, the Run Status dialog box appears as shown below. You can choose between displaying the data in the Data Analysis module or returning to the Workshop Module.

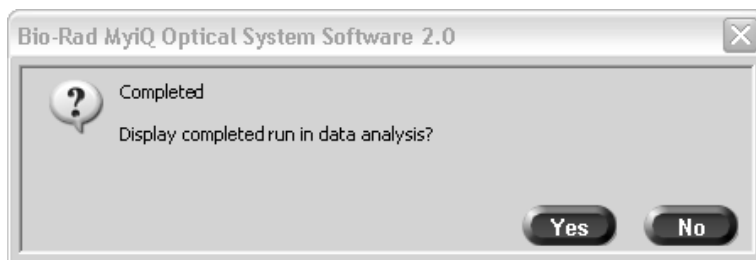


Fig. 2.1. The Run Status dialog box.

Click Yes to proceed to the Data Analysis module.

2.5 Data Analysis Quick Guide

The Data Analysis module is where data is presented and analyzed. When the MyiQ software opens, the Data Analysis module is gray and the module is not active. To analyze a data file, open the data file in the Data File tab of the Workshop module by selecting the data file and then clicking Analyze.

The Data Analysis module consists of five tabs:

- **PCR Quant**
- **Melt Curve/Peak**
- **End Point**
- **Gene Expr**
- **Edit Plate**

2.5.1 PCR Quant Tab Quick Guide

The PCR Quant screen is where conditions are set for the analysis of amplification data. Once the wells to be used in the analysis are selected and the baseline and threshold set, the software determines the threshold cycle for each well and, if standards are present, estimates the starting quantity of each unknown sample. The software will automatically set the baseline individually for each well and then automatically calculate a single threshold value for each dye. If standards are present, the software searches for a threshold that gives the statistically best standard curve. The baselines and threshold may also be set manually.

Use the PCR Quant tab to set the analysis conditions for the data file. The analysis conditions include:

- Setting the PCR baseline.
- Setting the threshold.
- Determining which wells to exclude or include in the experiment.

2.5.2 Analyzing a Data File

To analyze a data file:

1. Click the Data File tab in the Workshop module.
2. Select a data file, and then click Analyze. The file opens in the PCR Quant tab within the Data Analysis module. MyiQ automatically chooses the Data Analysis conditions including baselines and thresholds.
3. You may select or deselect wells to be included in the analysis by selecting the Analyze Wells check box.
4. If the data open in any screen other than the PCR Quant screen, click PCR Quant. If the data file is being opened for the first time, an automated analysis of baselines and threshold will be conducted, including every defined well. If the file was previously saved after an analysis, the last set of analysis conditions are applied again.
5. Make any manual adjustments to the baselines.

6. Make any manual adjustments to the threshold.
7. You may return to the automated analysis at any time by choosing Baseline Threshold from the context menu that you access by right clicking on the amplification chart.

2.6 Melt Curve/Peak Quick Guide

1. In the Workshop, click Data File, navigate the directory to find the desired data file, and then double click the file name to open the data file in the Data Analysis module.
2. Click the Melt Curve/Peak tab.

The Melt Curve and Melt Peak plots appear. The identified melt peaks will be listed in the Melt Curve/Peak Spreadsheet, along with the RFU and $-d(\text{RFU})/dT$ data.

There are many parameters you can edit.

3. Click Analyze Wells to select wells for analysis.
4. Right click on either plot to bring up a context menu that will allow you to:
 - Control Digital Filtering
 - Adjust Graph
 - Define Trace Style
 - Restore Graph
 - Show All Traces
 - Copy Graph
 - Print Graph
5. Before a peak is listed, it must cross the threshold bar. You can drag the threshold bar to another position to change the peaks listed.
6. Another way to remove a peak is to highlight the peak in the spreadsheet and then click Delete Selected Peak. You can use the SHIFT key to define a range of peaks to delete.
7. Click Edit Melt Peak Begin/End Temp to modify those default values.
8. At any time, click Restore Defaults to undo all modifications to peaks.

2.7 End Point Quick Guide

You can implement End Point Analysis in two ways:

- Click Run End Point to initiate the collection of End Point data from a sample plate.
- Click the Endpoint tab in the Data Analysis module for an existing data file.

2.7.1 End Point Run

To initiate an end point run:

1. Insert the experimental plate into the iCycler reaction module.
2. Select or Create the Plate Setup in the Workshop module Plate Setup tab.
3. Click Run End Point.
4. In the Run-Time Central/Initiate Run tab, specify the setpoint for data collection, and then click Begin Run.

Note: You must use Persistent Well Factors for every End Point Run.

5. Enter a name for the data file, and then click Save.
6. Once MyiQ completes the run, the End Point tab is displayed.
7. Make selections for the following parameters:
 - Method
 - End Point Tolerance and Tolerance Parameter

8. Select the wells to analyze by clicking Analyze Wells.
9. Define the positive and/or negative controls in the Define Controls column within the End Point Analysis table.
10. Click Recalculate. The End Point analysis table displays a positive, negative, or blank label for each unknown under the Unknowns Call column.
11. Click Reports to obtain customized reports of the End Point analysis.

2.7.2 End Point Analysis of an Existing Data File

To conduct an end point analysis of an existing data file:

1. Select the data file to be analyzed in the Workshop module Data Analysis tab.
2. Click Analyze.
3. Click the End Point tab.
4. Make selections for the following parameters:
 - Method
 - End Point Tolerance and Tolerance Parameter
5. Select the wells to analyze by checking the Analyze Wells button. Click on Analyze Selected Wells, and close the Select Wells for Analysis floating window when finished.
6. Define the positive and/or negative controls in the Define Controls column of the end point analysis table.
7. Click Recalculate. The End Point analysis table displays a positive, negative, or blank label for each unknown under the Unknowns Call column.
8. Click Reports to obtain customized reports of the End Point analysis.

2.8 Gene Expression Quick Guide

There are two ways to analyze Gene Expression results. Choose a method based on your experimental design.

- No Reference Genes (Relative Quantity, ΔC_T)
- Reference Genes (Normalized Expression, $\Delta\Delta C_T$)

2.8.1 Relative Quantity (ΔC_T) Quick Guide

To calculate Relative Quantity (ΔC_T):

1. In the PCR Quant tab, establish desired experimental conditions such as baseline and threshold and which wells are to be included in the analysis.
2. Click the Gene Expr tab.
3. Select **Relative Quantity** as your analysis method.
4. Assign Gene and Condition Names in the Gene Expression Plate Interface.
5. Assign Attributes in the Gene List
6. Assign Attributes in the Condition List
7. Click **Recalculate** to see your results.

Relative Quantity is graphed in the Gene Expression Graph Interface. You can access the data in the Gene Expression Settings Interface in the Data Table.

2.8.2 Normalized Expression ($\Delta\Delta C_T$) Quick Guide

To calculate Normalized Expression ($\Delta\Delta C_T$):

1. In the PCR Quant tab, establish desired experimental conditions such as baseline and threshold and which wells are to be included in the analysis.
2. Click the Gene Expr tab.
3. Select **Normalized Expression** as your analysis method.

4. Assign Gene and Condition Names in the Gene Expression Plate Interface.
5. Assign Attributes in the Gene List.

In Normalize Expression Analysis you must assign reference gene(s).

6. Assign Attributes in the Condition List.
7. Click **Recalculate** to see your results.

Normalized Expression is graphed in the Gene Expression Graph Interface. The data can be accessed in the Gene Expression Settings Interface within the Data Table.

2.9 Edit Plate Setup Quick Guide

For post-run editing of the Plate Setup saved in a Data File:

1. From the Workshop module:
 - a. Click Data File above the directory of the home workshop. Navigate the directory until the desired data file is found. Double click the file name to bring the file directly into the Data Analysis module.
 - or
 - b. Click Data File above the directory of the home workshop. Navigate the directory until the desired data file is found. Click the file name once to open the plate setup associated with the data file in the bottom right pane of the Workshop window. Click Analyze to bring the data to the Data Analysis module.
2. At the top of the Data Analysis window, click Edit Plate.

Edit Plate

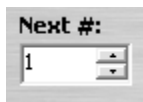
3. A modified version of the Plate Setup Editor opens. In this modified window, you cannot add fluorophores to or remove fluorophores from the fluorophore list. Also, you cannot add a previously-defined fluorophore or remove a previously-defined fluorophore from a well.

The screenshot shows the 'Edit Plate' window with the following components:

- Setup / Plate Summary** tabs at the top.
- Editing Plate:** A text box containing 'BioRadDelaultWellPlate.plt'.
- Notes:** A text area for additional notes.
- Sample Volume:** A dropdown menu set to '25'.
- Seal Type:** A dropdown menu set to 'Film'.
- Vessel Type:** A dropdown menu set to 'Plates'.
- Buttons:** 'Save & Exit Plate Editing' and 'Cancel & Exit Plate Editing'.
- Spreadsheet** tab with a grid of 12 columns (1-12) and 8 rows (A-H).
- Replicates:** A section on the left with icons for replicates and a 'Size' dropdown.
- Experiment Name:** A text box with 'My experiment'.
- Experiment Type:** A dropdown menu set to 'General'.
- Fluorophore:** A dropdown menu set to 'FAM'.
- Probe/Primer:** A text box with 'FAM'.
- Select/Add Fluorophore** button.
- Units:** A dropdown menu set to 'copy number'.
- Apply units to:** Radio buttons for 'Whole Plate' and 'Fluorophore'.
- Scientific Notation:** A checked checkbox.
- Dilution Series:** An unchecked checkbox.
- Table:** A table with columns: Row, Column, Sample Type, Rep #, Identifier/Condition, Quantity, Units.

4. Edit any notes about the plate setup in the Notes box.
5. Edit the name of the experiment in the Experiment Name box.
6. To edit a sample type.

- a. Click a Fluorophore.
- b. Click the correct sample type icon.
- c. Select the Next # check box to be sure that the desired number will be assigned to the next standard you define.



- d. Select the type of Replicate loading desired.
 - e. Click a previously defined well to overwrite its original sample type definition. Replicates of the well are not affected.
 - f. Repeat steps b-e above for each additional sample type to be edited for this fluorophore.
 - g. Repeat steps a-f above for each additional fluorophore.
7. To edit the concentrations of the standards:
 - a. Click the fluorophore for the standards to be defined.
 - b. Click Dilution Series and calculate the concentration, or enter it directly in the spreadsheet.
 8. Select the Units for the standards.
 9. Click Apply Plate Changes to make the changes. To see the effect on analysis, go to one of the other Data Analysis windows.



10. The original plate setup is retained with the data file and may be restored at any time by clicking Restore Original Plate



Section 3

Workshop Module

The Workshop module consists of two tabs: the Setup tab and the Plate Summary tab.

The primary tab in the Workshop module is the Setup window, shown below.

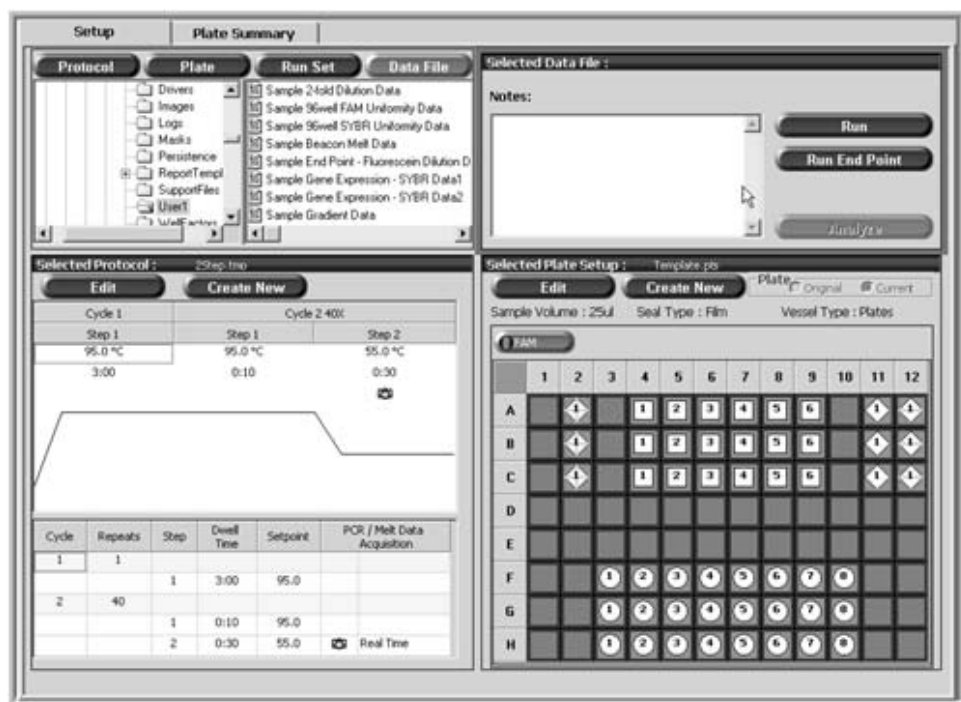


Fig. 3.1. The Setup window.

The Setup window contains four selections:

- **Protocol:** Used to select, edit, or create a protocol for running a real-time experiment.
- **Plate:** Used to select, edit, or create a plate setup for running a real-time experiment.
- **Run Set:** Consists of a linked protocol and plate setup. This tab is useful when you repeat the same experiment on a regular basis.
- **Data File:** Primarily used to select and open a data file. You can also use this tab to run a real-time experiment using the same protocol and plate setup that were used to create the data file. The selected plate setup can be either the Original plate setup or the Current (last saved) plate setup.

3.1 Setup Window Layout

The Workshop Setup window consists of four areas:

- **File Browser area:** Located in the top left of the Setup window, the File Browser area contains a folder tree on the left side and a file list on the right side. Between the folder tree and file list is a moveable dividing bar. Moving this bar can provide more space in either the folder tree or the file list, which can help with navigation.

The files displayed in the file list depends upon the selected Setup window tab.

- If the Protocol tab is selected, then MyiQ displays protocol files, which have the extension .tmo. The selected protocol appears in the Selected Protocol window.
- If the Plate tab is selected, then MyiQ displays plate setup files, which have the extension .pts. The selected plate appears in the Selected Plate Setup window.

- If the Run Set tab is selected, then MyiQ displays run set files, which have the extension .run. MyiQ displays the selected run set, which is a linked protocol and plate setup, in the Selected Protocol and Selected Plate Setup windows.
- If the Data File tab is selected, then MyiQ displays data files created from previous experimental runs, which have the extension .opd. MyiQ displays the selected data file name and any associated notes in the Selected Data File window. The Notes box is editable only after you open the data file. MyiQ displays the protocol and plate setup used in creating this data file in the Selected Protocol and Selected Plate Setup windows, respectively.
- **Selected Protocol area:** Located in the bottom left of the Setup window, the Selected Protocol area displays the details of the protocol selected in the File Browser area. The selected protocol file name appears at the top of this window. If you have selected a data file, then MyiQ displays the protocol used to create the data file and the data file name. MyiQ displays the selected protocol both graphically and in spreadsheet format in the Selected Protocol area.
- **Selected Plate Setup area:** Located in the bottom right of the Setup window, the Selected Plate Setup window displays the details of the plate setup selected in the File Browser area. The selected plate setup name appears at the top of this window. If you have selected a data file, then the plate setup within the data file and the data file name are both selected in MyiQ. When you select the Data File tab and a data file, then MyiQ displays the Original and Current plate setup. The Original plate setup is the plate setup used to create the data file. The Current plate setup is the plate setup last saved with the data file.
- **Selected Data File area:** Located in the top right of the Setup window, the Selected Data File area displays the selected data file or the run set file name that you selected in the File Browser area. The Notes box displays the notes associated with either the run set or data file. Buttons in this area include:
 - **Run:** Used to initiate a real time PCR experimental run.
 - **Run End Point:** Used to initiate an End Point run.
 - **Analyze:** Used to open a data file. You can also open the data file by double-clicking on the data file name in the File Browser area.

3.2 Plate Summary Window

The Plate Summary window provides a more detailed view of the plate including standard quantities and identifiers, as shown below. It is useful to check the Plate Setup after editing or creating the Plate and also before performing a run. It can also be used to check the plate layout in a Data File.

If you select the Data File tab, the plate shown in the Plate Summary window depends on whether you selected Original or Current in the Selected Plate Setup window. Original is the plate setup that the data file was created with. Current is the last saved plate setup.

Setup		Plate Summary											
Viewing Plate:		BioRadDef\util\NewPlate.xls											
Experiment:		My experiment											
		Copy To Clipboard Print											
		FAM1 FAM2 FAM3 FAM4 FAM5											
		1	2	3	4	5	6	7	8	9	10	11	12
A	SampleType	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8	Std-9	Std-10	Std-11	Std-12
	copy number	1.00e-03	5.00e-04	2.50e-04	1.25e-04	6.25e-05	3.13e-05	1.56e-05	7.81e-06	3.91e-06	1.95e-06	9.77e-07	4.88e-07
	Identifier	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel
B	SampleType	Std-13	Std-14	Std-15	Std-16	Std-17	Std-18	Std-19	Std-20	Std-21	Std-22	Std-23	Std-24
	copy number	2.44e-07	1.22e-07	6.10e-08	3.05e-08	1.53e-08	7.63e-09	3.81e-09	1.91e-09	9.54e-10	4.77e-10	2.38e-10	1.19e-10
	Identifier	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel
C	SampleType	Std-25	Std-26	Std-27	Std-28	Std-29	Std-30	Std-31	Std-32	Std-33	Std-34	Std-35	Std-36
	copy number	5.96e-11	2.98e-11	1.49e-11	7.45e-12	3.73e-12	1.86e-12	9.31e-13	4.66e-13	2.33e-13	1.16e-13	5.82e-14	2.91e-14
	Identifier	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel	squirrel
D	SampleType												
	Concentration												
	Identifier												
E	SampleType												
	Concentration												
	Identifier												
F	SampleType												
	Concentration												
	Identifier												
G	SampleType												
	Concentration												
	Identifier												
H	SampleType												
	Concentration												
	Identifier												

Fig. 3.2. The Plate Summary tab.

The spreadsheet displays data one filter mate at a time. You use the fluorophore buttons to select the plate summary of the desired filter mate.

To print the selected plate setup display, click Print. To copy and paste the entire plate setup, click Copy to Clipboard. You can also copy only a portion of the plate setup by clicking and dragging your mouse over the desired wells, then pressing CTRL+C in the MyiQ software, and then pressing CTRL+V in the desired application.

3.3 Thermal Protocol

MyiQ 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.

Protocol files contain the information necessary to direct the operation of the iCycler and also direct the MyiQ camera when to collect data.

- A Protocol is made up of as many as 9 cycles and a cycle is made up of as many as 9 steps.
- A Step is defined by specifying a setpoint temperature and the dwell time at that temperature.
- A Cycle is a collection of steps that are repeated (up to 600 times).
- Every protocol must have at least one data collection step. This may be a real-time data collection step or a melt curve data collection step.

Thermal protocols can be selected, created and edited from the Protocol tab in the Workshop module.

3.3.1 Protocol Viewer

The Protocol Viewer is shown below. It is selected by clicking on the Protocol button. When active, a green border appears around the Graphical Display and the spreadsheet.

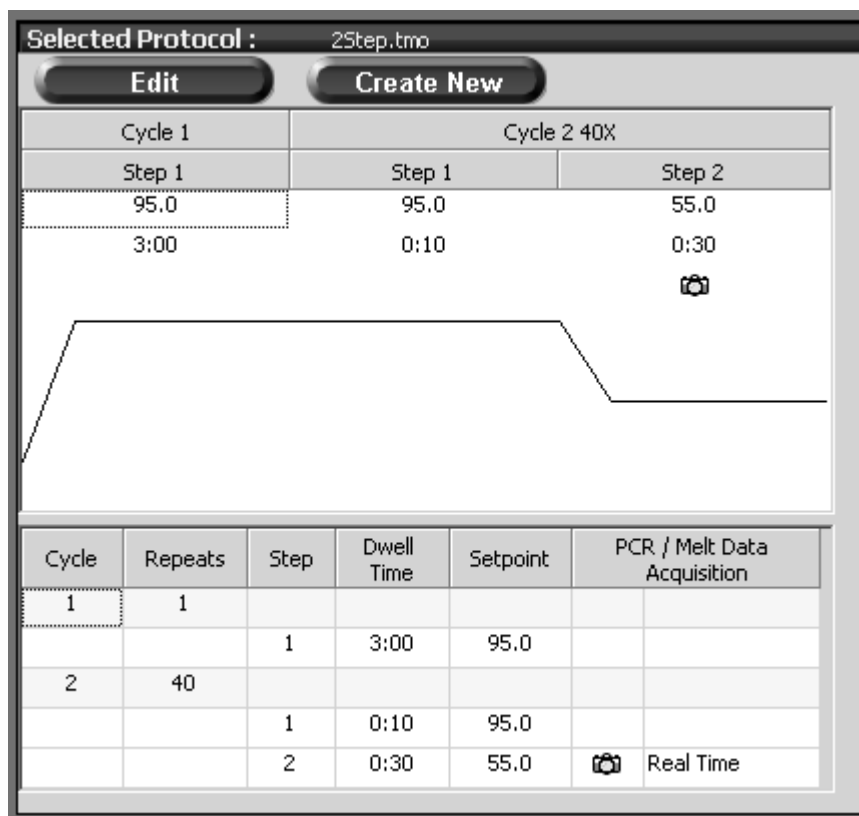


Fig. 3.3. Protocol Viewer.

When the Protocol button is selected (as indicated by the green active button) the browser displays protocol files (which have the suffix .tmo).

The browser may be used to select a protocol that is displayed in both the Graphical Display and the Spreadsheet.

The size of the folder browser and the file pane can be changed by moving the bar that separates them.

3.3.2 Selecting a Protocol

- Use the browser below the Protocol button to locate the folder the Protocol is located in, and then click on the protocol name to select it.
- The selected protocol appears in the Selected Protocol window.
- The MyiQ software has a number of sample protocol files you can use.

3.3.3 Editing or Creating a Protocol

To edit a selected protocol, double click the protocol file or click Edit in the Selected Protocol window.

To create a protocol from a protocol template, click Create in the Selected Protocol window.

Clicking Edit or Create in the Viewing Protocol window will transfer the software to the Protocol Editor. You can edit the protocol in the spreadsheet at the bottom of the Editing Protocol window.



Fig. 3.4. Protocol Editor.

Editing Dwell Time and Setpoint Temperature

Click in the spreadsheet in the Dwell Time or Setpoint cell, then enter the Dwell Time or Setpoint temperature.

Temperature and Dwell Time Ranges

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

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

Zero Dwell Times: When the dwell 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 attain the next setpoint temperature.

Data Acquisition

Click in the spreadsheet in the Data Acquisition column and select Real-Time data collection for any step.

Note: Ensure that every thermal protocol has at least one Data Acquisition step.

Click Melt Curve if data from a melt curve is required.

Inserting Cycles and Steps

To insert a cycle, click in the Insert column on the Cycle row. Cycles are indicated by a blue background.

To insert a step, click in the Insert column on a Step row. Steps are indicated by a white background.

