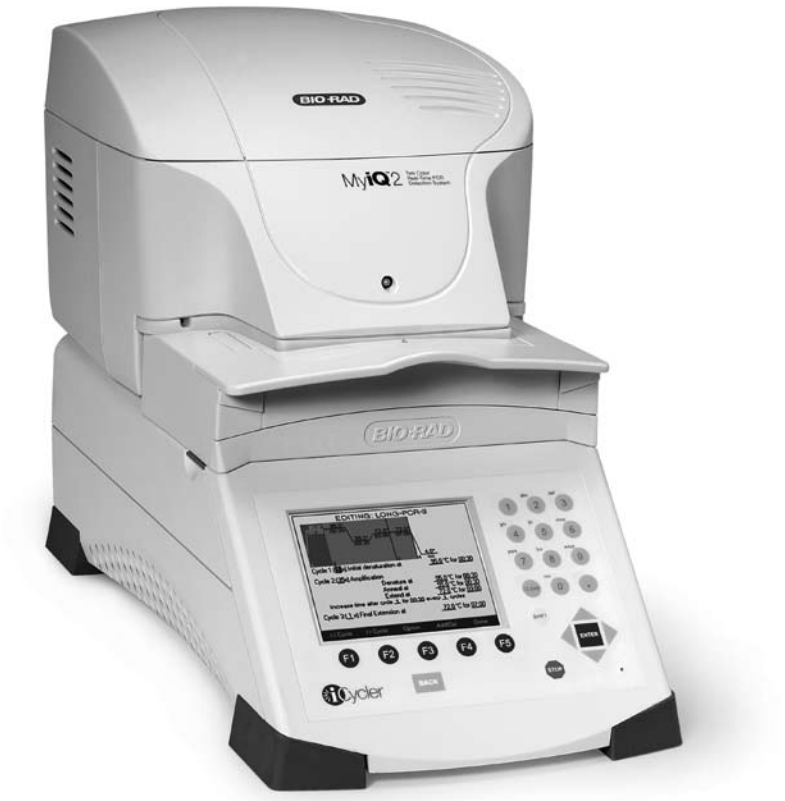
iQ™5 Optical System Software

Instruction Manual

Compatible with the MyiQ™2, MyiQ™, and iQ™5 real-time PCR detection systems



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Safety Information

Grounding

Always connect the MyiQ™2, MyiQ™, or iQ™5 optics module to a three-prong, grounded AC outlet using the AC power cord provided with the system. Do not use an adaptor 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 real-time PCR detection 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 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 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 real-time PCR detection system while the camera case is open. Do not open casing of the optics module when the instrument is in use.

Operating 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 MyiQ2, MyiQ, or iQ5 optics module. Do not block the fan vents near the lamp, as this may lead to improper operation or cause physical damage to the detector. Do not operate the optics module in extreme humidity (that is, greater than 90 percent) or where condensation can short internal electrical circuits or fog optical elements.

Notice

The MyiQ2, MyiQ, and iQ5 instruments are 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. These instruments should not be modified in any way. Alteration of the instruments 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 these instrument for purposes other than those for which they are intended, or by modifications to the instruments not performed by Bio-Rad or an authorized agent.

Table of Contents

Section 1. Getting Started

This section contains information on the following topics:

1.1 The MyiQ™2 Real-Time PCR Detection System

The MyiQ2 Real-Time PCR Detection System is the latest addition to a family of real-time PCR detection systems that are comprised of an optical detection module and iCycler® thermal cycler.

The two-color MyiQ™2 features a broad-spectrum light source (tungsten-halogen lamp) and paired filter optical design to ensure optimal excitation and emission. This results in excellent sensitivity and discrimination between multiple fluorophores. A CCD detector captures a simultaneous image of all 96 wells of the block resulting in a comprehensive data set illustrating the kinetic behavior of the data during each cycle.

The iQ™5 Optical System software (version 2.1) is used to control and collect and analyze data from the MyiQ2 Real-Time PCR Detection System. The software is also capable of controlling, and collecting and analyzing data from the single color MyiQ™ and five color iQ™5 Real-Time PCR detection systems. This manual provides instructions on use of the software applicable to each of these three systems. The online Help manual is available at all times by pressing the F1 key.

1.2 Setting Up the System Hardware

The MyiQ2, MyiQ, or iQ5 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. Handle the optics module and iCycler thermal cycler with care and with clean, dry hands during unpacking and assembly.