Deleting Cycles and Steps

To delete a cycle, click in the Delete column on the Cycle row. Cycles are indicated by a blue background.

To delete a step, click in the Delete column on a Step row. Steps are indicated by a white background.

To save a protocol, click Save & Exit Protocol Editing. Type the name of the protocol in the Save As dialog box and then click Save again.

The Protocol Editor may only be exited by clicking **Save & Exit Protocol Editing** or **Cancel & Exit Protocol Editing**.

3.3.4 Add Protocol Options

If you want to add any protocol options, first enable them by clicking in the check box next to its description in the Show Options box. The following options are available:

1. Gradient
2. Infinite Hold
3. Ramp Rate
4. Temperature Change
5. Time Change
6. Cycle Description
7. Step Process

Infinite Hold

When a cycle is not repeated, you can specify the dwell time at any step in that cycle as infinite by using the Infinite Hold option. This means that the instrument will maintain the specified temperature until execution is interrupted. 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 setpoint temperature until the Continue Running Protocol button in the Thermal Cycler tab of the Run-Time Central Module is selected.

To program an infinite hold:

1. Click Infinite Hold in the Show Options box. A new column titled Hold appears in the spreadsheet.
2. Select the Hold check box for the step that you want to maintain at a constant temperature and enter the desired temperature in the Setpoint cell of the spreadsheet.

Ramp Rate

The ramp rate is the speed with which the iCycler 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. The iCycler allows you to change temperatures at a fixed rate less than the maximum.

Ramp rates are adjustable to 0.1°C /sec and must fall within the range of 0.1–3.3°C per second for heating and 0.1–2.0°C per second for cooling. Invalid ramp rate entries are adjusted to the nearest valid entry.

To program a ramp rate:

1. Click Ramp Rate in the Show Options box. A new column titled Ramp Rate appears in the spreadsheet.
2. Double 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 rate is input, it is adjusted to the nearest valid ramp rate automatically.

Temperature Change

You may program an automatic periodic increase or decrease in the step temperature in a repeated cycle. Temperature increments or decrements may be as little as 0.1°C per cycle. You may make the increase or decrease as frequently as every cycle, and the increase or decrease can begin following any cycle. The temperature increment or decrement may be as large as desired, as long it does not result in temperatures which are outside the temperature limits of 4°–100°C.

To program a temperature increment or decrement:

1. Click Temperature Change 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 Temperature change column. To decrement the temperature enter the decremental change as a negative number (e.g., -0.5).
3. Enter the repeat in which you want the change to occur for the first time in the Begin Repeat column. Usually it is cycle 2, but it can be any cycle greater than 1.
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.

Time Change

You may program an automatic periodic increase or decrease in the step dwell time in a repeated cycle. Time increments or decrements may be as little as 0.1 sec per cycle. You may make the increase or decrease as frequently as every cycle, and the increase or decrease can begin following any cycle. 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.

To program a time increment or decrement:

1. Click Time Change 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 change desired in the Time Change column. To decrement the time enter the decremental change as a negative number (e.g., -0.5).
3. Enter the cycle in which you want the change to occur for the first time in the Begin Repeat column. Usually it is cycle 2, but it can be any cycle greater than 1.
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.

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, respectively.

To enter a cycle description or step process in the spreadsheet:

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 then either choose one of the listed names from the pull down menu or enter one of your own.

3.3.5 Gradient

A thermal gradient may be programmed across the reaction block at any step of a protocol. The gradient runs from the back of the instrument to the front, with the hottest temperature in Row A and the coolest temperature in Row H. 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 different temperature. The gradient may be as large as 25°C or as small as 1°C. The gradient is not linear, but is highly reproducible. No row can be at a temperature higher than 100°C or lower than 40°C during the gradient step.

You can program the gradient the following way:

1. Click Gradient in the Show Options box. Two columns will appear in the spreadsheet and a representation of the gradient will appear on the right side of the window.
2. Click the Gradient checkbox in the spreadsheet for the desired step.

3. The temperature listed in the Setpoint cell of the spreadsheet will be the coolest temperature on the block during the gradient step (Row H). Enter the desired difference between the coolest and hottest temperatures during the gradient step in the Range cell of the spreadsheet. The Gradient Display will update with the temperatures at each row.

Alternative Gradient Display

To obtain a specific temperature at any one row:

1. Click Gradient in the Show Options box. Two columns appear in the spreadsheet and a representation of the gradient appear on the right side of the window.
2. Click the Gradient checkbox in the spreadsheet for the desired step.
3. Type the desired temperature into that row on the gradient display.
4. Press ENTER.
5. 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.

You can change the range in the spreadsheet or you can make a direct entry of the range in the gradient display. Press ENTER. The display updates with the new calculated temperature for each row.

Note: A gradient cannot be applied to any step which also has a Melt Curve.

3.3.6 Melt Curve/Peak

Melt Curve/Peak analysis 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.

Two major applications for melt curve analysis are:

1. Peak identification (number of amplified products), and
2. Characterization of molecular beacons, and

The two applications listed above are typically used as a guide for improving real-time PCR assay development.

A Melt Curve cycle may follow an amplification cycle or can be conducted independently of the amplification. The melt curve may be programmed in the following manner:

1. Melt curves are programmed as a repeated cycle containing only one step. The temperature is programmed to increase or decrease incrementally with each repeat of the cycle. 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.
2. Insert a cycle into the protocol at the point that you want the melt curve. The step generated in this cycle will be used to generate the melt curve data.
3. Enter the temperature at which you wish to begin the Melt Curve in the Setpoint cell (4°C–100°C).
4. Enter an appropriate dwell time for data collection under the Dwell Time column. Dwell times for melt curve will vary based on the number of fluorophores in the experiment. To enter 10 seconds, type 0 followed by : then 10 (that is, as the time appears in the spreadsheet). Alternatively 10 seconds can be entered as 0.10.
5. Click in the PCR/Melt Data Acquisition column, and then select Melt Curve. A green camera will appear in the Data Acquisition cell as will add four additional columns to the spreadsheet entitled Temperature Change, End Temperature,

Begin Repeat and How Often? The Begin Repeat and How Often? columns are not editable in this form. By default Temperature Change is 0.5°C and the End Temperature is 95°C (unless the setpoint is 95°C). The number of repeats to achieve this melt curve is automatically calculated. The Temperature Change can be as low as 0.1°C increments. Typical Temperature Change values are 0.3–0.5°C for SYBR Green I.

6. Anneal Curves can be also created by entering the Temperature Change as a negative value, e.g., -0.5.
7. Save the protocol by entering a file name and then clicking Save & Exit Protocol Editing.

The minimum dwell times necessary for data collection are 10 seconds for 1 fluorophore. We recommend using a slightly higher dwell time than the minimum values so that more data points are collected at each repeat.

3.3.7 Sample Protocol Files

2Step.tmo: This protocol can be used for most single fluorophore or SYBR Green I real-time PCR experiments.


Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition	
1	1					
		1	3:00	95.0		
2	40					
		1	0:10	95.0		
		2	0:30	55.0		Real Time

Fig. 3.5. Basic 2-step Sample Protocol.

2StepAmp+Melt.tmo: This protocol can be used for most SYBR Green I real-time PCR experiments. The melt curve that follows the qPCR experiment can be used to determine the number of amplicons produced in the qPCR reaction.




Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition		Temperature Change	End Temperature	Begin Repeat	How Often?
	1									
		1	3:00	95.0			▼			
2	40									
		1	0:10	95.0			▼			
		2	0:30	55.0		Real Time	▼			
3	1									
		1	1:00	95.0			▼			
4	1									
		1	1:00	55.0			▼			
5	81									
		1	0:30	55.0		Melt Curve	▼	0.5	95.0	

Fig. 3.6. Sample Protocol for Amplification and Melt Curve.

Gradient.tmo: This protocol can be used to determine the most optimal temperature to perform real-time PCR experiments.

Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition	Gradient	Range
1	1						
		1	3:00	95.0		<input type="checkbox"/>	
2	40						
		1	0:10	95.0		<input type="checkbox"/>	
		2	0:30	55.0	<input checked="" type="checkbox"/> Real Time	<input checked="" type="checkbox"/>	10.0

Cycle 2
Step 2

◀ ▶

A 65.0

B 64.5

C 63.3

D 61.4

E 58.9

F 57.1

G 55.8

H 55.0

Range

10.0

Fig. 3.7. Gradient Protocol and the Gradient Viewer.

3.4 Plate Setup

The MyiQ monitors only the wells defined to have some type of sample and a fluorophore. In the Plate Setup Editor, you specify the type of sample in each well and the fluorophores present in each well. If you define standards on the plate, the Plate Setup Editor can calculate the concentration of each standard in a dilution series given the appropriate information, or you can enter them manually. You can import a unique Identifier for each well from a CSV file or enter them manually. You can get to the Plate Setup Editor from the main Workshop module window.

3.4.1 Well Definition Icons

To select an existing Plate Setup File in the Workshop module:

1. Click Plate and then negotiate the directory to find the desired plate setup file. The vertical gray bar next to the directory tree can be dragged to the right to widen the window.

Note: You can also select and view a plate setup within a Data File.

2. Click on the file name. The plate setup displays in the bottom right pane.

The sample type is displayed for every well one dye layer at a time. The buttons over the plate are used to select the currently displayed fluorophore. You can also determine what other fluorophores are in each well, though you cannot tell what the sample type is in those other dye layers.

The experimental sample volume as well as the type of vessel and sealing mechanism are displayed above the plate as shown below.

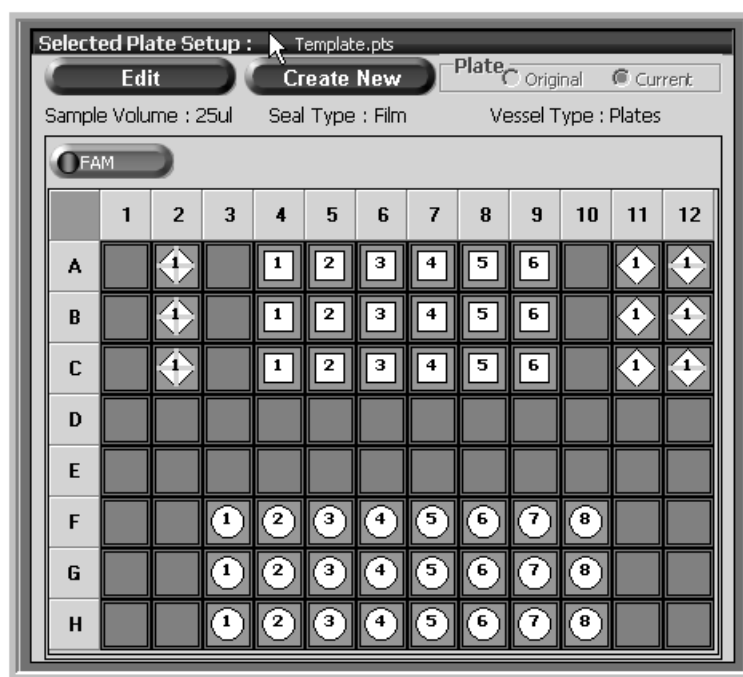


Fig. 3.8. The Selected Plate Setup information.

If you are looking at a data file that contains a plate setup that was edited and saved after the experimental data were collected, you can revert to the original plate setup by clicking Original in the Plate box. To go back to the present definition, click Current.

None of the information displayed on the plate in the Workshop home window can be edited from this screen.

3.4.2 Editing or Creating a Plate Setup

Editing a Plate Setup File

1. Select a Plate Setup.
2. Click Plate or Data File, and then negotiate the directory to find the desired plate setup file. The vertical gray bar next to the directory tree can be dragged to the right to widen the window.
3. Click on the file name and the plate setup will be displayed in the bottom right pane.
4. Click Edit to bring the plate setup into the Plate Setup Editor.

Alternatively, you can double click on the file name in the directory and bring the plate setup directly into the Plate Setup Editor.

5. Use the Plate Setup Editor to edit the Plate, then click Save & Exit Plate Setup.
6. Confirm that the Plate is defined correctly using the Plate Summary.

Creating a New Plate Setup File

1. Click Create New in the bottom right pane of the home screen in the Workshop. It does not matter what plate setup is displayed at the time.
2. The software will transfer to the Plate Setup Editor.
3. Use the Plate Setup Editor to edit the Plate, then click Save & Exit Plate Setup.
4. Confirm that the Plate is defined correctly using the Plate Summary.

3.5 Plate Setup Editor

The Plate Setup Editor is comprised of a 96-well plate layout, functions for specifying the sample type and fluorophores in each well, and a spreadsheet displaying the definition in each dye layer for any individual well.

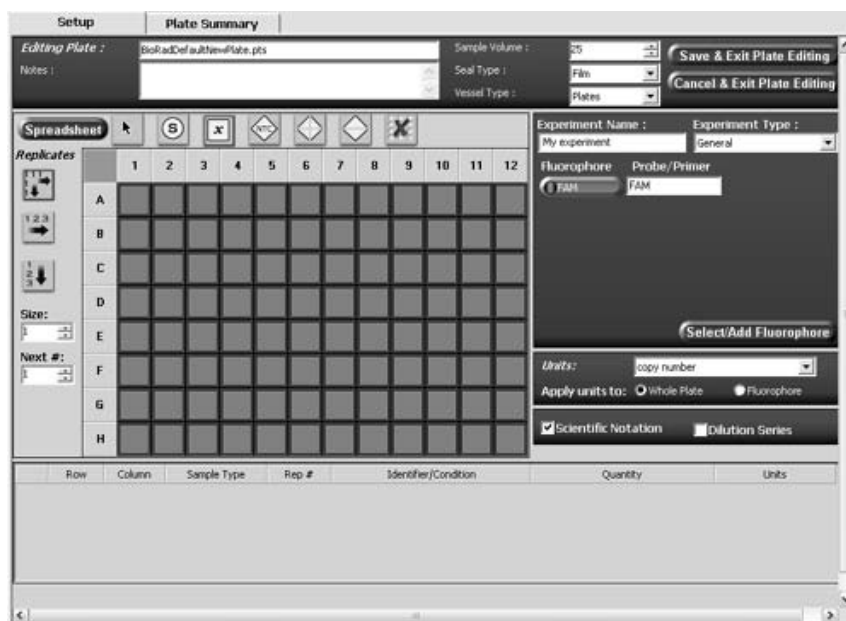


Fig. 3.9. The Plate Setup Editor.

There are two other windows accessible from the Plate Setup Editor:

1. Plate Summary shows well definition one dye layer at a time.
2. Plate Setup Editor Spreadsheet is a comprehensive set data for all 96 wells at once, shown one dye layer at a time.

3.5.1 Well Definition Icons



Fig. 3.10. The Well definition icons.

There are seven icon buttons running across the top of the representation of the experimental plate. These buttons are used to provide the two pieces of information required for each well: sample type and fluorophore(s) to be monitored.

The active button is always surrounded by a red box.



Standard Pointer: Used to select, but not alter, wells



Standards: Defines standards



Unknowns: Defines unknowns



No Template Controls: Defines no template controls



Positive Controls: Defines positive controls.



Negative Controls: Defines negative controls



Delete All — When the Delete All icon is active and you click a well, all information from that well is removed from that well. The other members of a replicate group are not affected.

3.5.2 Editing a Plate Setup

1. From the Workshop module:
 - a. Click Create New in the plate setup display window to enter the Plate Setup Editor.
or
 - b. Click Plate. Open the desired plate setup file in the appropriate directory. Double click the file name to go directly to the Plate Setup Editor.
or
 - c. Click Plate. Open the desired plate file in the appropriate directory. Click the file name to open the plate setup in the bottom right pane of the Workshop window. Click Edit to open the plate setup in the Plate Setup Editor.
or
 - d. Click Data File. Open the desired data file in the appropriate directory. Click the file name to open the plate setup associated with the data file in the bottom right pane of the Workshop window. Click Edit to open the plate setup in the Plate Setup Editor.
2. Enter or edit any notes about the plate setup in the Notes box.
3. Enter or edit the Sample Volume, Seal Type, and Vessel Type.
4. Enter or edit a name for the experiment.
5. Click Select/Add Fluorophores and select or edit the fluorophores to be used on the plate. Associate each fluorophore with a unique color.
6. Click a sample type icon.
7. Select the type of replicate loading desired.
8. Click a fluorophore.
9. Click or drag across the plate to define wells with the selected fluorophore and sample type.
10. Continue defining the remaining wells that will contain the first fluorophore by changing to the other sample type icons.
11. To delete a previously defined well, click the Delete All icon, and then click the well.
12. If there are standards in the first dye layer, click Dilution Series and calculate the concentrations of the standards, or type the concentrations in the spreadsheet manually.
13. Click Save & Exit Plate Setup. Give the plate setup file a new name or overwrite the current version of the file. The program adds the .pts extension automatically.

3.5.3 Fluorophore Selection

The MyiQ monitors only the wells defined to have a fluorophore within it. You may specify as many as five different fluorophores on a single plate, however each well may contain only one fluorophore.

3.5.4 Filter Mates

A new feature in the MyiQ software allows you to choose to monitor as many as five different sets of wells with the same filter pair as shown below. For example, you may have five different experiments on the same plate, all using SYBR Green, and each experiment will be treated independently. When more than one fluor is independently monitored by a single filter pair, those fluors are called filter mates.

Note: Filter mates have to be in separate wells. For example, you may not put FAM and SYBR Green in the same well.

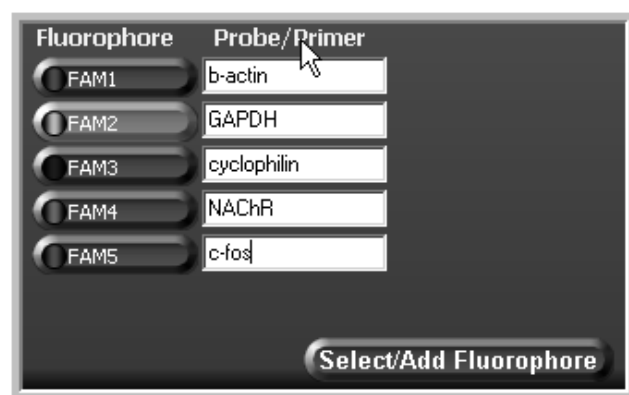


Fig. 3.11. List of five sets of wells.

Click Select/Add Fluorophore to open the Fluor Selector box. From this box, choose up to five fluorophores to be monitored. To remove a fluor from the list to be monitored, uncheck the box next to its name.

To change the default color associated with a fluor, click the color box on the row with the fluor name and choose a new color as shown below.

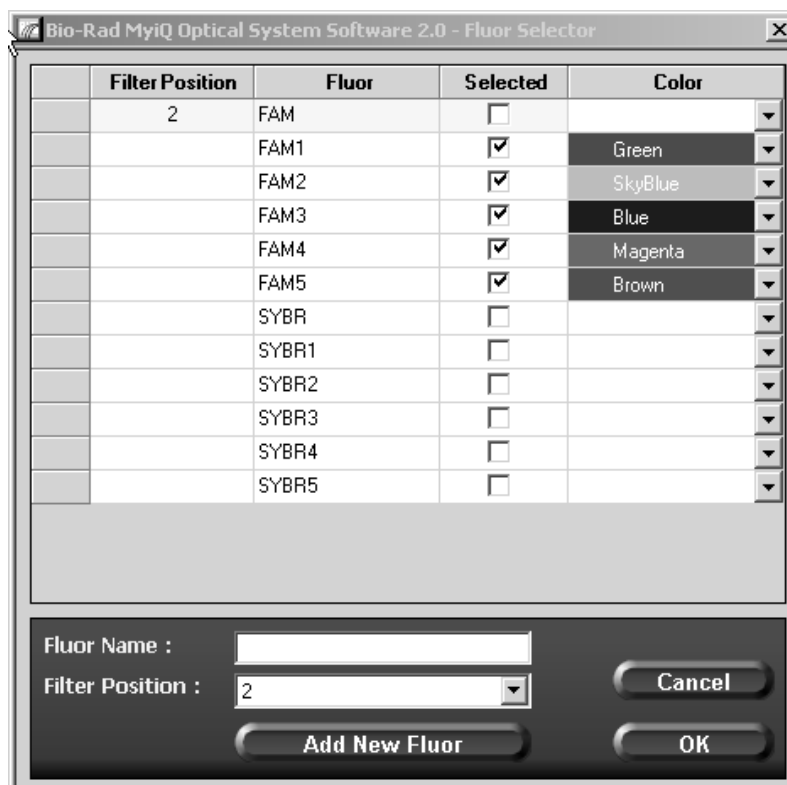


Fig. 3.12. The Fluor Selector window.

You may add new dyes to the list at any time by typing the fluorophore name in the **Fluor Name** text box and then clicking **Add New Fluor**.

3.5.5 Specifying Replicates

The buttons for specifying replicates run down the left side of the representation of the experimental plate.



This is the default setting and indicates that each sample is a single replicate.

With this button active, if you:

- Click a well, the sample type is incremented by one.
- Click a row letter or column number, the sample type for every member of the row or column is identical.
- Drag across a selection, the sample type for every member within the selection is identical.



The Horizontal and Vertical Replication buttons, together with the number in the Size box, specify the direction and number of replicates to be automatically defined.

With the horizontal direction button active and the size set at 5, if you:

- Click a well, the sample type is incremented by one for the next five wells in the horizontal direction. If there are not five wells left in the row, it will wrap around to the next row.
- Click a row letter, the sample number of the first five wells will be incremented by one, the next five wells incremented by two, the last two wells will be incremented by three. The software does not automatically wrap around so that the last set of replicates has the same size as the others.
- Drag across a selection, the first five members of the selection are incremented by one, the second five members are incremented by two, etc. Numbering goes in a horizontal direction and wraps around to the next row within the selection. The last set of replicates may have a smaller size than the others, depending on the number of wells within the selected area.



With the Vertical direction button active and the size set at 3, if you:

- Click a well, the sample type is incremented by one for the next three wells in the vertical direction. If there are not three wells left in the column, it will wrap around to the next column.
- Click a column number, the sample number of the first three wells will be incremented by one, the next three wells incremented by two, the last two wells will be incremented by three. The software does not automatically wrap around so that the last set of replicates has the same size as the others.
- Drag across a selection, the first three members of the selection are incremented by one, the second three members are incremented by two, etc. Numbering goes in a vertical direction and wraps around to the next column within the selection. The last set of replicates may have a smaller size than the others, depending on the number of wells within the selected area.



The **Next #** box allows you to overwrite the default replicate number for the next sample to be defined. This is an easy way to correct a mistakenly assigned replicate number. Use the up and down arrows to select the next desired replicate number, or highlight the field and type the desired number.

3.5.6 Sample Volume, Seal Type, and Vessel Type

In Plate Setup, you must specify the sample volume, the type of sealing, and the type of vessel to be used in the experiment. Use the up and down arrow keys to specify the sample volume, or highlight the field and type in the volume. Select the seal type and vessel type from the pull down menus as shown below.

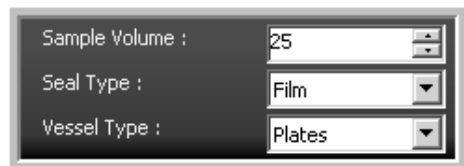


Fig. 3.13. Plate Format Selection Window.

The sample volume may be entered directly or input through the up and down arrows.

3.5.7 Exiting the Plate Setup Editor

You can return to the Workshop at any time by pressing Cancel & Exit Plate Setup, though you will lose your current work.

To save your work before returning to the home screen, click Save & Exit Plate Setup and assign a name to your new plate setup in a standard Windows **Save** dialog box. All plate setup files are automatically assigned an extension of .pts.

Note: Plate setup files from previous versions of MyiQ software can be opened in the new software, but before it can be saved you must assign a vessel type and sealing mechanism.

3.6 Dilution Series

MyiQ can automatically calculate the concentrations of each member of a dilution series, given the dilution factor and the concentration of either the most-concentrated or least-concentrated sample, in the Dilution Series box as shown below.

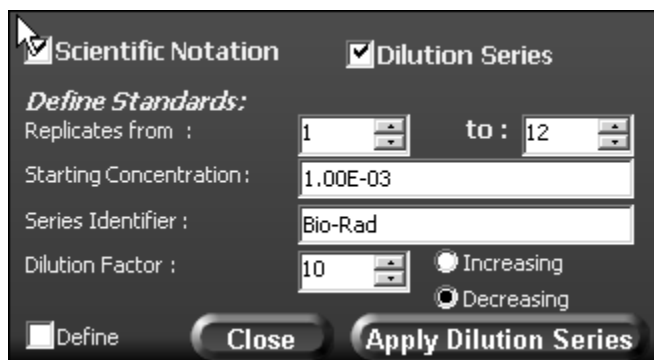
The image shows a software dialog box titled "Plate Summary" with a "Dilution Series" tab selected. At the top, there are two checkboxes: "Scientific Notation" (checked) and "Dilution Series" (checked). Below these, the "Define Standards:" section contains several input fields: "Replicates from :" with a value of 1, "to :" with a value of 12, "Starting Concentration :" with a value of 1.00E-03, "Series Identifier :" with a value of Bio-Rad, and "Dilution Factor :" with a value of 10. To the right of the dilution factor is a radio button group with "Increasing" selected and "Decreasing" unselected. At the bottom left is a "Define" checkbox, and at the bottom right are "Close" and "Apply Dilution Series" buttons.

Fig. 3.14. The Plate Summary tab.

3.6.1 Defining a Dilution Series of Standards

1. Define the range of standard Replicates to be defined. By default, the beginning and ending standards are the lowest- and highest-numbered standards, but you may define only a subset of all standards by clicking on the up or down arrows.
2. Enter the Starting Concentration, the concentration of the lowest-numbered standard to be defined. You may enter the concentration using scientific notation, even if the Scientific Notation button is unselected. The reverse is also true—you may make an entry *without* using scientific notation if the Scientific Notation button is selected. The Scientific Notation button controls only how the data are displayed in the spreadsheet.
3. Enter a Series Identifier for the standard if desired. It will be shown in the spreadsheet and in the reports.
4. Enter the fold Dilution Factor. The default value is 10.
5. Indicate whether the concentrations decrease or increase as the standard replicates increase in number. The default is Decreasing.
6. Click Apply Dilution Series and all standard concentrations will be automatically calculated.

3.6.2 Adding Additional Standards

When Define is selected (at the bottom of the dialog box), any standards added to the set after you close the Dilution Series box, will have concentrations automatically calculated.

If Define is not selected, then in order to assign values to any newly added standards, you must open the Dilution Series dialog box again, make any necessary edits and click Apply Dilution Series.

3.7 Plate Summary

3.7.1 Spreadsheets

There are two different spreadsheets available from the plate setup editor. One spreadsheet, shown below, opens beneath the representation of the experimental plate any time that the pointer tool is clicked on a well. This spreadsheet simultaneously displays information about each dye layer in the active well.

In this spreadsheet you may edit the Sample Type, Identifier, and Quantity. Any changes are carried out through all members of a replicate group. It is also possible in this spreadsheet to change the replicate group assignment of a well, but it will also automatically change the assignment of all other members of the original replicate group.

Note: In order to edit the replicate group assignment of a single well, without changing the other members of the same replicate group, make the change on the plate, not in the spreadsheet.

	Row	Column	Sample Type	Rep. #	Identifier/Condition	Quantity	Units
	A	1	Standard	1	squirrel	1.00E-03	copy number

Fig. 3.15. The spreadsheet that opens beneath the experimental plate.

3.7.2 Plate Setup Editor Spreadsheet

The second spreadsheet can be opened by clicking the Spreadsheet button in the plate setup editor window. This spreadsheet displays information about all wells simultaneously, one dye layer at a time. Dye layer selection is made at the top of the spreadsheet.

Setup		Plate Summary					
Plate Spreadsheet Edit Mode:		FAM1		Import Identifiers		Exit Plate Spreadsheet Editing	
	Row	Column	Sample Type	Rep. #	Identifier/Condition	Quantity	Units
	A	1	Standard	1	squirrel	1.00E-03	copy number
	A	2	Standard	2	squirrel	5.00E-04	copy number
	A	3	Standard	3	squirrel	2.50E-04	copy number
	A	4	Standard	4	squirrel	1.25E-04	copy number
	A	5	Standard	5	squirrel	6.25E-05	copy number
	A	6	Standard	6	squirrel	3.13E-05	copy number
	A	7	Standard	7	squirrel	1.56E-05	copy number
	A	8	Standard	8	squirrel	7.81E-06	copy number
	A	9	Standard	9	squirrel	3.91E-06	copy number
	A	10	Standard	10	squirrel	1.95E-06	copy number
	A	11	Standard	11	squirrel	9.77E-07	copy number
	A	12	Standard	12	squirrel	4.88E-07	copy number
	B	1	Standard	13	squirrel	2.44E-07	copy number
	B	2	Standard	14	squirrel	1.22E-07	copy number
	B	3	Standard	15	squirrel	6.10E-08	copy number
	B	4	Standard	16	squirrel	3.05E-08	copy number
	B	5	Standard	17	squirrel	1.53E-08	copy number

Fig. 3.16. The spreadsheet that displays information about all wells.

Within this spreadsheet you may change the Sample Type, the Identifier, and the Quantity. Note that changes made to one member of a replicate group are carried through to all members of the replicate group. Replicate group assignment changes should be made on the plate, not in the spreadsheets.

The single-dye layer spreadsheet has another feature that differentiates it from the single-well spreadsheet. From this spreadsheet you may Import Identifiers from an external CSV (comma separated values) file. The number of identifiers must match the number of wells (Well Sample Identifier Import.xls, located in the User 1 folder of the MyiQ SW directory) before the import can be carried out. The simplest way to use the import feature is to fill out the Identifier template file provided with the software and then save the file in the CSV format.

3.8 Run Set

A Run Set is a linked Plate Setup and Protocol and MyiQ run set files are stored with the extension .run. Run sets are useful for experiments that are repeated on a frequent basis.

3.8.1 Selecting a Run Set

To select a run set:

1. Click Run Set.
2. Use the browser below the Run Set button to locate the folder the run set file is located in and then click on the run set file name to select it.
3. The selected run set will appear in the Selected Protocol and Selected Plate Setup windows.

See an example Run Set.

3.8.2 Creating a Run Set

To create a run set:

1. Select the plate setup and protocol that are to be linked.

The plate setup and protocol can be stand alone files (plate setup files have the extension .pts while protocol files have the extension .trno) or they can be already together in a data file (with the extension .opd). A data file has two plate setups: Original is the plate set up the data file was created with, and Current is the plate setup the data file was last saved with. The run set will be created with the plate setup displayed in the Selected Plate Setup window.

2. Once you have chosen the protocol and plate setup files, click Create Run Set from the File menu.

3.8.3 Run Set Example

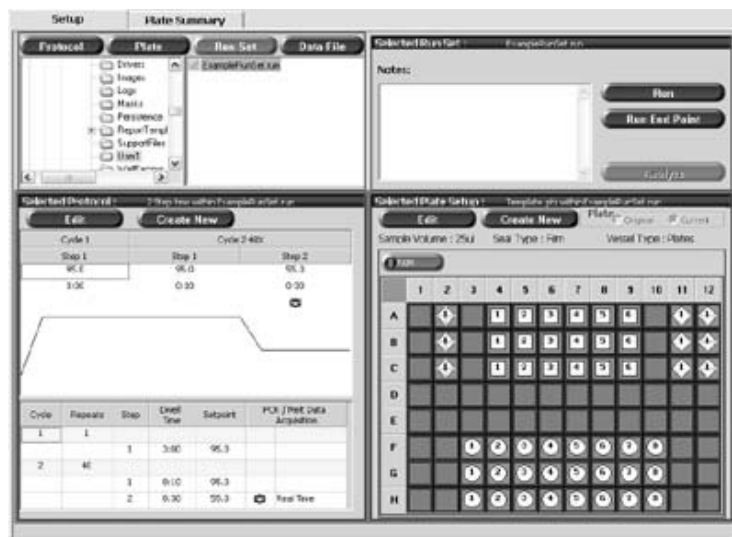


Fig. 3.17. Sample Run Set File.

When the Run Set Tab is selected, run set files display in the File Browser.

A Run Set is a linked protocol and plate set up and run set files have the extension .run.

The protocol defined in the run set is shown in the Selected Protocol window and the plate setup defined in the run set is shown in the Selected Plate Setup window.

3.9 Data File

Primarily used to select and open a data file. Can also be used to run a real-time experiment using the same protocol and plate setup that where used to create the data file. The plate setup that is selected can be either the Original plate setup or the Current (last saved) plate setup.

3.9.1 Selecting a Data File

1. Click the Data File button.
2. Use the file browser below the Data File button to locate the folder the data file is located in and then click on the data file name to select it
3. The protocol and plate setup used in the data file will appear in the Selected Protocol and Selected Plate Setup windows. The plate setup that is shown can be either the Original plate setup or the Current (last saved) plate setup. If the data file protocol and plate setup are being used as the conditions for running a new experiment, then the plate setup that is shown in Selected Plate Setup window will be the one that is used to run the new experiment.

3.9.2 Opening a Data File

To open an optical data file from an amplification or melt curve experiment, select the name of the data file in the File Browser, and then click Analyze (or double click the file name).

3.9.3 Modifying a Data File

Optical data is saved to the data file along with the Calibration Files. It is possible to overwrite the calibration files stored within the OPD data file thus changing the analysis of the experiment. This feature protects you from losing valuable experimental data if the calibrations were conducted incorrectly.

3.9.4 Applying Alternate Well Factors

To apply alternate Well Factors to a data file first select the data file in the File Browser. Then select Apply Alternate Well Factors from the Tools menu. Use the Select Well Factors File Name dialog box to navigate to, then open the Well Factors calibration file. After the Well Factors calibration file has been opened a Save Optical Data File dialog box will appear. The existing well factor calibration for this data file will be overwritten. For this reason it is recommended to save the optical data file with a different name from the original to ensure that the original file, with the original calibration data, is maintained for reference.

Do not use the well factor file containing dynamic well factors (identified by the name Dynamic_vessel_seal_volume_MyiQCameraSerial#_iCyclerSerial#) since this file contains only the well factors for the wells that were used in the experiment that generated this file. If the well list does not match, the operation will fail. Hence, it is a good practice to only use the Persistent Well Factor files when applying alternate well factors.

Section 4

Run-Time Central Module

This module is entered automatically after clicking Run or Run End Point in the Workshop module. There are two windows in this module: Initiate Run and Monitor Run.

4.1 Initiate Run Window

Use the Initiate Run window, shown below, to confirm run conditions and then to initiate the optical data run. The protocol to be run is in the bottom left corner of the window, and the plate setup that will be used appears in the bottom right corner. The type of well factors to be used in the run can be selected in the top left section of the window. You can record details of the experiment in the Notes box, and these notes will be incorporated into the experimental file.

Initiate Run | **Monitor Run**

☐ Use Persistent Well Factors

☒ Collect Well Factors from Experimental Plate

Notes:

Running user selected Protocol and Plate

Begin Run

Selected Protocol : 2Step.tmo

Cycle 1		Cycle 2 40X	
Step 1	Step 2	Step 1	Step 2
95.0	55.0	95.0	55.0
3:00	0:30	0:10	0:30

Selected Plate Setup : Template.plt

Sample Volume : 25ul Seal Type : Film Vessel Type : Plates

FAM

	1	2	3	4	5	6	7	8	9	10	11	12
A				1	2	3	4	5	6		1	1
B				1	2	3	4	5	6		1	1
C				1	2	3	4	5	6		1	1
D												
E												
F				1	2	3	4	5	6	7	8	
G				1	2	3	4	5	6	7	8	
H				1	2	3	4	5	6	7	8	

Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition
1	1				
		1	3:00	95.0	
2	40				
		1	0:10	95.0	
		2	0:30	55.0	Real Time

Fig. 4.1. The Initiate Run window.

To begin a run, click Begin Run. The Save dialog box opens. Type a name for the optical data file. MyiQ saves data automatically during the experiment.

4.1.1 Initiate Run Window—End Point Run

Initiate an End Point run by selecting a Plate Setup, then clicking Run End Point. The software transfers to the Initiate Run window in the Run-Time Central module. An End Point run uses a canned protocol in which only the temperature of the data collection step can be modified. Enter the desired temperature of the data collection step into the Setpoint box as shown below. Click Enter.

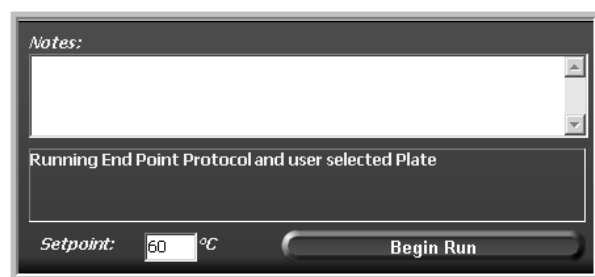


Fig. 4.2. The Setpoint Box.

Changes entered into the Setpoint: box appear in the Selected Protocol region of the Initiate Run window as shown below.

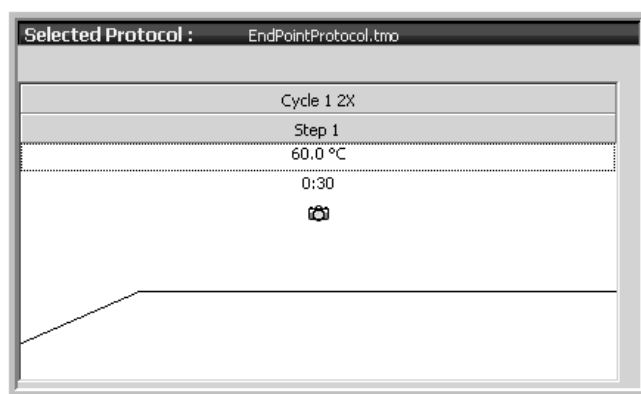


Fig. 4.3. The Selected Protocol region of the Initiate Run window.

To begin a run, click Begin Run. The Save dialog box opens. Type a name for the optical data file. MyiQ saves data automatically during the experiment.

4.2 Monitor Run Window

Open the Monitor Run window by clicking Monitor Run. The window appears as shown below.

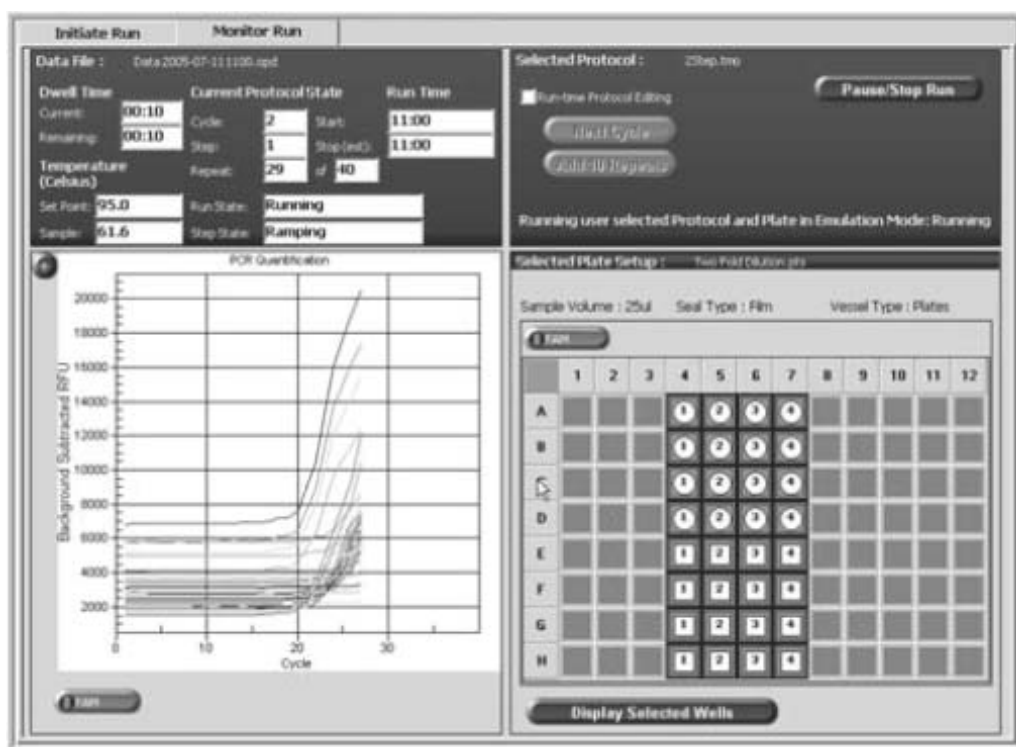


Fig. 4.4. The Monitor Run window.

4.3 Run-Time Protocol Editing

There are two choices available for modifying a protocol during a run: **Next Cycle** and **Add 10 Repeats**.

During a run, you can access these options by selecting the Run-time Protocol Editing check box as shown below.

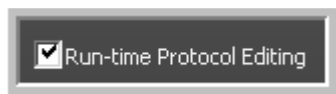


Fig. 4.5. The Run-time Protocol Editing check box.

Selecting this check box activates the Next Cycle and Add 10 Repeats buttons as shown below.



Fig. 4.6. The Next Cycle and Add 10 Repeats buttons.

4.3.1 Next Cycle

Click Next Cycle to complete the current repeat of the present cycle before skipping to the next cycle. For example you could use this feature when your samples have clearly crossed threshold and you want to skip to the melt cycle of your protocol.

4.3.2 Add 10 Repeats

Click Add 10 Repeats to add additional repeats to the current cycle. You can click this button multiple times; however, the total number of repeats is limited to 600. For example, it may be necessary to add repeats to a run in an experiment amplifying low copies of DNA to allow all samples to cross threshold. Click Add 10 Repeats to an amplification cycle of 30 repeats to make it 40 repeats.

Note: Modifications to the protocol are updated on the protocol displayed on the iCycler base module.

4.3.3 Pause/Stop Run

The Pause/Stop Run button allows you to pause a thermal cycling protocol. If you click Pause/Stop Run when the iCycler is at a setpoint temperature, the iCycler will hold at the setpoint temperature and stop counting down the dwell time. If you click Pause/Stop Run when the iCycler is ramping to the temperature, the iCycler will continue ramping until it reaches the next setpoint temperature then pause at that step.

Clicking Pause/Stop Run activates two new buttons. Click Resume Run to resume the thermal cycling protocol. Click End Run to terminate the experiment.

Section 5

Data Analysis Module

The Data Analysis module is where data is presented and analyzed. When the MyiQ software opens, the Data Analysis module is gray and the module is not active. To analyze a data file, open the data file in the Data File tab of the Workshop module by selecting the data file and then clicking Analyze.

The Data Analysis module consists of five tabs:

- **PCR Quant**

The PCR Quantification tab is used to set the analysis conditions for the data file. The analysis conditions include setting the PCR baseline, the threshold and which wells to exclude or include in the experiment. The analysis conditions should be set before using the Gene Expression or End Point tabs. For experiments with standards of known quantities, the PCR Quant tab is also where absolute quantities can be determined. The efficiency of the PCR reaction can also be determined using standard curves with either known quantities used to produce the standard curve or by using a serial dilution of the template under investigation.

- **Melt Curve/Peak**

Melt Curve/Peak 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 (for example, SYBR Green I) or by hybridization with fluorescently labeled probes. Three major applications for Melt Curve/Peak are peak identification (number of amplified products), characterization of molecular beacons in the MyiQ software.

- **End Point**

End Point Analysis provides a convenient method of analyzing final RFU (Relative Fluorescence Unit) values. This can be useful when PCR Analysis is to determine if a given sample is Positive or Negative for a particular nucleic acid sequence.

- **Gene Expr**

The Gene Expression screen has flexible tools for the determination of the fold induction of one gene relative to another gene or relative to itself under different conditions.

- **Edit Plate**

In the Edit Plate screen, you may make changes to the sample type assignment or to the quantities of the Standards in the plate setup used to run the experiment. This is a simple way to salvage your experiment should you make mistakes in the sample type assignment. While you may make changes to the plate setup, the original plate setup is never discarded and always remain with the data file so that you may revert to it at any time.

5.1 PCR Quantification Tab

The PCR Quant tab is the tab that is first displayed after opening a data file that contains amplification data, as shown below. For experiments that lack amplification data, such as Melt Curve only or End Point Only experiments, the PCR Quant tab is grayed out and unavailable for analysis.

Use the PCR Quant tab to set the analysis conditions for the data file. The analysis conditions include setting the PCR baseline, setting the threshold, and determining which wells to exclude or include in the experiment. The analysis conditions should be set before using the Gene Expression or End Point tabs.

For experiments with standards of known quantities, the PCR Quant tab is also where you can determine absolute quantities for unknown samples. You can also determine the efficiency of the PCR reaction using standard curves with either known quantities used to produce the standard curve, or by using a serial dilution of the template under investigation.

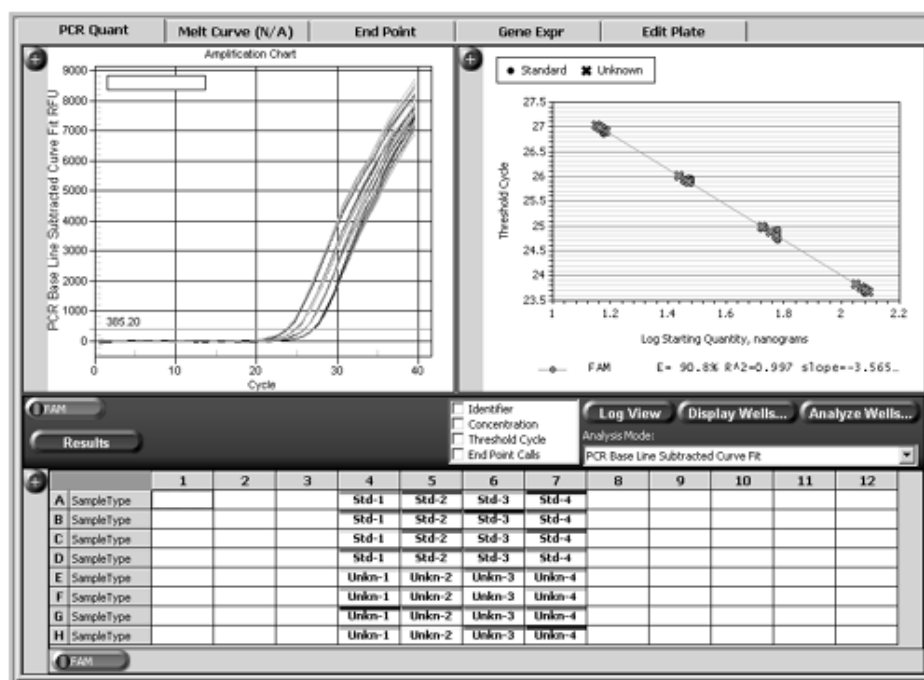


Fig. 5.1. The PCR Quant tab.

The PCR Quant tab consists of three panes:

1. Amplification Chart
2. Standard Curve Chart and
3. Results Section

5.1.1 Customizing the PCR Quant Display

You can customize the size of the panes in the PCR Quant tab in a number of ways. In the upper corner of each pane is a + (plus) button that enlarges the pane when clicked. The enlarged pane has a - (minus) button that reduces the pane when clicked. The amplification chart appears in the enlarged pane and includes an inactive - (minus) button when no standard curve is present. You can also move divider bars between each pane by clicking and dragging on the divider bar you want to move.

5.2 Amplification Chart

The Amplification Chart displays the relative fluorescence for each well at every cycle. Each trace represents the fluorescence of a given fluorophore for a single well and at each cycle a single data point is plotted which is the calculated mean of the data collected for that well during the particular cycle. The data that is used to determine this mean point is set by the Set Data Analysis Window dialog box. The data can be plotted in Background Subtracted, PCR Base Line Subtracted or PCR Base Line Subtracted Curve Fit mode.

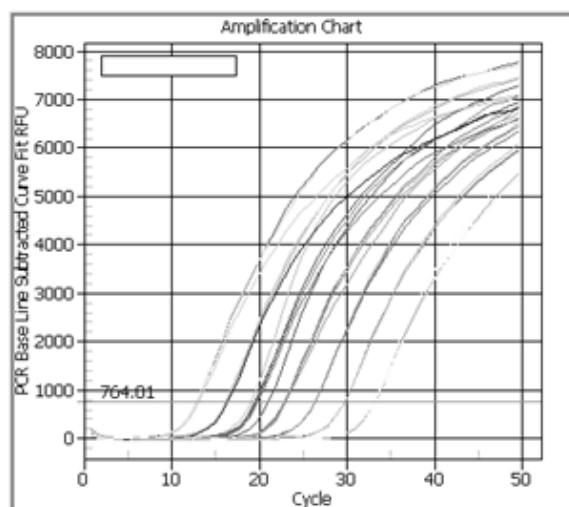


Fig. 5.2. The Amplification Chart.

5.2.1 Fluorophore Selector Buttons

You can select from three analysis modes in the Analysis Mode drop-down list box as shown below.

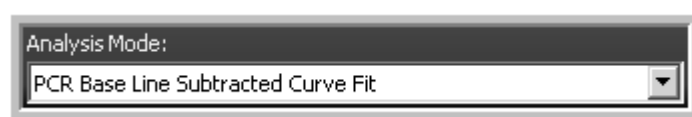


Fig. 5.3. The Analysis Mode drop-down list box.

The three analysis modes include:

- **Background Subtracted:** The background subtracted data is the relative fluorescence of each fluorophore after normalizing for exposure time, background factors, and well factors. No further analysis is possible on background subtracted data, therefore, the End Point and Gene Expr tabs are unavailable.
- **PCR Base Line Subtracted:** To determine threshold cycles, construct standard curves, and determine the concentration of unknown samples, the data must be PCR baseline subtracted. MyiQ determines the PCR baseline subtracted trace by fitting the best straight line through the recorded fluorescence of each well during the baseline cycles. MyiQ then subtracts the best fit data from the background subtracted data at each cycle to generate the PCR baseline subtracted trace. By default, MyiQ automatically chooses the beginning and end baseline cycles. You can override this default and manually give each trace a beginning and ending baseline cycle.
- **PCR Base Line Subtracted Curve Fit:** MyiQ fits the PCR Baseline Subtracted data 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.

5.2.2 Log View Button

You can click Log View, shown below, to change between a semi-logarithmic and linear display of the Amplification Chart data.



Fig. 5.4. The Log View button.

5.2.3 Selecting Fluorophores to be Displayed

You can use the **Amp Chart Fluorophore Selector** buttons, which are located under the amplification chart, to display which fluorophores appear in both the amplification chart and standard curve chart. Selecting a single fluorophore to display is useful for determining the analysis parameters for that fluorophore. Selecting all fluorophores can be useful for ensuring that the efficiencies of each fluorophore set are approximately equal.

Fluorophore Selector Buttons

Use the fluorophore selector buttons to select and deselect which fluorophores to display. Deselecting a fluorophore does not remove the fluorophore from the analysis, only from the display.



Fig. 5.5. Fluorophore selector buttons.

Selected fluorophore buttons display with the button highlighted bright green. Deselected fluorophores display in a darker green. In the example below, the SYBR1, SYBR3, and SYBR4 fluorophores have been selected, and SYBR2 has been deselected.



Fig. 5.6. Selected and deselected fluorophore selector buttons.

5.2.4 Setting Analysis Conditions for Threshold Cycle Calculation

In the amplification chart, MyiQ uses the two major analysis conditions that determine the threshold cycle as the baselines used for each individual trace and the threshold that is set for the fluorophore. By default, MyiQ calculates the baseline cycles and threshold when in PCR Base Line Subtracted or PCR Baseline Subtracted Curve Fit mode.

You can override the automatic conditions for baseline and threshold in the Base Line Threshold dialog box. Before you access this dialog box, ensure that only one fluorophore from the Amp Chart Fluorophore Selector buttons is selected, then right click on the Amplification Chart, and then click BaseLine Threshold. For other analysis condition settings, see the **Amplification Plot Context Menu**.

5.2.5 Analyze Wells

Click Analyze Wells, shown below, to select the wells that you wish to include in data analysis.



Fig. 5.7. The Analyze Wells button.

After you click Analyze Wells, the Select Analyzed Wells for Display dialog box appears as shown below.

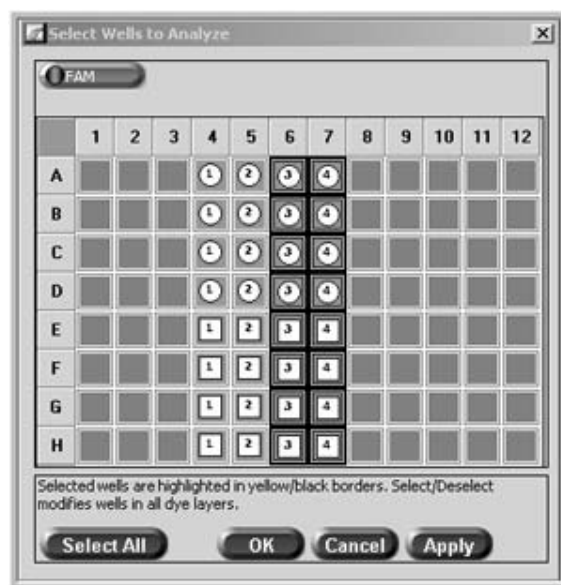


Fig. 5.8. The Select Analyzed Wells for Display dialog box.

You can include or exclude wells for analysis in this dialog box. The original data are always preserved and excluded wells can always be added back to the data analysis in this dialog box.

Removing wells from data analysis changes the calculation of the threshold location. Changing the calculation of the threshold location may result in a change in the Threshold crossing (Ct) location thus affecting all subsequent data

analyses that depend on Ct values. This would include standard curve calculations and quantification of unknowns and Gene Expression using Threshold Crossing values.

In addition removing wells from analysis will change the statistics for replicates.

To select the wells to include in data analysis:

1. In the PCR Quant tab, click Analyze Wells. The Select Analyzed Wells to Display dialog box appears. A set of fluorophore selector buttons appears at the top of this dialog box. The dialog box indicates the presence of a fluorophore in a well by that well having the fluorophore button color in the well. When you click a fluorophore button, the dialog box displays the sample type present in that well for that fluorophore.
2. To select or deselect an individual well, click inside that well. Wells included in the analysis appear with a yellow and black border. Wells excluded from the analysis appear with a pale border (columns 4 and 5 in the above example). When a well is excluded from analysis, all fluorophores in that well are excluded.
3. To select all wells, click Select All.
4. To toggle the current selection so all wells currently selected will be unselected and vice versa, click the upper most left cell of the spreadsheet; this cell has the color of the currently selected fluorophore. You can also perform this action on a subset of the spreadsheet by clicking the appropriate row or column header. Only the wells in that row or column are toggled.
5. After you determine which wells to include in analysis, click Apply if you want the Select Analyzed Wells to Display dialog box to remain open after MyiQ re-analyzes the wells. If you are satisfied with your selection, click OK to close the dialog box and update the wells to include in the analysis. If you click Cancel, MyiQ closes the dialog box and discards all changes.

Note: This procedure does not permanently remove data. This procedure only removes that data from the current analysis. You can add the excluded wells back to the analysis at any time by including them in the Select Analyzed Wells to Display dialog box.

5.2.6 Display Wells

Click Display Wells to select the wells that you wish to include in the data display as shown below.



Fig. 5.9. The Display Wells button.

After you click Display Wells, the Select Analyzed Wells for Display dialog box appears as shown below.

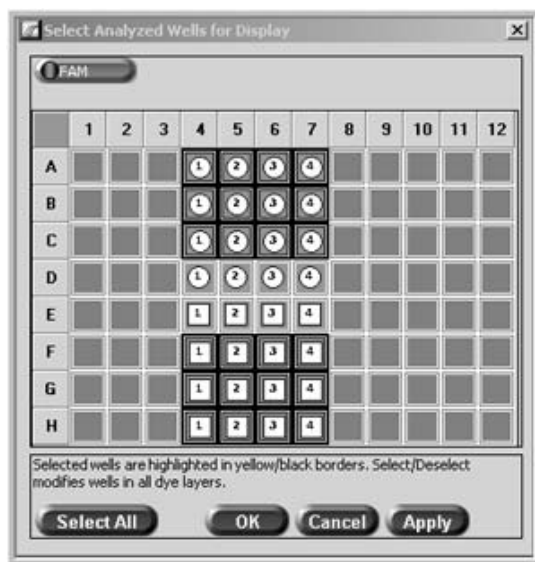


Fig. 5.10. The Select Analyzed Wells for Display window.

You can include or exclude wells for display in the Select Analyzed Wells for Display dialog box. You can only choose from analyzed wells. Wells that have been excluded from analysis in the Select Analyzed Wells for Display dialog box do not appear in the Select Analyzed Wells for Display dialog box.

Selecting wells for display does not change the underlying data analysis. Therefore, the calculation of thresholds and replicate statistics does not change.

To select the wells to include in data display:

1. In the PCR Quant tab, click Display Wells. The Select Analyzed Wells for Display dialog box appears. Only those wells that are included in the analysis appear in the dialog box. A set of fluorophore selector buttons appears at the top of this dialog box. The dialog box indicates the presence of a fluorophore in an analyzed well by that well having the fluorophore button color in the well. After you click a fluorophore button, the dialog box displays the sample type present in that well for that fluorophore.
2. Select or deselect an individual well by clicking inside that well. Wells included in the data display appear with a yellow and black border. Wells excluded from the data display appear with a pale border (rows D and E in the above example). When a well is excluded from analysis, the dialog box does not display the well (wells G8 and G9 in the above example).
3. To select all wells, click Select All.
4. To toggle the current selection so all wells currently selected will be unselected and vice versa, click the upper most left cell of the spreadsheet; this cell has the color of the currently selected fluorophore. You can also perform this action on a subset of the spreadsheet by clicking the appropriate row or column header. Only the wells in that row or column are toggled.
5. After you determine which wells to include in analysis, click Apply if you want the Select Analyzed Wells for Display dialog box to remain open after MyiQ re-analyzes the wells. If you are satisfied with your selection, click OK to close the dialog box and update the wells to include in the analysis. If you click Cancel, MyiQ closes the dialog box and discards all changes.

The Select Analyzed Wells for Display dialog box displays traces for fluorophores with parameters per the selected Amp Chart Fluorophore Selector buttons. You can use this dialog box to select all wells for display. Restore all traces for display by right clicking on the Amplification Chart and clicking Show All Traces in the menu.

Identifying a Specific Amplification Trace

Identify a specific trace by moving the mouse pointer along the trace until the hand icon appears. The dialog box identifies the trace, by both well name and fluorophore, in the top left corner of the Amplification Chart.

Selecting a Specific Amplification Trace

Select a specific trace by moving the mouse pointer on the trace until the hand icon appears, and then double click. The dialog box displays the selected trace. If you click Amplification Chart Fluorophore Selector, traces from other fluorophores in the selected well also appear.

Zoom

- To zoom in on a section of the plot, click and drag with the mouse on the desired region.
- To zoom out, select the plot and then type R, or right click on the plot and then click Restore Graph in the menu.

5.3 Standard Curve Chart

The Standard Curve Chart appears when, for a given fluorophore, more than 2 standards (with different quantities) are defined in the plate setup. The Amp Chart Fluorophore Selector buttons determine which fluorophores appear in the Standard Curve Chart, shown below.

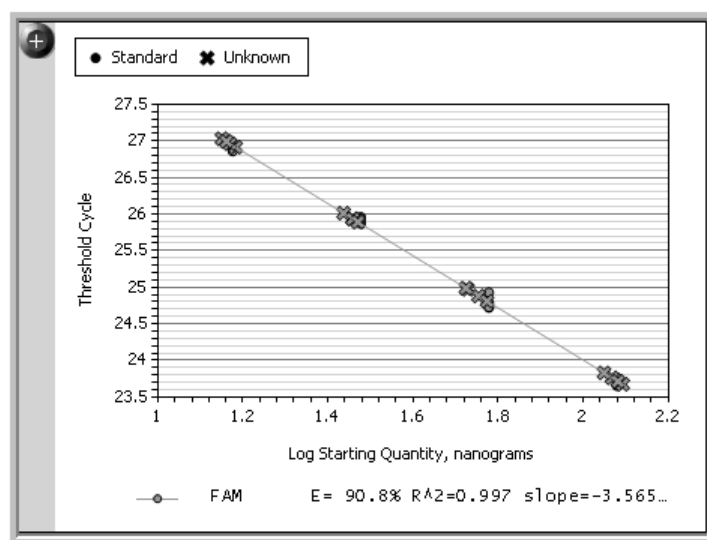


Fig. 5.11. The Standard Curve Chart.

The bottom of the chart displays a legend that includes:

- The color used to plot each fluorophore
- The name of the fluorophore
- The efficiency of the reaction
- The coefficient of determination (R^2)
- The slope of the line
- The y-intercept

You can enlarge the Standard Curve Chart by clicking on the plus (+) sign in the upper left corner of the pane.

5.3.1 Standard Curve Chart Menu

Right clicking on the Standard Curve Chart opens the Standard Curve Chart menu as shown below.

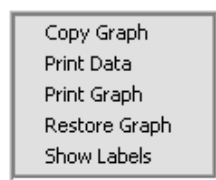


Fig. 5.12. The Standard Curve Chart menu.

This menu includes the following options:

Copy Graph

Copies the Standard Curve Chart to the clipboard. To copy the entire graph:

1. Enlarge the chart by clicking the + button in the upper left corner of the Standard Curve Chart.
2. Click Copy Graph in the Standard Curve Chart menu.

Print Data

Prints the Standard Curve/Ct Results spreadsheet to your specified printer in Windows.

Print Graph

Prints the graph to your specified printer in Windows.

Restore Graph

Active only after you zoom in on the chart. Clicking Restore Graph returns the Standard Curve Chart to its un-zoomed state.

Show Labels

Labels standards and unknowns with the well name as shown below.

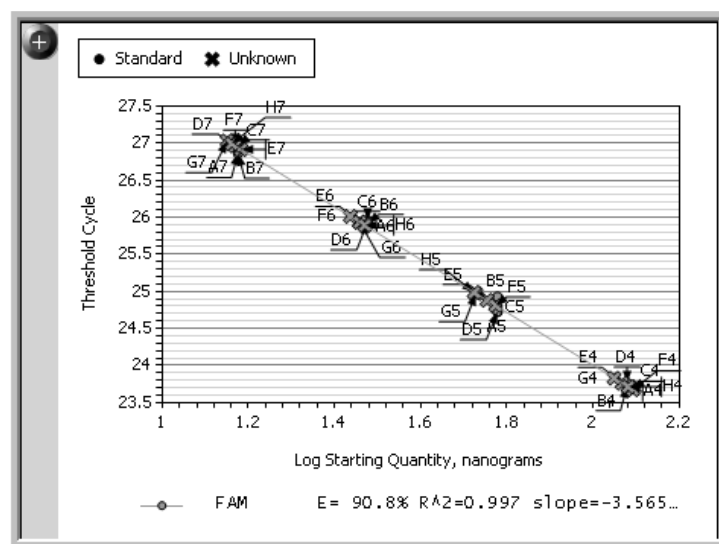


Fig. 5.13. The Standard Curve Chart with labels.

5.4 Results Section

The Results Workbook consists of three spreadsheets:

1. Plate Spreadsheet
2. Amplification Data (RFU) Spreadsheet and the
3. Standard Curve/Ct Results Spreadsheet

Click Results to toggle between the Plate Spreadsheet and the Amplification Data (RFU)/Standard Curve/Ct Results spreadsheets.



5.4.1 Plate Spreadsheet

The Plate spreadsheet displays data for each well in a grid fashion that represents the plate setup used in the experiment.

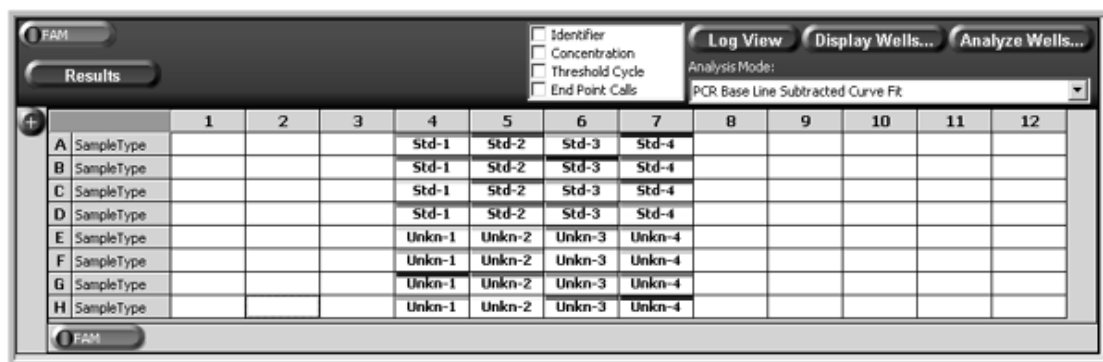


Fig. 5.14. The Plate spreadsheet.

The spreadsheet displays only one fluorophore at a time. You can use the Plate Spreadsheet Fluorophore Selector buttons found under the plate spreadsheet to determine which fluorophore displays in the spreadsheet. Each well has a colored bar at the top of the cell, which is the color used in the Amplification Chart to display the amplification trace for this well and fluorophore. The sample type is always displayed in this spreadsheet.

Above the spreadsheet is a set of check boxes you can use to display the Identifier, Concentration, Threshold Cycle, and End Point Call in the plate spreadsheet, as shown below.

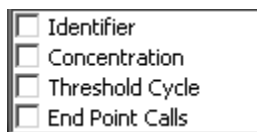


Fig. 5.15. Plate spreadsheet check boxes.

5.4.2 Amplification Data (RFU) Spreadsheet

You can view the individual RFU readings for each amplification trace at every cycle in the Amplification Data (RFU) spreadsheet. The Amp Data Fluorophore Selector buttons appear below the spreadsheet; these buttons control which fluorophore displays in the Amplification Data (RFU) spreadsheet. The spreadsheet can display data for only one fluor.

The well number appears at the top of the spreadsheet, and the cycle number appears down the side of the spreadsheet. In Single Point mode, each cycle is represented by one point that is the mean of all the data points analyzed during that cycle. In All Candidates mode, each individual data point displays.

5.4.3 Standard Curve/Ct Results Spreadsheet

The Standard Curve/Ct Results spreadsheet, shown below, displays the well, fluorophore, sample type, identifier, replicate number, threshold cycle, starting quantity, and statistics for replicate groups. None of the data in this spreadsheet may be edited. You can copy the data to the clipboard for export into other software programs.

	Well	Fluor	Type	Identifier	Replicate #	Threshold Cycle (Ct)	Log Starting Quantity	Starting Quantity (SQ)	SQ Mean	SQ Std. Dev.	Ct
1	A04	FAM	Std		1	23.65	2.079	1.200e+002	1.20E+02	0.00E+00	23
2	A05	FAM	Std		2	24.72	1.778	6.000e+001	6.00E+01	0.00E+00	24
3	A06	FAM	Std		3	25.87	1.477	3.000e+001	3.00E+01	0.00E+00	25
4	A07	FAM	Std		4	26.87	1.176	1.500e+001	1.50E+01	0.00E+00	26
5	B04	FAM	Std		1	23.73	2.079	1.200e+002	1.20E+02	0.00E+00	23
6	B05	FAM	Std		2	24.93	1.778	6.000e+001	6.00E+01	0.00E+00	24
7	B06	FAM	Std		3	25.92	1.477	3.000e+001	3.00E+01	0.00E+00	25

Fig. 5.16. Standard Curve Results Spreadsheet.

5.5 Amplification Plot Context Menu

You can access PCR amplification plot data analysis and display parameters by right clicking on the PCR amplification plot. After you right click on the plot, the amplification plot menu displays as shown below.



Fig. 5.17. The amplification plot menu.

5.5.1 Raw Data Analysis Options

Set Data Analysis Window

After you click Set Data Analysis Window... in the amplification plot menu, the Set Data Analysis Window appears as shown below. The number of data points collected per cycle depends on the exposure time and the dwell time of the cycle. You can change the percentage of data points used in determining the RFU value and you can choose to select this percentage from either the beginning or the end of the cycle or from a window in the middle of the cycle.

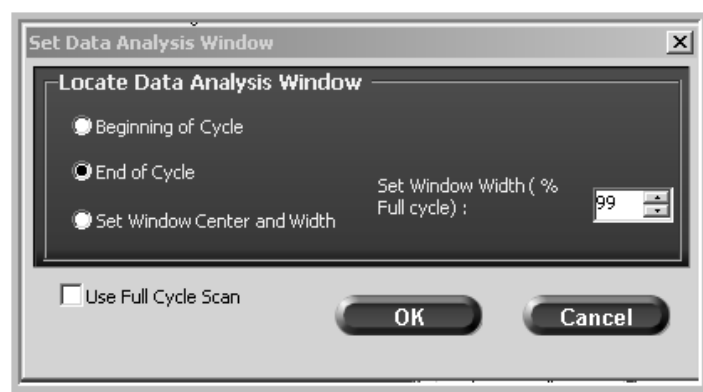


Fig. 5.18. The Set Data Analysis Window.

To set the Data Analysis Window:

1. Right click on the PCR amplification plot and then click Set Data Analysis Window in the amplification plot menu.
2. Click the Beginning of Cycle or End of Cycle option buttons to select data from the beginning of the cycle or the end of the cycle, respectively.
3. Set the window width in the Set Window Width scroll box.
4. To use all data points in the cycle select Use Full Cycle Scan
5. Click OK to return to the PCR Quant screen.

Note: To center the analysis around data collected with a window in the middle of the cycle select the Set Window Center and Width radio button. Use the up or down arrows of the Set Window Width scroll box to select the percentage of data points. These data points will be chosen around the value set in the Window is Centered at % of Full Cycle text box. Click OK to return to the PCR Quant screen.

Digital Filter

After you click Digital Filter... in the amplification plot menu, the Set Digital Filters window appears as shown below. There are two intra-cycle data filtering options available: Rolling Boxcar and Weighted Mean. The default filter is the weighted mean as this filter is the only one available during data acquisition.



Fig. 5.19. The Set Digital Filters window.

The weighted mean is determined by the equation:

$$O_i = (R_i + c \cdot M) / (1 + c)$$

Where:

- O_i is the filtered value for data point i
- R_i is the unfiltered value for data point i
- c is a weighted factor with a value of 2
- M is the arithmetic mean of all data points for the well within the given cycle.

The rolling box car filter is the arithmetic mean of the data readings $i - (w - 1)$, where w is the filter width. For example if you want to calculate the 20th data point ($i=20$) and the width is 4 ($w=4$) then you take the mean of the data points 17–20, data point 21 is the mean of the data points 18–21, etc.

The above filters only apply within a cycle. A global filter that smoothes data from cycle to cycle is also available by clicking the Enable Global Filter option button. The global filter operates on the trace for a given well and fluorophore using all cycles together in a single pass. Global filtering should be reserved for data that appears very noisy and should not be applied routinely.

BaseLine Threshold

After you click BaseLine Threshold... in the amplification plot menu, the Base Line Threshold Parameter window appears and displays the fluorophore of the traces to be adjusted as shown below. In the example below, the window shows the FAM traces are to be adjusted.



Fig. 5.20. The BaseLine Threshold Parameter window.

Manual Baseline Definition

Baselines are auto-calculated by default. You can override auto-calculation by clicking User Defined in the Base Lines Cycles area within the Base Line Threshold Parameter window. After you click User Defined, MyiQ activates the Select All and Edit Range buttons.

To edit a single trace, select the cell to the left of the well name, and then click Edit Range. Use the Edit Baseline Cycle Range dialog box to determine the beginning and ending baseline cycles. The dialog box indicates wells with manually determined baseline cycles with an asterisk.

Edit Baseline Cycle Range

The Edit Base Line Cycle Range dialog box has three modes.

Both Start and Ending Cycle

In the Both start and ending cycle mode, you can edit both the Start Cycle and Ending Cycle boxes are editable. Enter the desired values for the start cycle and ending cycle for the selected traces and then click OK, as shown below.

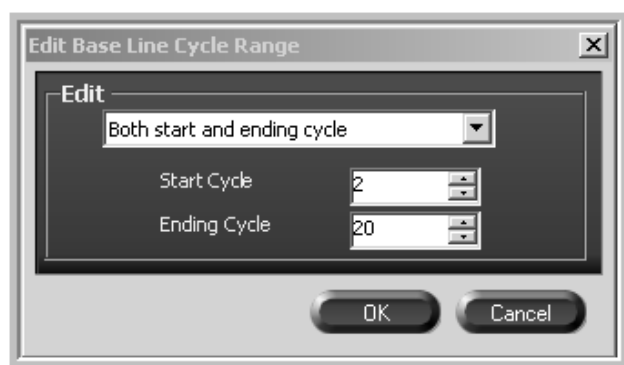


Fig. 5.21. The Both start and ending cycle mode.

Start Cycle Only

In the Start cycle only mode, you can only edit the Start Cycle box. This mode is useful if you want to retain the automatically determined end cycle but wish to change the value of the start cycle. Enter the desired value for the start cycle for the selected traces and then click OK, as shown below.

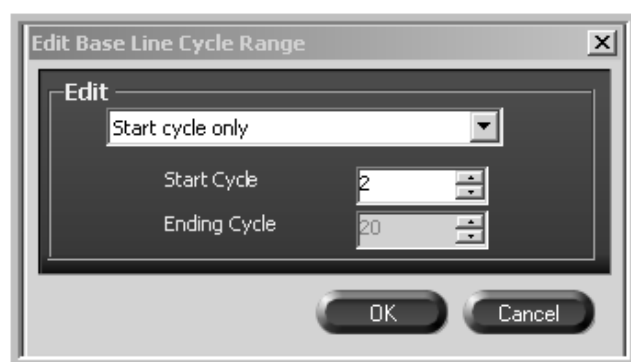


Fig. 5.22. The Start cycle only mode.

Ending Cycle Only

In the Ending cycle only mode, you can only edit the Ending Cycle box. This mode is useful if you want to retain the automatically determined start cycle but wish to change the value of the end cycle. Enter the desired value for the end cycle for the selected traces and then click OK, as shown below.

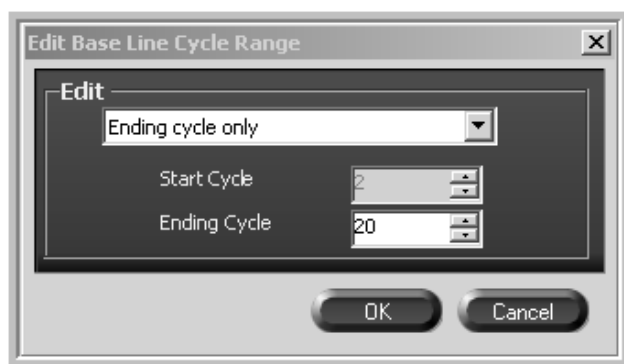


Fig. 5.23. The Ending cycle only mode.

Manual Threshold Definition

By default, MyiQ automatically calculates the threshold. You can override auto calculation by clicking User Defined in the Crossing Threshold section within the Base Line Threshold Parameter dialog box. Enter the desired value for the threshold position and then click OK.

5.5.2 PCR Amplification Data Display Options

Single Point

The Single Point mode of data presentation, in which MyiQ averages all data collected at a particular step and then plots the average, is the default mode. For example, if MyiQ collects four data points during the third repeat of an amplification cycle, the mean of those 4 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.

All Candidates

In this mode, MyiQ plots every single data point that is collected. You cannot use automated data analysis features when the data is in All Candidates mode. This mode is unavailable when the analysis mode is PCR Base Line Subtracted Curve Fit. The alternative to viewing the data in All Candidates mode is to view the data in Single Point mode.

Adjust Graph

You can re-scale or change the amplification plot from linear to log, or vice versa, by clicking Adjust Graph... in the menu. After you click Adjust Graph..., the Chart Axes Range Definition dialog box appears as shown below.

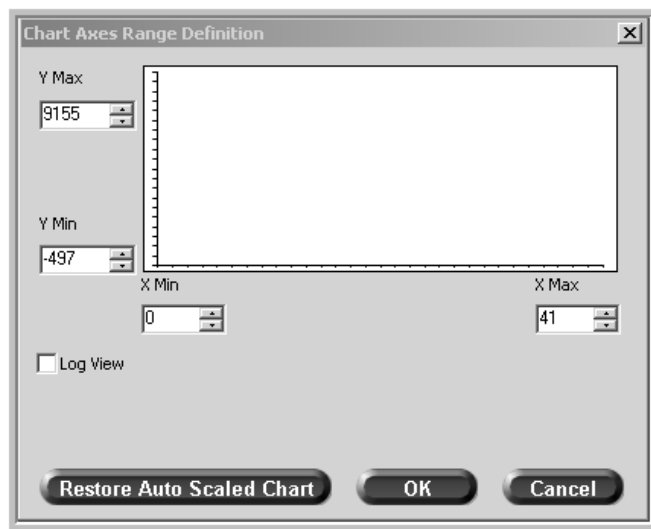


Fig. 5.24. The Chart Axes Range Definition dialog box.

You can type the maximum and minimum values for either axis into the Y Max, Y Min, X Min, and X Max scroll box(es), or use the up and down arrows in each scroll box.

You can change the display to a semi-logarithmic view by selecting the Log View check box. Clear this check box to revert to a linear plot.

Revert the plot settings to the default by clicking Restore Auto Scaled Chart.

Note: You can also change the display to a semi-logarithmic view by selecting the Log View button in the PCR Quant tab.

Define Trace Style

You can customize the display by clicking Define Trace Style... in the menu. After you click Define Trace Style... the Define Trace Style dialog box appears as shown below.

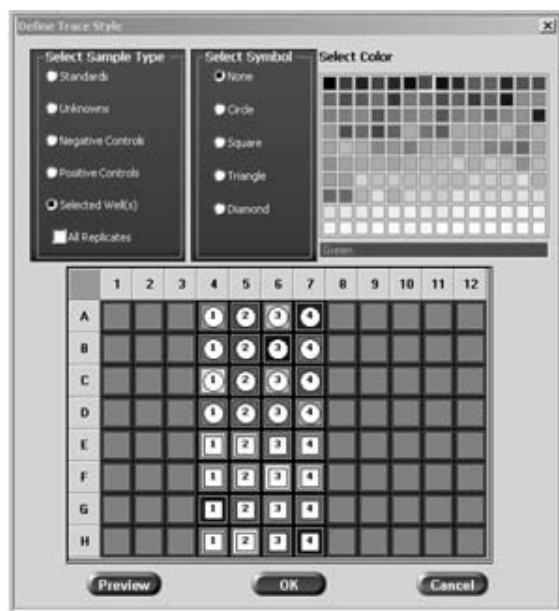


Fig. 5.25. The Define Trace Style dialog box.

You can customize the display on a well by well basis or in groups of sample types. You can also preview all changes before you apply them.

To modify a trace style:

1. Choose the color of the trace.
2. Chose the symbol type.
3. Chose the type of traces to be modified, for example, All Standards. If you want to choose on a well by well basis, select the wells in the plate grid at the bottom of the Define Trace Style dialog box. Click a column or row header to change an entire row or column. The dialog box outlines the edge of each well in the grid in the selected trace color. Select the All Replicates check box to apply the selected color to all selected replicates of the wells.
4. Click Preview to see your changes, and if those changes are satisfactory, click OK.

Display Data

Click Display Data to change the view of the results spreadsheet as follows:

- When Display Data is unselected, the results spreadsheet displays the plate spreadsheet view.
- When Display Data is selected, the results spreadsheet displays two tabs: the Amplification Data (RFU) tab and Standard Curve/Ct Results tab. You can perform the same function by clicking Results.

Restore Graph

Click Restore Graph to redraw the graph to its original size after you zoom in on the graph.

Show All Traces

Click Show All Traces to show all traces after you single out one or more using Display Wells or by clicking on a trace in the Amplification Plot.

5.5.3 Data Export Options

Copy Graph

Click Copy Graph to copy the Amplification Plot into the clipboard to export into other programs.

Print Graph

Click Print Graph to print the graph to your default Windows printer.

Print Amplification Data

The Print Amplification Data option is only available when MyiQ displays the Amplification Data (RFU) spreadsheet. You can display this spreadsheet by clicking Results in the PCR Quant tab, or by clicking Display Data in the Amplification Chart menu.

When you click Print Amplification Data, the Print Preview dialog box appears so you can print the amplification data.

Print Std Curve Data

The Print Std Curve Data option is only available when MyiQ displays the Standard Curve/Ct Results spreadsheet. You can display this spreadsheet by clicking Results in the PCR Quant tab, or by clicking Display Data in the Amplification Chart menu.

When you click Print Std Curve Data, the Print Preview dialog box appears so you can print the amplification data.

Note: If you alter the width of the columns in the spreadsheet in the Standard Curve/Ct Results spreadsheet, the printed spreadsheet will reflect that change.

5.6 Melt Curve Analysis

The RFU data collected during the melt curve part of the experiment are plotted as a function of temperature, as shown below.

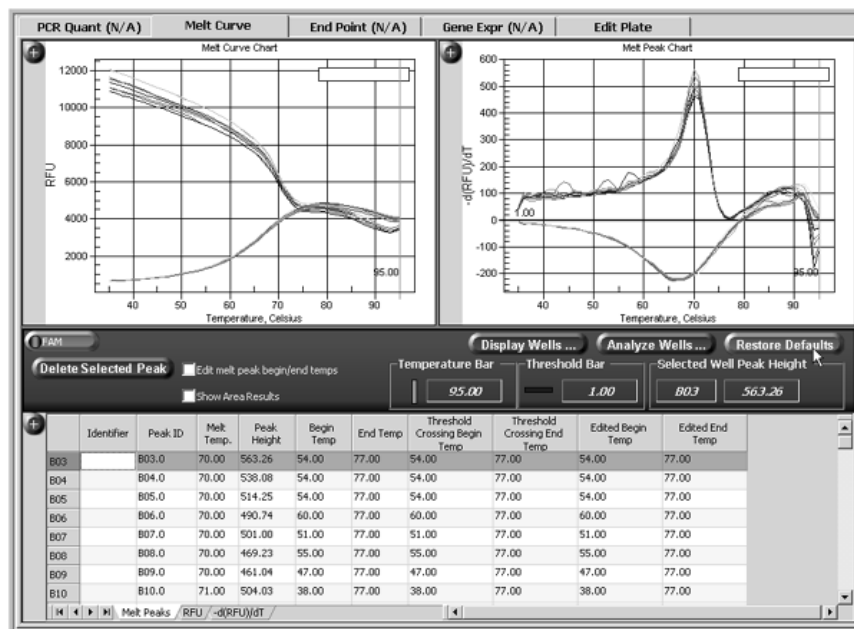


Fig. 5.26. The Melt Curve Analysis Screen.

The melt curve form has four areas as shown in the figure above. Each area is discussed in detail in the following sections.

1. Melt Curve Chart. This is a plot of the RFU data as a function of temperature.
2. Melt Peak Chart. This is a plot of the negative rate of change in RFU as temperature changes, that is, a first derivative plot, or $-d(RFU)/dT$ where T is temperature.
3. Spreadsheet. The spreadsheet at the bottom of the form displays the melt peak data, the RFU data used to make the melt curve plot and the first derivative data used to make the melt peak plot.
4. Control Area. This is the space between the charts and the spreadsheet where various control and modification functions are accessed.

Multiple Melt Curves in the Same Experiment

If more than one melt curve is conducted within the same experiment, use the selector box above the spreadsheet (shown below) to choose the melt curve to be displayed and analyzed.

Stage Cycle 5

5.6.1 Melt Curve and Melt Peak Charts

The RFU data collected during the melt curve part of the experiment are plotted as a function of temperature, as shown below.

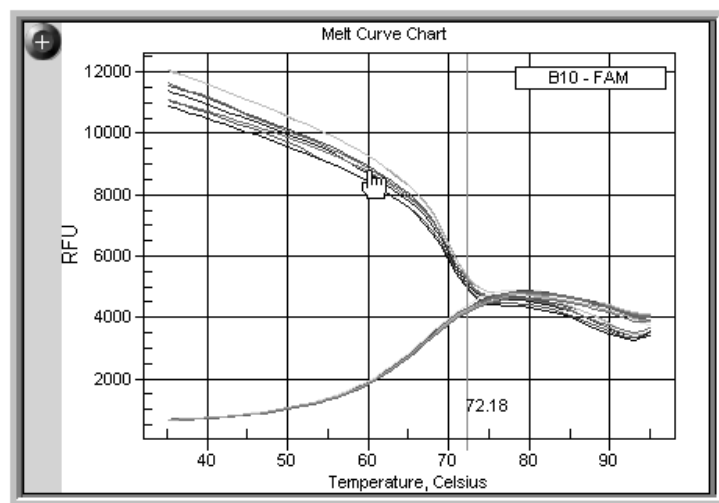


Fig. 5.27. Melt Curve Chart.

The vertical temperature bar may be dragged to any position on the plot. The temperature bar on the melt peak plot moves along to the same position as the temperature bar on the melt curve plot is moved. If you place the cursor over a trace, so that the pointer turns into a hand, the trace will be identified in the small box placed in the top right corner of the plot.

The chart can be expanded to fill the window by clicking the + box in the top left corner of the plot. Once expanded, it can be returned to its original size by clicking the – box in the top left corner.

The data in the melt peak chart are derived from the melt curve chart, as shown below.

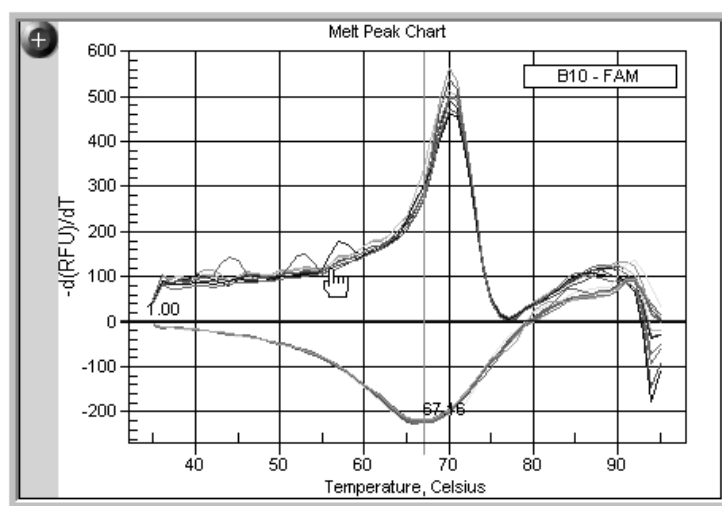


Fig. 5.28. Melt peak chart.

They represent the negative rate of change in fluorescence with changing temperature, that is:

$$-d(\text{RFU})/dT$$

Where T is temperature.

In analyzing the melt peak data, the software assigns Begin and End temperatures to each peak and then calculates an area beneath that curve. The floor of the peak area is specified by the position of the threshold bar.

In order to be identified as a valid peak, a peak must have a certain minimum height relative to the distance between the threshold bar and the height of the highest peak. Therefore, if the threshold bar is dragged upward, reducing the distance between the threshold bar and the highest peak, previously unidentified peaks may show up in the spreadsheet as their height becomes significant in relation to the highest peak. Similarly, dragging the threshold bar down can cause previously-identified minor peaks to drop off the spreadsheet.

The vertical temperature bar may be dragged to any position on the plot. The temperature bar on the melt curve plot moves along to the same position as the temperature bar on the melt peak plot is moved. If you place the cursor over a trace, so that the pointer turns into a hand, the trace will be identified in the small box placed in the top right corner of the plot. The chart can be expanded to fill the window by clicking the + box in the top left corner of the plot. Once expanded, it can be returned to its original size by clicking the - box in the top left corner.

5.6.2 Melt Curve and Melt Peak Chart Menu

There are a number of features of the plot that may be modified as well as control of data filtering. All changes specified by the context menu accessed on the Melt Curve plot are also carried out on the Melt Peak plot. Those features are accessed by a right click on the chart, which brings up the following menu.



Fig. 5.29. Chart menu.

5.6.3 Delete Selected Peaks

To remove a peak from the analysis, highlight the peak in the spreadsheet and then click Delete Selected Peaks as shown below. You can press the Shift key to highlight multiple peaks and delete them all at once.



Fig. 5.30. The Delete Selected Peaks button.

5.6.4 Edit Melt Peak Begin/End Temperature

You may edit the begin and end temperatures for a melt peak by clicking the Edit melt peak begin/end temps option button as shown below.

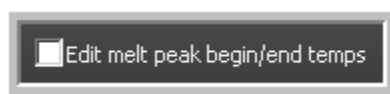


Fig. 5.31. The Edit melt peak begin/end temps option button.

After you click this option button, the currently-active peak displays along with a Begin Temp bar and an End Temp bar. Drag the bars to the desired begin and end temperatures. As you drag the begin and end bars along the Melt Peak plot, MyiQ tracks the movement on the Melt Curve chart and updates the movement in the spreadsheet. When the Edit melt peak begin/end temps option button is activated, the Display Wells and Analyze Wells dialog boxes are unavailable.

In the figure below, the peak begin temperature is 59°C, the peak end temperature is 79°C, and the threshold is 1.

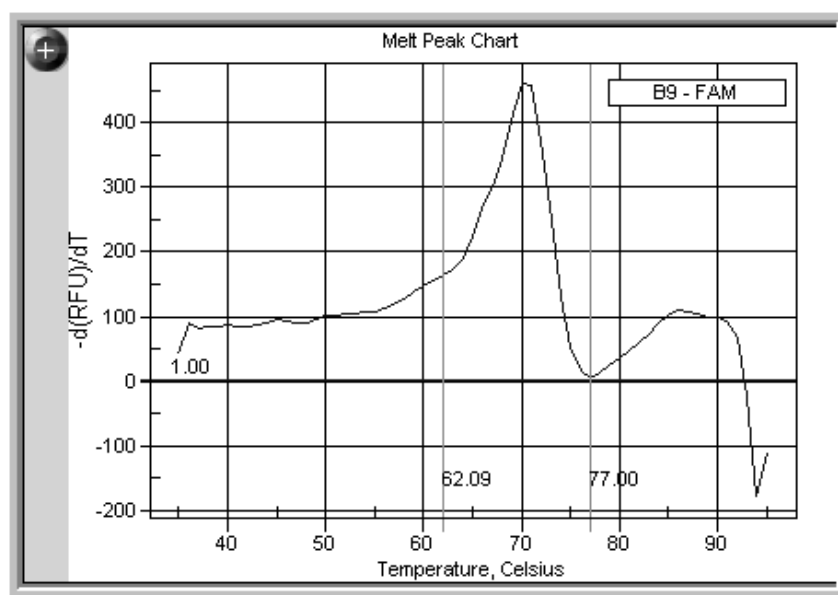


Fig. 5.32. Single Well Melt Peak View.

5.6.5 Melt Curve/Peak Spreadsheet

The spreadsheet below the plots displays information about the melt peaks, the RFU data or the $-d(RFU)/dT$ data for each well. None of the fields may be edited, though you may eliminate peaks from the spreadsheet by either highlighting the peak in the spreadsheet and clicking Delete Selected Peak, or by dragging the threshold bar up to a position above the peak.

	Peak ID	Melt Temp.	Peak Height	Begin Temp	End Temp	Area	Area Fraction %	Threshold Crossing Begin Temp	Threshold Crossing End Temp	Edited Begin Temp	Edited End Temp	Edited Area	Edited Area Fraction %
B03	B03.0	70.00	563.26	54.00	77.00	5296.01	100.00	54.00	77.00	54.00	77.00	5296.01	100.00
B04	B04.0	70.00	538.08	54.00	77.00	5037.94	100.00	54.00	77.00	54.00	77.00	5037.94	100.00
B05	B05.0	70.00	514.25	54.00	77.00	5290.81	100.00	54.00	77.00	54.00	77.00	5290.81	100.00
B06	B06.0	70.00	490.74	60.00	77.00	4092.19	100.00	60.00	77.00	60.00	77.00	4092.19	100.00
B07	B07.0	70.00	501.00	51.00	77.00	5310.12	100.00	51.00	77.00	51.00	77.00	5310.12	100.00
B08	B08.0	70.00	469.23	55.00	77.00	4600.71	100.00	55.00	77.00	55.00	77.00	4600.71	100.00
B09	B09.0	70.00	461.04	47.00	77.00	5462.93	100.00	47.00	77.00	62.09	77.00	3704.35	100.00
B10	B10.0	71.00	504.03	38.00	77.00	6232.16	100.00	38.00	77.00	38.00	77.00	6232.16	100.00

Fig. 5.33. The Melt Curve spreadsheet.

The spreadsheet displays the following information for each peak:

Column

Description

Peak ID

A unique identification number in the format RCC.N where R is a row letter, CC is a column number, and N is a number beginning with 0, 1, 2, etc. In the example spreadsheet above, the first peak for well E3 is identified as E03.0 and the second peak as E03.1.

Melt Temp.

The temperature at the highest point of the melt peak.

Peak Height

The highest point of the melt peak.

Begin and End Temp.

Starting and ending point for melt peak. Area calculations are based on these starting and ending points. These values may be edited, but not in the spreadsheet.

Area

The area beneath the melt peak curve, bounded by the default peak begin and end temperatures and the default position of the threshold bar. Because this area calculation is defined by the default values of begin and end temperature and threshold bar, it does not change as the threshold bar is moved or if the begin or end temperatures are edited.

Area Fraction %

When there is more than one peak associated with a well, the contribution of each well to the total area beneath all melt peaks for that well is calculated. MyiQ makes this calculation using default begin and end temperatures and the default position of the threshold bar, so is not affected by edits to any of those parameters.

Edited Begin Temp.

When the peak begin temperature bar is dragged to a new position, it is reflected here.

Edited End Temp.

When the peak end temperature bar is dragged to a new position, it is reflected here.

5.6.6 Melt Curve/Peak Control Area

The Control Area appears between the spreadsheet and the plots. This area contains buttons to access the Display Wells and Analyze Wells dialog boxes, as shown below.



Fig. 5.34. Melt Curve/Peak Control Area.

The position of the temperature bar, threshold bar, the height and well of the currently selected peak are also displayed here. You cannot edit any of these fields. When the Edit melt peak begin/end temp option button is active, the most-recently modified temperature (begin or end) displays in the Temperature Bar field.

The buttons at the top of the Control Area are:

- Display Wells
- Analyze Wells
- Restore Defaults

5.7 End Point Analysis

The End Point Analysis module is a convenient method of analyzing final RFU, or Relative Fluorescence Unit, values.

Endpoint analysis can be performed in two ways:

- Selecting the Endpoint tab in the Data Analysis module for an existing data file.
- Click Run End Point to initiate the collection of end point data from a sample plate.

5.7.1 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 tab. However, the corresponding PCR quantification data must be analyzed in either the PCR Base line Subtracted Mode or the PCR Base line Subtracted Curve Fit mode before the End Point tab becomes available. 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 tab.

5.7.2 Initiating an End Point Run

When initiating an End Point run, two repeats of a 30-second step will be run. Programmed to collect only end point analysis data, only the setpoint temperature of the End Point protocol can be modified. To initiate the collection of end point data from a sample plate:

1. Insert the plate into the iCycler reaction module.
2. Create or open a plate setup file.

Note: Any positive or negative controls defined in the loaded plate setup will be exported to the endpoint analysis table after the run. You may always define all your controls post-run from the End Point or the Edit Plate tab in the Data Analysis module.

3. Click on Run End Point. The Initiate Run tab appears.
4. Specify the setpoint for data collection in the Setpoint box.

Note: You must use Persistent Well Factors for every End Point Analysis Run.

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 tab appears and displays the results.

5.7.3 Analyzing Endpoint Data

End point data is displayed immediately following an End Point 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 tab. Melt curve-only experiments, which neither contain amplification data nor end point data, may not be analyzed in the End Point tab.

The End Point tab is comprised of several sections; each one described in detail below.

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 based on RFU values. The Method box consists of the following three choices:

- **Negatives:** This is the default method. 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.
- **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.
- **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. Samples are considered negative if their RFU value is less than the negative control average plus the tolerance.

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 drop-down list box consists of 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 value minus the absolute value of the lowest RFU value. The default RFU tolerance value is 10% of the total RFU range.
- **Percent of Range:** Select this setting if you would like to use a percentage of the RFU range for the tolerance value. The minimum percent of range is 1 percent, whereas the maximum percent of range is 99 percent. The default percent of range tolerance is 10 percent.

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

- For the Negatives method, the range is the highest RFU value minus the Negative control average.
- For the Positives method, the range is the positive control average minus the Lowest RFU value.
- For the Positives & Negatives method, the range is the positive control average minus the Negative control average.

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

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 MyiQ to analyze non-End Point Only data in the End Point Analysis tab, at least 6 repeats at data acquisition must be performed.

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 minimum 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.

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 but also includes sorting and color coding the Positive and Negative Calls. The ranking function is disabled in this mode.

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 within 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 appears.
- **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 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.

5.7.4 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 Recalculate 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 Analyze Wells button above the spreadsheet.
- **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 RFU's:** Lists the absolute RFU averages for each well, as calculated from the end cycles, which are specified in the End Cycles to Average field. You may click on the End RFU's 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. You may click on the Define Controls column header to sort the column by the controls.
- **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 Recalculate 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 RFU's 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. You may click on the Identifier column header to sort the table by identifier.

Recalculate Button

Clicking Recalculate performs the final calculations used to determine unknown sample calls. The Recalculate button is active when sufficient controls have been specified for the selected Method and End Point Tolerance settings. The following conditions result in an error message:

- If the Positives method is selected and there is not at least one positive control defined in the End Point analysis spreadsheet.
- If the Negatives method is selected and there is not at least one negative control defined in the End Point analysis spreadsheet.
- If the Positives & Negatives method is selected and there is not 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 (that is, the negative control RFU average is larger than the positive control RFU average).

End Point analysis cannot be performed if one or more of these error conditions is present when you click Recalculate.

After you are satisfied with your End Point analysis, you may choose to view, save, or print a customized End Point Analysis report. Click the Reports menu to obtain customized reports for the End Point data. Click Print to print the End Point spreadsheet.

5.8 Gene Expression Analysis

Gene expression analysis is the most common MyiQ application. Therefore, this manual uses terminology that is specific to this kind of study. You can safely substitute gene with the word sequence or target when you read about the Gene Expression tab.

Without stringently quantified controls, you cannot use this feature of MyiQ to evaluate target concentrations of particular sequences relative to each other. MyiQ can only evaluate relative differences of a sequence between a group of samples.

Activate the Gene Expression tab from the Data Analysis Module (1) by clicking Gene Expr (2) as shown below.

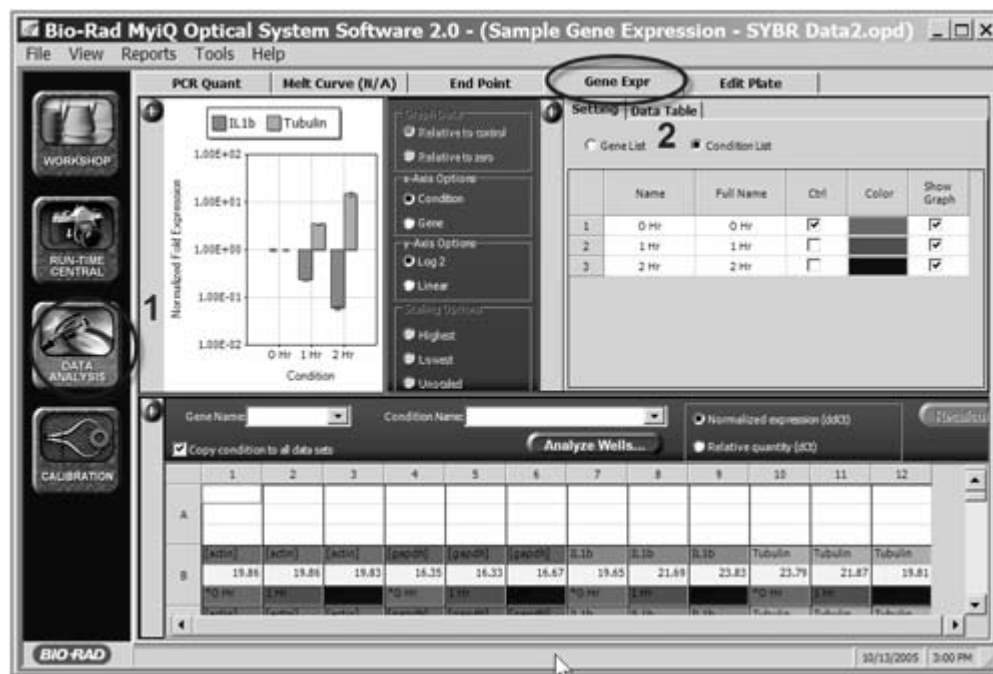


Fig. 5.35. The Gene Expression Analysis Tab.

You can use the Gene Expression tab to evaluate relative differences in any target concentration. For example, you can evaluate relative numbers of viral genomes or stably transfected sequences. The most common application is evaluating target concentration in cDNA samples to infer steady state messenger RNA levels.

5.8.1 Gene Expression Analysis Steps

1. In the PCR Quant tab, establish desired analysis conditions such as baseline and threshold and which wells are to be included in the analysis.
2. Click the Gene Expr tab.
3. Choose an analysis method:
 - a. Normalized Expression ($\Delta\Delta C_T$) is the default; or
 - b. Relative Quantity (ΔC_T).
4. Assign Gene and Condition Names in the Gene Expression Plate Interface.
5. Assign Attributes in the Gene List

If you choose Normalized Expression ($\Delta\Delta C_T$), assign reference gene(s).

6. Assign Attributes in the Condition List
7. Click Recalculate to see your results.

Step 1: Establish PCR Quant Tab Analysis Conditions

In the **PCR Quant** tab, establish desired analysis conditions such as baseline and threshold and which wells are to be included in the analysis.

Step 2: Choose the Gene Expression Tab

Click the Gene Expr tab.

Step 3: Choose an Analysis Method

- a. Normalized Expression ($\Delta\Delta C_T$) is the default; or
- b. Relative Quantity (ΔC_T).

Step 4: Assign Gene and Condition Names in the Gene Expression Plate Interface

1. For easier viewing, maximize the Gene Expr Plate Interface in order to view all wells.
2. Highlight the wells in the Gene Expr Plate Interface you wish to edit
3. Change the gene and condition assignments in these wells by typing in the gene pull-down menu and the condition pull-down menu. You may use the default values which already populate these lists.
4. Minimize the Gene Expr Plate Interface when finished.

Step 5: Assign Attributes in the Gene List

Setting | Data Table

☒ Gene List ☐ Condition List

	Name	Full Name	Ref	Color	Show Graph	Auto Efficiency	Efficiency (%)
1	Gapdh	Gapdh	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
2	IL1b	IL1b	<input type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
3	Tubulin	Tubulin	<input type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
4	Actin	Actin	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
1 2 3 4 5 6							

The following attributes can be assigned to genes in the Gene List:

1. Assign a longer name to a gene if
 - a. it is too long for the plate setup interface
 - b. you would like to display something different in the legend with the gene names are displayed on the x-axis
2. Assign one or more genes as Reference Genes.
3. Assign each gene a color for presentation on the graph and plate interface

4. Indicate whether to show each gene on the graph
5. Indicate whether to use Auto Efficiency (imported from standard curves in experiment) or
6. Type in the efficiency for the gene.

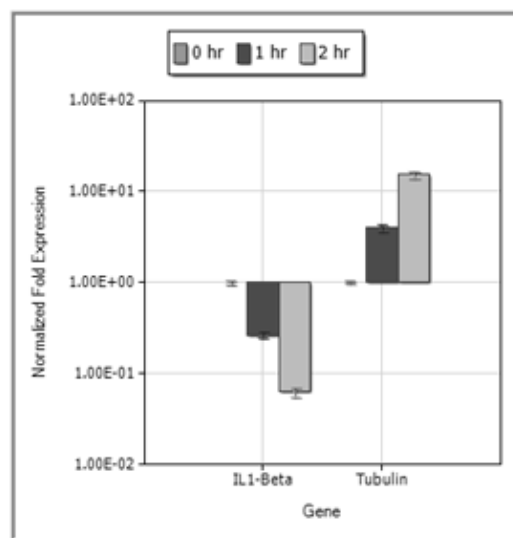
Step 6: Assign Attributes in the Condition List

Setting		Data Table			
<input type="radio"/> Gene List <input checked="" type="radio"/> Condition List					
	Name	Full Name	Ctrl	Color	Show Graph
1	0 Hr	0 Hr	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>
2	1 Hr	1 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
3	2 Hr	2 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>

1
2
3
4

1. Assign a longer name to a condition if
 - a. it is too long for the plate setup interface
 - b. you would like to display something different in the legend when conditions are displayed on the x-axis
2. Assign one condition the Control condition (optional)
3. Assign each condition a color for presentation on the graph and plate interface
4. Indicate whether to show each condition on the graph.

Step 7: Press Recalculate and view your data



5.8.2 Relative Quantity Gene Expression (ΔC_T)

Relative Quantity is the target sequence concentration relative to other samples in the experiment. This is sequence quantity without taking into account reference genes for normalization. No calculations are made to account for loading differences or differences in the number of cells represented in each of your samples. Relative Quantity can be viewed as Non-Normalized Expression. This value is sometimes referred to as ΔC_T because of the equation used to calculate relative quantity.

Workflow for Relative Quantity (ΔC_T) Analysis

To calculate Relative Quantity (ΔC_T):

1. In the **PCR Quant** tab, establish desired experimental conditions such as baseline and threshold and which wells are to be included in the analysis.
2. Click the **Gene Expr** tab.
3. Select **Relative Quantity** as your analysis method.
4. Assign Gene and Condition Names in the Gene Expression Plate Interface.
5. Assign Attributes in the Gene List
6. Assign Attributes in the Condition List
7. Click **Recalculate** to see your results.

Relative Quantity is graphed in the Gene Expression Graph Interface. You can access the data in the Gene Expression Settings Interface in the Data Table.

Not using Reference Genes (Relative Quantity, ΔC_T)

By definition, Relative Quantity data is not normalized. Typically researchers that do not use reference genes are confident in one of the two following considerations.

1. Each condition (sample) assayed represents the same amount of biological sample. Typically they choose to load the same mass of RNA or cDNA in each well and feel that mass of nucleic acid is an effective way of normalizing the resulting data. No modification of Relative Quantity data is needed too obtain normalized data. The data is normalized by experimental design.
2. They prefer to normalize post PCR analysis by some method that will account for the amount of biological sample. They would simply divide the relative quantity value by the normalizing factor indicated after each of the examples listed below. Options may include but are not limited to:
 - a. Mass of nucleic acid loaded for each sample
Rel Quant/ng RNA represented in each sample
 - b. Number of cells from which nucleic acid was isolated
Rel Quant/number of cells represented in each sample
 - c. Mass of tissue from which nucleic acid was isolated
Rel Quant/mass of tissue represented in each sample

5.8.3 Normalized Gene Expression ($\Delta\Delta C_T$)

Normalized expression is the relative quantity of your gene normalized to the relative quantities of the reference gene(s). Simply stated, the quantities of the reference genes are used to normalize the values of your genes of interest.

Provided the reference genes are not regulated in your system, this will account for loading differences or variations in cell # represented in each of your samples. This value is sometimes referred to as $\Delta\Delta C_T$ because of the equation initially introduced as part of the Livak method, to evaluate normalized expression. The software uses a modification of this equation. View the equation used to calculate normalized expression.

Normalized Expression can be viewed as normalized target sequence quantity if you are using the software for other purposes.

Workflow for Normalized Gene Expression ($\Delta\Delta C_T$) Analysis

To calculate Normalized Expression ($\Delta\Delta C_T$):

1. In the **PCR Quant** tab, establish desired experimental conditions such as baseline and threshold and which wells are to be included in the analysis.
2. Click the **Gene Expr** tab.
3. Select **Normalized Expression** as your analysis method.
4. Assign Gene and Condition Names in the Gene Expression Plate Interface.
5. Assign Attributes in the Gene List

In Normalize Expression Analysis you must assign reference gene(s).

6. Assign Attributes in the Condition List

- Click **Recalculate** to see your results.

Normalized Expression is graphed in the Gene Expression Graph Interface. The data can be accessed in the Gene Expression Settings Interface within the Data Table.

Using Reference Genes (Normalized Expression, $\Delta\Delta C_T$)

Rather than using some other method to normalize data, you may use one or more reference gene's message levels as assayed in your qPCR experiment as a normalization factor. Reference genes should be genes which are not regulated in the biological system being studied.

Why should I normalize my data?

Relative Quantity data that is not normalized by some means is difficult to interpret. Imagine the case where you load 1 μ g of RNA in one well and 10 ng in the other well. If you perform a relative quantity analysis on the results from such an assay the fact that the 10 ng sample has a smaller Relative Quantity value is irrelevant. It is likely the result is to do with using less RNA and not the result of some biological response.

5.8.4 Specifying Gene and Condition Labels for Gene Expression Analysis

Specifying Gene Names Using the Gene List Option

To access the Gene List:

- Make sure the Settings button is active (see below).
- Click the Gene List option button (see below).

The Gene List appears in the spreadsheet directly below the Gene List option button as shown below.

	Name	Full Name	Ref	Color	Show Graph	Auto Efficiency	Efficiency (%)
1	Gapdh	Gapdh	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
2	IL1b	IL1b	<input type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
3	Tubulin	Tubulin	<input type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
4	Actin	Actin	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0

Fig. 5.36. Gene List View.

The Gene List is the list of valid genes (sequences) in your experiment. Access the Gene List to assign attributes like colors or Ref (Reference gene) to genes.

A gene can be as simple as "Gene 1" or complex as "CMVP/IL1B/SV40s", though the latter example is often too long to present aesthetically on a graph.

Genes which have no valid Threshold Crossing values (NaN) for every condition in the experiment are considered invalid. The software excludes such genes from the experiment.

The following attributes can be assigned to genes in the Gene List. Attribute columns are listed as they appear in the gene list from right to left:

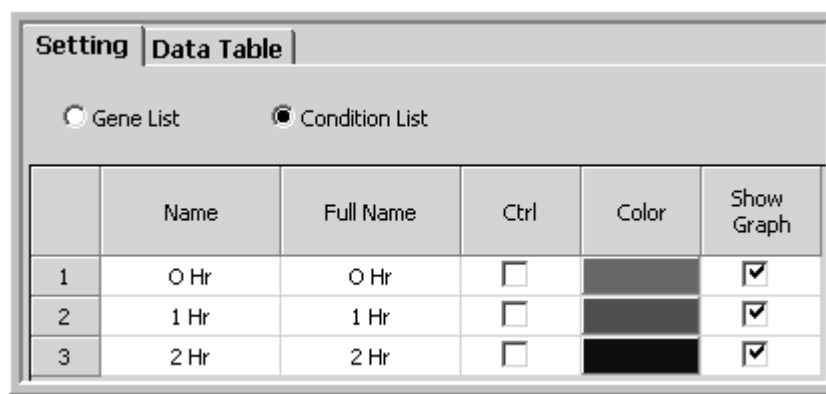
- Assign a longer name to a gene if
 - it is too long for the plate setup interface
 - you would like to display an alternative in the legend when conditions are displayed on the x-axis
- Assign one or more genes as reference genes.
- Assign each gene a color for presentation on the graph and plate interface.
- Indicate whether to show each gene on the graph.
- Indicate whether to use Auto Efficiency (from standard curves in experiment) or not.
- Type in the efficiency for the assay that is being used to measure each gene.

Specifying Assay Conditions Using the Condition List Option

To access the Condition List:

1. Make sure the Settings button is active (see below).
2. Click the Condition List option button (see below).

The Condition List appears in the spreadsheet directly below the Condition List option button as shown below.



	Name	Full Name	Ctrl	Color	Show Graph
1	0 Hr	0 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
2	1 Hr	1 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
3	2 Hr	2 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>

Fig. 5.37. The Condition List spreadsheet.

The Condition List is the list of valid conditions (samples) in your experiment. Access the Condition List to assign attributes like colors or control to conditions.

A condition can be as simple as "sample 1" or complex as "mouse #123558 liver + PMA", though the latter example is often too long to present aesthetically on a graph.

Invalid conditions have no valid Threshold Crossing values (NaN) for every gene in the experiment. The software excludes such conditions from the experiment.

The following attributes can be assigned to conditions in the Condition List. Attribute columns are listed as they appear in the gene list from right to left:

1. Assign a longer name to a condition if
 - a. it is too long for the plate setup interface
 - or
 - b. you would like to display something different in the legend when genes are displayed on the x-axis.
2. Assign one condition the control condition.
3. Assign each condition a color for presentation on the graph and plate interface.
4. Indicate whether to show each condition on the graph.

5.8.5 Assigning Control Conditions and Reference Genes

You can assign one condition as the control condition by selecting a check box next to the sample in the Condition List.

MyiQ assigns the control sample a value of 1 for every gene and all other conditions will be presented with values relative to this one. This makes it simple to evaluate fold expression relative to the chosen (control) sample.

Note: The control condition will have a value of one for all genes.

Access the Condition List

To access the condition list:

1. Make sure the Settings button is activated (1). If not, click Settings. See the screen example below.
2. Click the Condition List option button (2).

Setting | **Data Table**

1 ☐ Gene List ☒ Condition List 2

	Name	Full Name	Ctrl	Color	Show Graph
1	0 Hr	0 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
2	1 Hr	1 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
3	2 Hr	2 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>

Fig. 5.38. Condition List Settings.

Assign a Control Condition

To assign a control condition:

1. Click the Condition List option button (1). See the screen example below.
2. Select the check box(es) in the Ctrl column next to the condition(s) which you want to assign as control conditions (2).

Setting | **Data Table**

☐ Gene List 1 ☒ Condition List 2

	Name	Full Name	Ctrl	Color	Show Graph
1	0 Hr	0 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
2	1 Hr	1 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>
3	2 Hr	2 Hr	<input type="checkbox"/>		<input checked="" type="checkbox"/>

Fig. 5.39. Assigning Control Conditions.

Access the Gene List

To access the gene list:

1. Make sure the Settings button is activated (1). See the screen example below.
2. Click the Gene List option button (2).

Setting | **Data Table**

☒ Gene List 2 ☐ Condition List

	Name	Full Name	Ref	Color	Show Graph	Auto Efficiency	Efficiency (%)
1	Gapdh	Gapdh	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
2	IL1b	IL1b	<input type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
3	Tubulin	Tubulin	<input type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0
4	Actin	Actin	<input checked="" type="checkbox"/>		<input checked="" type="checkbox"/>	<input type="checkbox"/>	100.0

Fig. 5.40. Gene List Settings.

Assign a Reference Gene

To assign a reference gene:

1. Click Gene List (1). See the screen example below.
2. Select the check box in the Ref column within the control condition(s) you want to assign a reference gene to (2). You can assign the role of reference gene to as many genes as you wish.

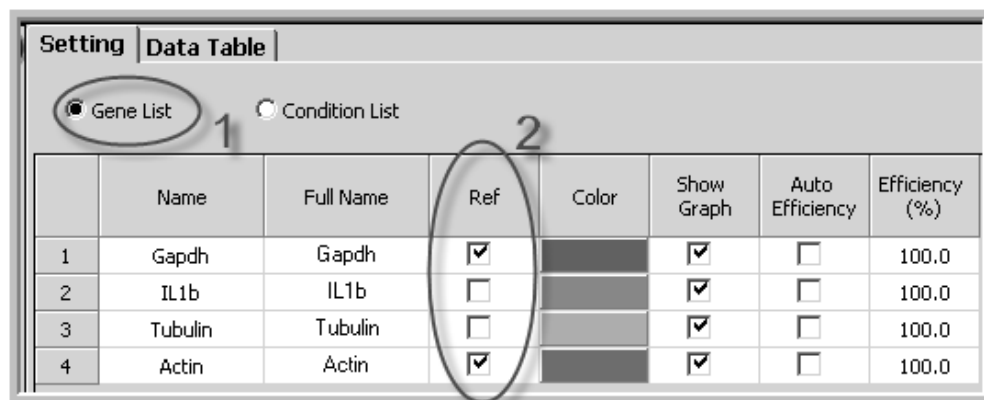


Fig. 5.41. Assigning Reference Genes.

Choose an Analysis Method

Choose an analysis method by clicking one of the option buttons as shown in the screen example below:

1. Normalized Expression ($\Delta\Delta C_T$) is the default. This option requires you to choose a reference gene.
2. Relative Quantity (ΔC_T)

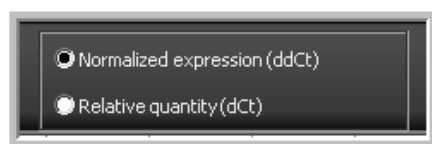
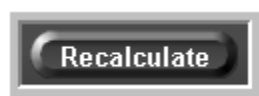


Fig. 5.42. Analysis Method Selection Box.

Recalculate

Many changes made to the plate setup and analysis options require that you recalculate. If you do not immediately see the change you expect to see, click Recalculate as shown below.



5.8.6 Reaction Efficiency Compensation in Gene Expression Analysis

Efficiency describes how much of your sequence of interest is being produced with each cycle. 100% efficiency means that you are doubling your sequence of interest with each cycle. People focus on efficiency for a number of reasons. There is evidence that using accurate efficiencies for each of your primer/(probe) pairs will give you more accurate results when using the mathematical modules used in the Gene Expression tab.

You set the efficiency for each of your genes (sequences, primer/probe set) in the Gene List. The default value is 100 percent efficiency.

How do I Determine Efficiencies?

Typically the efficiency for each primer/(probe) set is evaluated and recorded during assay development. Generate a standard curve using serial dilutions of a representative sample across a relevant dynamic range, and then record the efficiency for subsequent gene expression analysis.

Auto Efficiency

The Auto Efficiency function uses efficiency values from your experiment so that you do not have to type them in yourself. To use Auto Efficiency, you must have standards in your experiment that result in valid standard curves in the PCR Quant tab.

Auto Efficiency Standards

MyiQ only requires two standards at different concentrations though it is recommended to have at least four samples in triplicate across a relevant dynamic range.

Efficiencies in Calculations

The symbol E used in the equations that describe the Gene Expression calculations refers to Efficiencies used in the Pfaffl method and the Vandesompele method. In these publications an efficiency of 2 (a perfect doubling with every cycle) is equivalent to 100% efficiency used in the software. You can convert efficiencies by using the following relationships.

$$E = (\% \text{ Efficiency} * 0.01) + 1$$

$$\% \text{ Efficiency} = (E - 1) * 100$$

5.8.7 Graphing Options for Expression Data

Graph Expression Relative to Control or Relative to Zero

These options, shown below, allow you to present data with bars originating at 1 (relative to control) or at zero (relative to zero). If you assign a control graphing relative to control allows you to quickly visualize up-regulation and down-regulation. The values graphed are exactly the same.

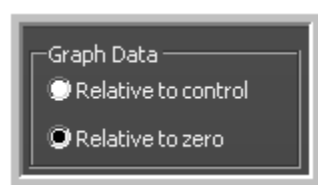


Fig. 5.43. Relative Graphing Options.

x-axis Options

You may graph either genes or conditions on the x-axis by changing these options.

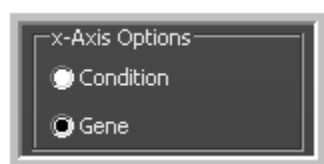


Fig 5.44. X-axis Graphing Options.

Graph y-axis linear or Log2 (Natural Log, Ln)

This option allows you to display the graph with the y-axis in log 2 or linear scale as shown below. The latter is useful when you evaluate samples across a large dynamic range.

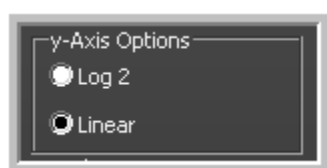


Fig 5.45. Y-axis Graphing Options.

Scaling Options

These options, shown below, are only active in Normalized Expression mode; they allow you to calculate and present your data in a manner that is best suited for your experiment.

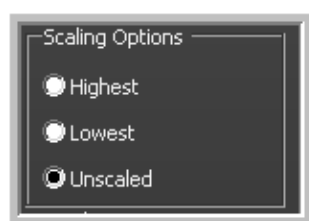


Fig 5.46. Bar Chart Scaling Options.

Scale to Highest

This option recalculates the Normalized expression for each gene by dividing the expression level of each condition by the highest expresser. The highest expresser for each gene has a value of 1.

Scale to Lowest

This option recalculates the Normalized expression for each gene by dividing the expression level of each condition by the lowest expresser. The lowest expresser for each gene has a value of 1.

Unscaled

This option does not scale to any sample in particular. It presents unscaled normalized expression.

Scale to Control

Scale to control is another scaling option which is accomplished by assigning a control in the Condition List.

Graph Error Options

The default presentation for the error bars is plus and minus one standard deviation. You can change the multiplier to get plus and minus 2 or 3 standard deviations.

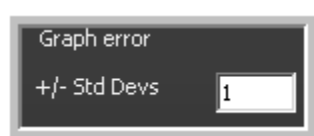


Fig 5.47. Error Bar Calculation Options.

5.8.8 Exporting Gene Expression Results

To export a graph:

1. Place the mouse pointer over the graph.
2. Click the right mouse button. A shortcut menu appears. See the screen example below.
3. Click Copy Graph.
4. Switch to or open the document into which you will paste the graph.
5. Paste the graph image by choosing Edit, then Paste, or by pressing CTRL+V.

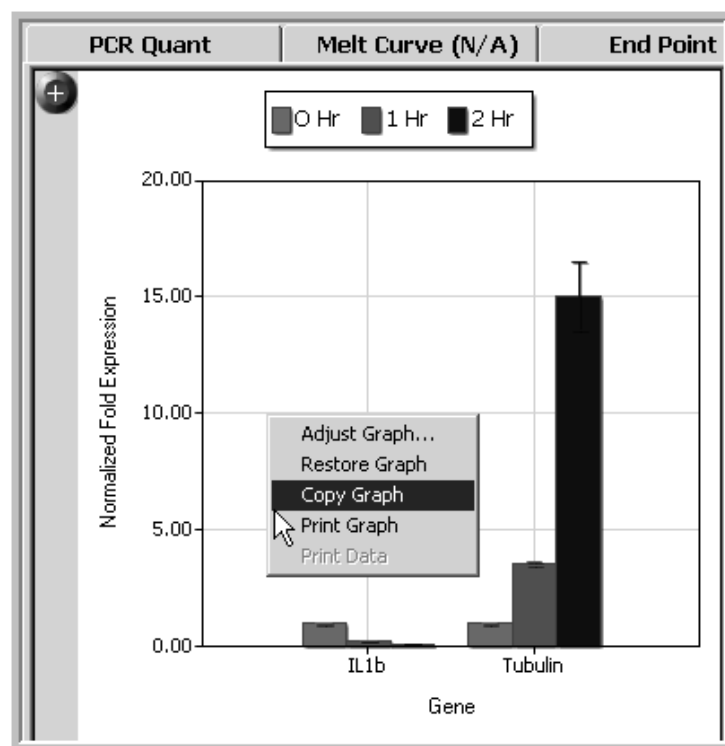


Fig 5.48. Exporting Gene Expression Bar Chart.

Export Data from the Data Table

1. Make sure the Data Table button is chosen. See the screen example below.
2. Highlight the data you wish to export.
3. Press CTRL+C.
4. Switch to or open the document into which you will paste the data.
5. Paste the graph image by choosing Edit, then Paste, or by pressing CTRL+V.

	Condition	Gene	Expression	Expression SD
1	0 Hr	Gapdh	NaN	NaN
2	0 Hr	IL1b	1.00000	0.03635
3	0 Hr	Tubulin	1.00000	0.03285
4	0 Hr	Actin	NaN	NaN
5	1 Hr	Gapdh	NaN	NaN
6	1 Hr	IL1b	0.23757	0.00728
7	1 Hr	Tubulin	3.56056	0.10845
8	1 Hr	Actin	NaN	NaN
9	2 Hr	Gapdh	NaN	NaN
10	2 Hr	IL1b	0.06109	0.00583
11	2 Hr	Tubulin	15.03916	1.45200
12	2 Hr	Actin	NaN	NaN

Fig 5.49. Exporting Gene Expression Data Table.

5.8.9 Calculations for Relative and Normalized Gene Expression Analysis

Relative Quantity Calculations

Relative Quantity Calculations with No Controls Identified

Relative Quantity (ΔC_T) for any sample for gene x is calculated as follows.

$$\text{Relative Quantity}_{\text{sample (Gene x)}} = E_{\text{Gene x}}^{(C_{T(\text{MIN})} - C_{T(\text{sample})})}$$

Where E = Efficiency of primer/(probe) set

this efficiency is calculated as follows (% Efficiency * 0.01 + 1)

where 100% = 2

$C_T(\text{MIN})$ = Average C_T for the sample with the lowest average C_T for Gene x

$C_T(\text{sample})$ = Average C_T for the sample.

Relative Quantity When a Control is Assigned

With a control assigned Relative Quantity (ΔC_T) for any sample for all genes is calculated as follows.

$$\text{Relative Quantity}_{\text{sample (Gene x)}} = E_{\text{Gene x}}^{(C_T(\text{Control}) - C_T(\text{sample}))}$$

Where E = Efficiency of primer/(probe) set

this efficiency is calculated as follows (% Efficiency * 0.01 + 1)

where 100% = 2

C_T (MIN) = Average C_T for the sample which has been assigned as a control

C_T (Sample) = Average C_T for the sample

This is where the calculations differ from those outlined by Vandesompele on the geNorm Web site. In the example on the geNorm Web site, the results are not scaled until the control normalized expression is calculated. This is referred to as rescaled normalized expression in the example spreadsheet.

Standard Deviation of Relative Quantity of Gene x for a Given Sample

$$SD_{\text{Relative Quantity}} = SD_{CT \text{ sample}} * \text{Relative Quantity}_{\text{sample}} * \ln(E_{\text{Gene x}})$$

Where:

SD Relative Quantity = Standard Deviation of the Relative Quantity

SD CT sample = Standard Deviation of the CT of the sample.

Relative Quantity sample = Relative Quantity of sample.

E = Efficiency of primer/probe set

this efficiency is calculated as follows (% Efficiency * 0.01 + 1)

where 100% = 2

Normalized Expression Calculations

The normalized expression calculation is stated below. To see how MyiQ calculates relative quantities, go to Relative Quantity Calculations.

$$\text{Normalized Expression}_{\text{sample (gene x)}} = \frac{\text{Relative Quantity}_{\text{sample (gene x)}}}{(\text{Rel Quant}_{\text{sample (Ref 1)}} * \text{Rel Quant}_{\text{sample (Ref 2)}} * \dots * \text{Rel Quant}_{\text{sample (Ref n)}})^{1/n}}$$

Normalized Expression when a Control is Chosen

When a control is chosen, MyiQ uses the same equation to calculate normalized expression. If a control is assigned, relative quantities for all genes is equal to 1 for the control sample. This results in normalized expression of 1 for the control with all other conditions presented relative to this sample. From the perspective of the calculations actually performed Normalized Expression when an assigned control is unscaled normalized expression. MyiQ does not perform any more operations.

Normalization Factor

The denominator of the normalized expression equation is referred to as the normalization factor. The normalization factor is the geometric mean of the relative quantities of all the reference genes for a given sample.

$$\text{Normalization Factor}_{\text{sample (gene x)}} = (\text{Rel Quant}_{\text{sample (Ref 1)}} * \text{Rel Quant}_{\text{sample (Ref 2)}} * \dots * \text{Rel Quant}_{\text{sample (Ref n)}})^{1/n}$$

Scaled Normalized Expression

Scaling to highest or lowest is achieved simply by dividing all unscaled normalized expression values for a given gene by the highest or lowest expresser respectively.

Scaled to Highest

$$\text{Scaled Normalized Expression}_{\text{sample (gene x)}} = \frac{\text{Normalized Expression}_{\text{sample (gene x)}}}{\text{Normalized Expression}_{\text{Highest (gene x)}}}$$

Scaled to Lowest

$$\text{Scaled Normalized Expression}_{\text{sample (gene x)}} = \frac{\text{Normalized Expression}_{\text{sample (gene x)}}}{\text{Normalized Expression}_{\text{Lowest (gene x)}}}$$

Standard Deviation for the Normalized Expression

Rescaling this value is accomplished by dividing the Standard deviation of the normalized expression by the normalized expression value for the highest or lowest expresser depending on which scaling option you choose.

$$\text{SD Normalization Factor}_{\text{sample}} = \sqrt{\left(\frac{\text{SD Rel Quant}_{\text{sample (Ref 1)}}}{n * \text{Rel Quant}_{\text{sample (Ref 1)}}}\right)^2 * \left(\frac{\text{SD Rel Quant}_{\text{sample (Ref 2)}}}{n * \text{Rel Quant}_{\text{sample (Ref 2)}}}\right)^2 * \dots * \left(\frac{\text{SD Rel Quant}_{\text{sample (Ref n)}}}{n * \text{Rel Quant}_{\text{sample (Ref n)}}}\right)^2}$$

When a control is assigned you need not perform this rescaling function on the standard deviation as illustrated below.

$$\text{SD Normalized Expression}_{\text{sample (Gene x)}} = \text{Normalized Expression}_{\text{sample (Gene x)}} * \sqrt{\left(\frac{\text{SD Normalization Factor}_{\text{sample}}}{\text{Normalization Factor}_{\text{sample}}}\right)^2 * \left(\frac{\text{SD Rel Quant}_{\text{sample (Gene x)}}}{\text{Rel Quant}_{\text{sample (Gene x)}}}\right)^2}$$

Standard Deviation of the Scaled Normalized Expression

Rescaling this value is accomplished by dividing the standard deviation of the normalized expression by the normalized expression value for the highest or lowest expresser depending on which scaling option you choose.

$$\text{SD Scaled Normalized Expression}_{\text{sample (Gene x)}} = \frac{\text{SD Normalized Expression}_{\text{sample (Gene x)}}}{\text{Normalized Expression}_{\text{MAX or MIN (Gene x)}}}$$

When a control is assigned you need not perform this rescaling function on the standard deviation.

5.8.10 Gene Expression Analysis - Frequently Asked Questions

Why would I have to assign gene names in the gene expression tab?

If you have not assigned gene names in your initial plate setup or if you are studying more than 5 genes you can use the plate interface in the gene expression tab to assign these relationships.

Can I customize my gene and condition names?

Yes. Both the gene pull-down menu and the condition pull-down menu will accept whatever test you type into them. If your names are very long, you can edit the long name field in the gene list and condition list. The long name appears only in the legend above the graph. The name displays on the x-axis.

Why would I use Normalized Gene Expression ?

In order to make valid comparisons between two genes, their measured expression levels must be normalized in some way. Consider the example of an experiment in which one well is loaded with 100 ng of cDNA and another with 1 ng of cDNA. There is an immediate bias by a factor of 100 if the resultant data are taken at face value.

Normalization can be part of the qPCR experiment by inclusion of a reference gene, presumed to have constant levels of expression over the variation under study. Otherwise, normalization takes place before the qPCR using some other measurement such as cell number, RNA mass, or tissue mass.

The MyiQ software can present expression data normalized to one or more reference genes, or, for data normalized before PCR, as a relative quantity. The final interpretation of relative quantitation data may require additional calculation outside the MyiQ software. For example, when normalization is based on number of cells and sample A comes from twice as many cells as sample B, if both A and B have identical threshold cycles, the software will report a relative quantity of $A/B = 1$. To obtain the correct relationship, divide by the normalizer, cell number. Since A started with twice as many cells as B, the normalizer for A:B is 2. Applying the normalization factor changes the final result to show the A is expressed at half the level of B.

How does normalized expression as calculated by this software compare to the $\Delta\Delta C_T$ equation?

If you leave efficiencies at 100% and only evaluate one reference gene and one gene of interest the software will generate the same results as you would get using the $\Delta\Delta C_T$ equation.

The standard deviations will be larger since the error propagation outlined in the Livak publication does not propagate error based on the derivative of the function used to calculate the results. The approach in the Livak publication though popular often underestimates the true variance.

How does normalized expression as calculated by this software compare to the model introduced by Dr. M. Pfaffl?

If you only evaluate one reference gene and one gene of interest you will get exactly the same results using MyiQ as you would using the model introduced in this paper.

Standard deviations may be slightly different.

How does normalized expression as calculated by this software compare to the model outlined by Dr. Jo Vandesompele on the geNorm Web site?

MyiQ uses the models outline on the geNorm Web site and will give you the same results.

5.9 Edit Plate

The Post-Run Edit Plate window allows you to edit the plate setup of a data file after an experiment has been performed. This feature allows you to make corrections for incorrect sample type, identifiers/conditions, probe/primer names, units, or standard concentration assignments and to add notes about the experiment.

You may not add or remove fluorophores from wells in post-run plate editing, nor may you delete a well from the plate setup by removing both its sample type and fluorophore assignments. (Use Analyze Wells to remove undesired wells from the analysis.) After editing the plate, you can reanalyze the experimental data with the new plate setup.

Note: You can always restore the original plate setup definitions by clicking Restore Original Plate.

For post-run editing of the Plate Setup saved in a Data File:

1. From the Workshop module:
 - a. Click Data File above the directory of the home workshop. Navigate the directory until the desired data file is found. Double click the file name to bring the file directly into the Data Analysis module.
 - or
 - b. Click Data File above the directory of the home workshop. Navigate the directory until the desired data file is found. Click the file name once to open the plate setup associated with the data file in the bottom right pane of the Workshop window. Click Analyze to bring the data to the Data Analysis module.

2. At the top of the Data Analysis window, click Edit Plate.



3. A modified version of the Plate Setup Editor opens. In this modified window, you cannot add fluorophores to or remove fluorophores from the fluorophore list. Also, you cannot add a previously-defined fluorophore or remove a previously-defined fluorophore from a well.

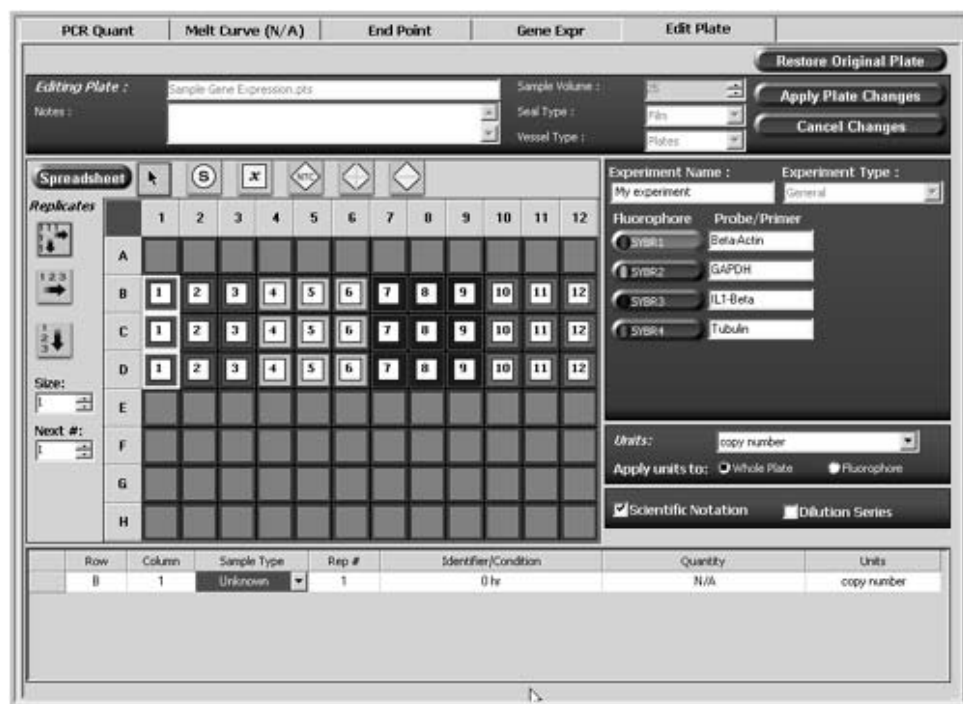


Fig. 5.50. Post-Run Edit Plate Screen.

4. Edit any notes about the plate setup in the Notes box.
5. Edit the name of the experiment in the Experiment Name box.
6. To edit a sample type:
 - a. Click a Fluorophore.
 - b. Click the correct sample type icon.
 - c. Select the Next # check box to be sure that the desired number will be assigned to the next standard you define.



- d. Select the type of Replicate loading desired.
 - e. Click a previously defined well to overwrite its original sample type definition. Replicates of the well are not affected.
 - f. Repeat steps b-e above for each additional sample type to be edited for this fluorophore.
 - g. Repeat steps a-f above for each additional fluorophore.
7. To edit the concentrations of the standards:
 - a. Click the fluorophore for the standards to be defined.
 - b. Click Dilution Series and calculate the concentration, or enter it directly in the spreadsheet.
8. Select the Units for the standards.

- Click Apply Plate Changes to make the changes. To see the effect on analysis, go to one of the other Data Analysis windows.



- The original plate setup is retained with the data file and may be restored at any time by clicking Restore Original Plate.



5.9.1 Edit Plate Sample Type Icons

There are six icon buttons running across the top of the representation of the experimental plate as shown below. These buttons are used to provide the two pieces of information required for each well: sample type and fluorophore(s) to be monitored. The active button is always surrounded by a red box.



Fig. 5.51. Sample type icons.

Beginning at the left, the arrow is the standard pointer. The next five icons define the type of sample in the well: Standard, Unknown, No-Template Controls, Positive Control, and Negative Control, respectively.

Section 6

Calibration for the MyiQ System

There are three calibrations required for the MyiQ system which must be done in the following order:

1. Mask Alignment
2. Background Calibration
3. Persistent Well Factor Generation

This order is the order that the Calibration tabs occur in the MyiQ Software (from left to right).

Choose Mask **Alignment** first, and then when you complete each calibration, choose the tab to the right of the completed calibration to do the next calibration.

Every experiment on the MyiQ System requires that the instrument be calibrated.

Calibration factors are specific for the optical path you are using which includes the Reaction vessel, sealing mechanism and volume used in the experiment you wish to run.

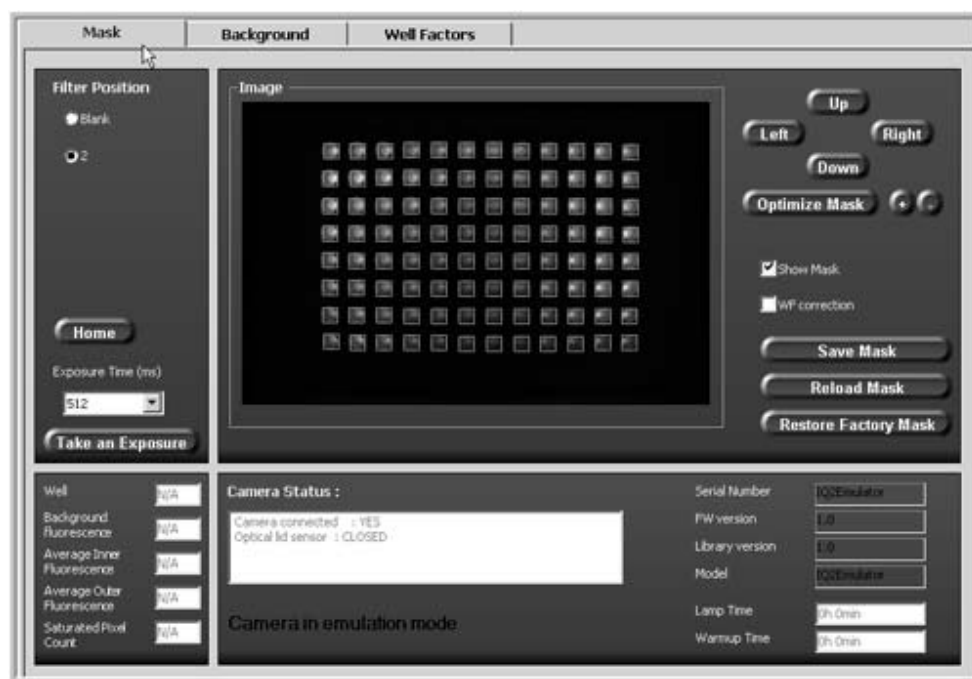


Fig. 6.1. The Mask window of the Calibration Module.

The main use of the Mask screen is to adjust the masks although there are other uses including capturing an image of the experimental plate to check the response of a probe or to assess the completion of a reaction. With a plate image you can obtain fluorescence readings for each of the 96 individual wells.

6.1 The Plate Image Screen

The Image screen displays the fluorescence detected by the CCD camera for each of the 96 wells on the plate. Pixels that are saturated will be shown in pink. The Masks are displayed as green boxes and each individual mask should be centered over the well so that all the fluorescent signal from the well falls within the mask.



Fig. 6.2 Mask Alignment on the Image Screen.

The masks can be displayed or hidden using the Show Mask button.

The masks can be moved as a group by clicking on the Up, Down, Left, or Right buttons. An individual well can also be moved by first clicking on that mask to select it, then using the Up, Down, Left, or Right buttons to move it.

The masks can be optimally positioned by clicking Optimize Mask. It can then be saved as the mask-data.xml file in the Mask folder of the MyiQ Folder by clicking Save Mask. The mask can be restored to the last saved mask by clicking Reload Mask or restored to the Factory mask by clicking Restore Factory Mask.

6.1.1 Filter Position

The Filter Position radio buttons are used to position the filter wheels. Home locates the filter wheel to the blank position.

The filters at each position are as follows:

- **Blank:** a non-transmissive blank filter
- **Position 2:** 485/20X 530/30M Fluorescein (FAM), SYBR Green I

The exposure time can be changed from this screen and an exposure taken from the MyiQ optics.



Fig. 6.3. Selecting Filter Position and Exposure Time.

6.1.2 Camera Status

The Camera Status screen provides feedback about the MyiQ Camera. In this screen you can determine:

1. If the camera is connected.
2. If the optical lid is closed.
3. The serial number, firmware version, library version and model of the camera.
4. The length of time the camera has been in use since it was installed.
5. The length of time the lamp has been on since the camera was last turned on.

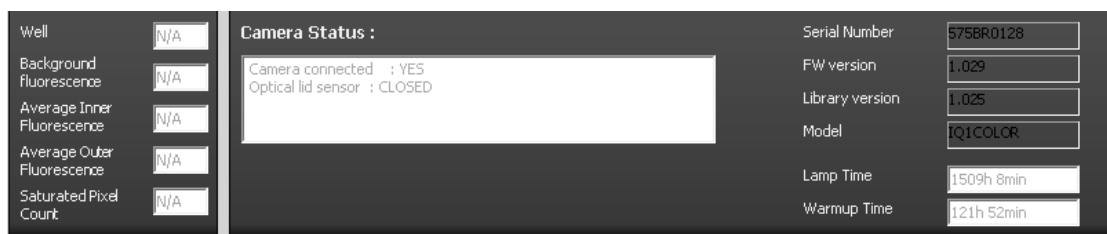


Fig. 6.4. The Camera Status window.

6.2 Calibration (Alignment) of the Mask

What You Will Need

You need the following to calibrate the MyiQ Camera:

1. External Well Factor Solution
2. 96-well PCR plate or preferred reaction vessel (referred to as 'plate').
3. Optical quality sealing tape or preferred sealing method.

6.2.1 Preparation of an External Well Factor Plate

To prepare an external well factor plate:

1. Dilute the 10x External Well Factor Solution to 1x with water.
2. In the 'plate' pipet equal volumes of the 1x external well factor solution into ALL 96 wells using the volume that you will be using in your experiment.
3. Seal the plate using the sealing method you will use in your experiment.

To align the mask:

1. Click Home.
2. Click the Filter Position 2 option button.
3. Click Take an Exposure.
4. Click Show Mask.
5. If any pink pixels are present in the mask, reduce the exposure time and retake an exposure. Keep retaking the exposure until no pink pixels are present.
6. Click Optimize Mask.
7. Click Save Mask.

6.3 Background Calibration

Background calibration is performed to account for fluorescence in the experimental system that is due to the reaction vessel and sealing mechanism. Open the Background window by clicking Background as shown below.



Fig. 6.5. The Background window.

What You Will Need

You need the following to calibrate the MyiQ Camera:

1. 96-well PCR plate or preferred reaction vessel (referred to as 'plate').
2. Optical quality sealing tape or preferred sealing method.

6.3.1 Preparation of an Background Calibration Plate

Prepare a background calibration plate by sealing the plate using the sealing method you will use in your experiment.

Background Calibration

To perform background calibration:

1. Select a well seal.
2. Select a vessel type.
3. Click Collect Background Factors.
4. When MyiQ completes the Background Calibration run, a dialog box appears with the message, "Background Calibration Run Complete."
5. Click OK to exit.

6.4 Well Factor Calibration

Well factors are used to compensate for any system or pipetting non-uniformity in order to optimize fluorescent data quality and analysis. Well factors may be collected directly from the experimental plate (dynamic well factors) or from an external well factor plate (persistent well factors).

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 be cycled for approximately five minutes extra.

Open the Well Factors window by clicking Well Factors as shown below. The Well Factors window is used to collect Persistent Well Factors.



Fig. 6.6. The Well Factors window.

6.4.1 Dynamic Well Factors

In order to collect Dynamic Well Factors one of the following must be true

1. All wells must have the same combination of fluorophores AND the same concentration of fluorophores.
2. No fluorophore is used both alone and in combination with other fluorophores, AND no fluorophore is used in more than one combination of fluorophores.

The collection of dynamic well factors is a completely automated process which begins as soon as the Begin Run button is clicked and the file name saved. When collecting dynamic well factors, the plate is held at 95°C for two and a half minutes prior to the first cycle with a setpoint of 90.0°C or higher. You may wish to adjust the dwell time/temperature for your first step of your thermal protocol accordingly.

6.4.2 Persistent Well Factors

Persistent well factors are not collected from the experimental plate but on a separate calibration plate containing the volume of 1x External Well Factor Solution in all 96 wells.

In addition the persistent well factors must be collected with the same reaction vessel and sealing mechanism as the experiment will be run with. Persistent Well Factors are stored in the Well Factors folder in the MyiQ Folder.

Persistent Well factor files can be viewed by selecting Calibration Data from the View menu.

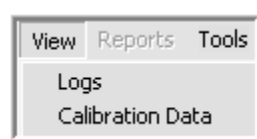


Fig. 6.7. The View menu for Calibration Data and System Log Information.

Files will be named:

Persistent_vessel_seal_volume_MyiQCameraSerial#_iCyclerSerial#

where:

- *vessel* is one of plates, (tube) strips, or tubes
- *seal* is one of film, flat caps, or domed caps

The plate setup used to run the experiment will have a reaction vessel, seal and volume associated with it. This plate setup will not be able to be run in an experiment unless it has the appropriate calibration files including a Persistent Well Factor calibration file.

In general persistent well factors can be used for about one month, but should be collected 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 may be used for any experimental run but there are situations in which they must be used.

6.4.3 Real-Time PCR Experiments using DNA Binding Dyes

In most real-time PCR experiments using DNA-binding dyes, like SYBR® Green I or ethidium bromide, dynamic well factors may not be collected. When the template DNA is denatured, the fluorescence of these dyes is not sufficiently high to calculate statistically valid well factors using the experimental plate.

There are three possible solutions to this problem:

1. Use iQ™SYBR® Green Supermix, which already includes fluorescein.
2. Use Persistent Well Factors.
3. For experiments with SYBR Green I, spike the master mix with fluorescein (the addition of fluorescein provides sufficient fluorescence at 95°C for the collection of well factors from the experimental plate while not interfering with the PCR reaction).

6.4.4 Spiking Real-Time PCR Experiments using DNA binding Dyes with Fluorescein

The Bio-Rad iQ SYBR Green Supermix is already 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 the experimental plate 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 in PCR buffer (10 mM Tris, pH 8.0, 50 mM KCl, 3 mM MgCl₂). Then add 10 µl of the 1 µM fluorescein with 990 ml of master mix to yield a final concentration of 10 nM fluorescein.

Section 7

Maintenance of the MyiQ Real-Time PCR Detection System

7.1 Cleaning the MyiQ Optical Unit

Take care not to spill liquids onto or into the iCycler thermal cycler or the MyiQ optical module. Cleaning may be done using a lint-free cloth or paper towel. The case may be cleaned using a soft, lint-free cloth and water.

7.2 MyiQ Filter Description and Installation Instructions

The filters designed for use in the MyiQ optics module are made of glass and mounted in plastic holders. The filter holders are held in either the excitation or emission filter wheel of the MyiQ optics module. The first position in each filter wheel is designated as the "home" position and must always contain an opaque filter blank. Filters can be removed for cleaning or replacement. If a filter shatters or breaks during the installation process, contact your local Bio-Rad office immediately for service, do not attempt to remove the broken components from the interior of the camera housing.

It is critically important that the excitation and emission filters are in the correct positions in the filter wheels, please confirm that the filters are in the proper location after cleaning or replacing filters in the MyiQ optics module. The table below summarizes the positions of the filter pairs, their optical characteristics, and the recommended fluorophores with which they are compatible.

Position	Excitation	Emission	Recommended Fluorophores
----------	------------	----------	--------------------------

- | | | | |
|-----|---------|---------|---------------------------------|
| • 1 | Blank | | |
| • 2 | 485/20X | 530/30M | Fluorescein (FAM), SYBR Green I |

Note: The filter designation 485/20X indicates that this filter will allow light between 475 and 495 nm to pass through. The first number, 485, indicates the center of the wavelength of light. The second number, 20 indicates the total breadth of wavelengths of light that can pass through it. The letter "X" indicates that the filter is specified for excitation only, and the letter "M" indicates emission only types of filters. Excitation and emission filters are not interchangeable.

7.2.1 Access the Existing Filters

To access the existing filters, proceed as follows:

1. Turn off the power to the optics module.
2. Reach behind the MyiQ optics module and unscrew the short fastener that secures the lid of the module in place.
3. Using gentle pressure and both hands, push inward on the rear vents located on the top half of the MyiQ casing. Lift upward to remove the cover of the optics module.
4. To access the excitation filter wheel, remove the black plug from the slot located near the lamp, at the right-hand side of the optics module. To access the emission filter wheel, remove the plug from the slot located at the top of the instrument.
5. Turn the filter wheels to the desired positions using the supplied ball-end hex driver. As long as the power to the optics module is off, the filter wheels may be turned freely in either direction.
6. To remove a filter, grasp it on both sides with the filter removal pliers and squeeze the tab in; gently pull the filter up and out.
7. To insert a filter, grasp the filter with the pliers and insert it into a vacant slot. For the excitation filters, the tab on the filter should face toward the front of the instrument. For the emission filters, the tab on the filter should face the right of the instrument.
8. After the filters or filter blanks have been inserted, replace the rubber plugs over the slots of the filter wheels.
9. Realign the tabs on the front end of the MyiQ optics module cover with the tabs on the main housing. Lower the cover until the top half of the camera housing snaps into place.
10. Replace the screw in the rear of the optics module to secure the casing.

7.2.2 Cleaning the Filters

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.

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 impair data quality. Contact Bio-Rad for replacement filters.

7.3 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 continuously on, we recommend the lamp 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. Installing a new lamp will alleviate this problem.

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

7.3.1 Lamp Replacement Procedure

1. Turn off the power to the optical module.
2. Reach behind the MyiQ optical module and unscrew the short fastener that secures the lid of the module in place.
3. Using a gentle pressure with both hands, push inward on the rear vents located on the top half of the MyiQ casing. Lift upward to remove the cover of the optical module.
4. Push up on the lamp spring clip to release the lamp from the bracket.
5. Lift the lamp out of the socket. The lamp is located on the right side of the optics module.
6. Install the new lamp using the reverse of steps 1–5. Hold the new lamp by the outer reflector and do not touch the bulb. Be sure the spring clip is down before inserting the lamp into the socket. Push the lamp firmly into the bracket, and then close the case and secure the lid.

Appendix A

Support

The MyiQ real-time PCR detection system is warranted against defects in materials and workmanship. For specific warranty information, contact your local Bio-Rad office.

If any defects should occur during the warranty period, Bio-Rad will replace the defective parts without charge. However, damage or defects resulting from any of the following causes are specifically excluded:

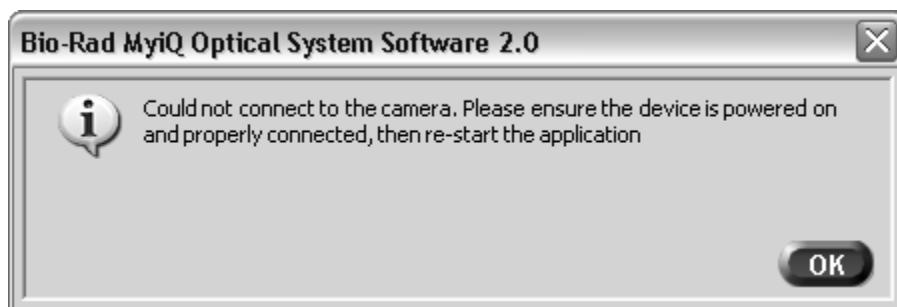
1. Improper operation.
2. Use of improper solvent or sample.
3. Use with tubes, plates, or sealing materials not specified by Bio-Rad Laboratories for use with the MyiQ real-time PCR detection system.
4. Deliberate or accidental misuse.
5. Repair or modifications done by anyone other than Bio-Rad Laboratories.
6. Natural disaster of any kind.

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 support, call your local Bio-Rad office, or in the US, call 1-800-4BIORAD (1-800-424-6723), or visit us on the Web at consult.bio-rad.com.

Appendix B Error Messages

B.1 Software Startup

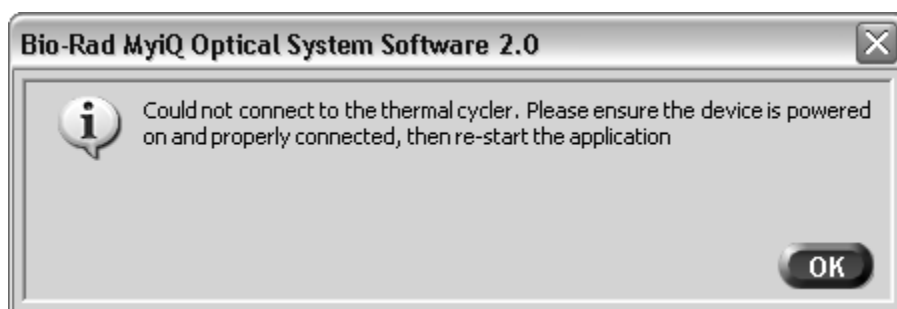


Cause: The software is unable to communicate with the camera.

Solution: Ensure that the camera is on.

Check that the USB cable is securely connected to both the camera and computer.

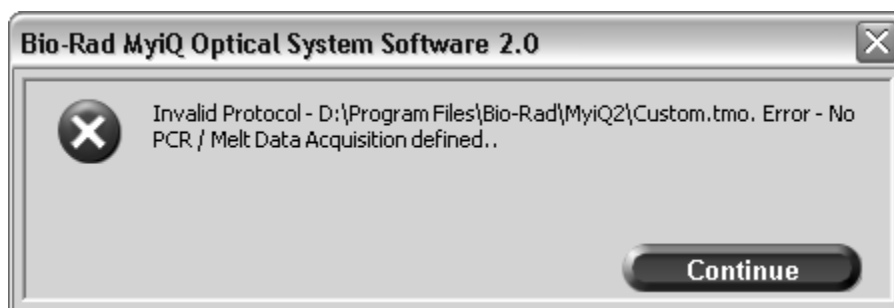
Ensure that the USB port in the computer is 2.0 High Speed.



Cause: The software is unable to communicate with the iCycler base.

Solution: Ensure that the iCycler base is turned on.

Check the serial cable connection between the iCycler base and the Camera.

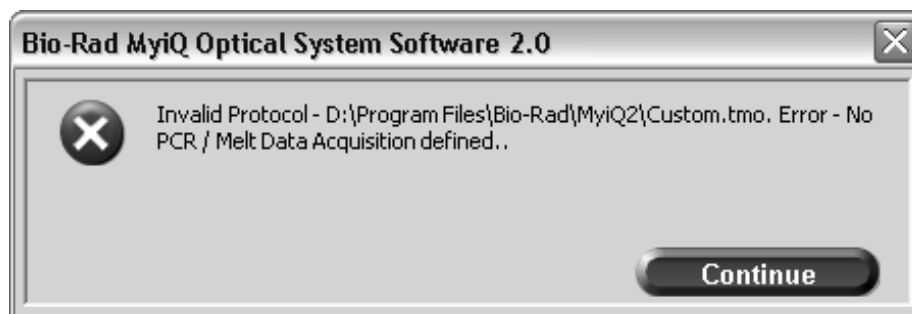


Cause: The MyiQ Software did not shutdown properly.

Solution: If the software had been controlling a run check the iCycler base to see if the run is still proceeding. If a run is still proceeding clicking Yes to this dialog box will abort the run, clicking No will allow the run to continue but will not re-start the software.

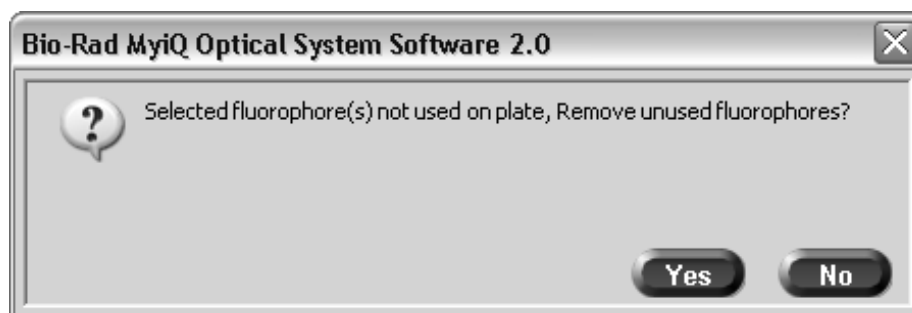
If the software is not connected to a camera, for example on a computer which is used only to analyze the data, clicking Yes will re-start the software.

B.2 Protocol and Plate Errors



Cause: There is no step defined for data collection or analysis in the thermal protocol. At least one data acquisition step must be present in the thermal protocol.

Solution: Specify a data collection at one step of the protocol. A data collection step is shown with a yellow or green camera icon at the appropriate step in the graphical display of the protocol.



Cause: You have selected fluorophores but have not defined any wells to contain these fluorophores.

Solution: This is a reminder to check your plate setup. Clicking No will return you to the Editing Plate window. Clicking Yes will remove the unused fluorophores and save the plate setup.

Appendix C

Product Information

Catalog # Description

Instruments

170-9770 MyiQ Real-Time PCR Detection System, includes MyiQ optical module, MyiQ optical system software on CD-ROM, preinstalled filter set, iCycler chassis, 96-well optical reaction block assembly, MyiQ calibration solutions, optical-quality 96-well PCR plates, communication cables, power cord, system accessories, instructions

Accessories

223-9441 96-well 200ml Thin Wall PCR Plates, 25 per box
223-9443 96-well 200ml PCR Plate Caps, for 223-9441, 300 per box
MSB-1001 Microseal B Optical Quality Adhesive Seal, 100 per package
170-8756 Replacement Halogen Lamp
170-9755 MyiQ 2.0 Software Installation Disk
170-8794 iCycler iQ External Well Factor Solution

Reagents

170-8860 iQ Supermix, 100 x 50 ml reactions, 2x mix
170-8862 iQ Supermix, 500 x 50 ml reactions, 2x mix
170-8880 iQ SYBR Green Supermix, 100 x 50 ml reactions, 2x mix
170-8882 iQ SYBR Green Supermix, 500 x 50 ml reactions, 2x mix
170-8890 iScript cDNA Synthesis Kit, 25 x 20 ml reactions, includes 5x iScript reaction mix, iScript enzyme, nuclease free water
170-8891 iScript cDNA Synthesis Kit, 100 x 20 ml reactions,
170-8896 iScript Select cDNA Synthesis Kit, 25 x 20 ml reactions, includes 5x iScript Select reaction mix, iScript reverse transcriptase, oligo(dT) mix, random primer mix, gene-specific primer (GSP) enhancer solution, nuclease free water
170-8897 iScript Select cDNA Synthesis Kit, 100 x 20 ml reactions



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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
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