1.2.1 System Checklist

MyiQ2 Optics Module (catalog #170-9758)

- Optics module
- Power cord
- iQ5 optical system software installation disk (170-9753)
- Amplification tech notes CD (version 1.0)

iCycler Chassis (catalog #170-8701)

- Power cord
- iCycler chassis
- iCycler thermal cycler instruction manual

iQ5 Optical Accessory Kit (catalog #170-9752)

- 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

MyiQ2 Calibrator Dye Solution Kit (catalog #170-8791)

- 1x calibration dye solutions (4 dyes, 3 tubes of each dye)

External Well Factor Solution (catalog #170-8794)

- External well factor solution (5 tubes)

Sample Consumables

- PCR plates and sealers
- Reagents

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

Accessories

The following accessories are required to complete the installation:

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

1.2.2 Installing the Optical Reaction Module on the iCycler Chassis

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.

1.2.3 Installing the Support Bracket

A support bracket with roller is provided for the MyiQ2, MyiQ, or iQ5 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.

1.2.4 Installing the 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 has been installed, 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.

1.2.5 Connecting Power and Communication Cables to the System

Before connecting any communication or power cables to the system, confirm that the power switch for the iCycler thermal cycler and the 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, 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 optics module. This connection enables communication between the iQ5 software and the iCycler thermal cycler
    - USB port connector — Connect the supplied USB cable between the 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 optics module and the iCycler thermal cycler by the iQ5 Optical System software
    - At the right rear corner of the optical reaction block is a single connector the Positive docking connector — This self-aligning connector is secured into place when the optics

module is installed on the iCycler chassis. This connection senses when the lid handle is lifted.

1.3 Installing the iQ5 Software

Locate the software installation disk for iQ5 Optical System software version 2.1 provided with the MyiQ2 system. This installation disk is compatible with computers running the Windows XP and Windows Vista 32-bit operating system.

1. Insert the iQ5 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:\iQ5\Setup**, where X is the drive letter of the CD-ROM drive. For example, if the CD-ROM is the E drive, type E:\iQ5\Setup.
3. Follow all screen prompts to finalize the installation. Certain configurations of Windows operating systems 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/iQ5 folder so that you can save protocol, plate setup, and data files.
4. If you still cannot write to the folders beneath the Program Files/Bio-Rad/iQ5 folder after changing the protection on either of these folders, check the properties of each folder. Specifically, in the Properties window Securities tab, ensure that the checkbox that allows inheritable permissions to propagate to that folder has been selected.
5. Confirm that the iQ5 system software is working properly by double-clicking on the shortcut icon located on the Windows desktop, or by selecting the iQ5 program icon from the Bio-Rad folder in the Windows Start menu.

1.3.1 Installing the Camera Drivers

Before using the real-time PCR detection system for the first time the camera drivers must be installed.

1. Power on the iCycler thermal cycler and the optics module.
2. Windows will display a "Found New Hardware Wizard" dialog box. To install the camera drivers, select the option to **Install from a list or specific location**. Click **Next** to continue.
3. In the new window that appears, select **Search for the best driver in these locations**, and then click the **Include this location in the search** checkbox. Use the browse button to navigate to the "iQ5\Drivers" folder. The default location of the drivers is: C:\Program Files\Bio-Rad\iQ5\Drivers. Click Next to continue.
4. Next Windows will display a Hardware Installation dialog box regarding Windows Logo Testing, a service offered by Microsoft. To complete driver installation for the iQ5 or MyiQ systems, click **Continue Anyway**.

1.4 Recommended Computer Settings

For the MyiQ2, MyiQ and iQ5 systems to communicate properly with the iQ5 Optical System software, version 2.1, the computer settings should be set as described below.

Computer Power Management Settings on Windows Vista

1. From the Start menu, choose Control Panel. Switch the Control Panel view to Classic View.
2. Choose Power options, and then choose Create a Power Plan.
3. Choose the High Performance plan, then enter the Plan name iQ5 software, and press Next.
4. Change the Turn Off The Display and Put the Computer to Sleep options to "Never".
5. Click Save Changes.
6. Select your new iQ5 software power plan by clicking on the radio button, open Change Advanced Power Settings to set the following conditions.
    a. Adjust settings to turn off hard disk after 180 minutes.
    b. For Sleep Settings:
        i. Set Sleep After setting to "Never"
        ii. Set Hybrid Sleep setting to "On"
        iii. Set Hibernate After setting to "Never"
    c. For USB Settings, set USB Selective Suspend setting to "Disabled".
    d. Set Search and Indexing, Power Savings Mode setting to "Balanced",
    e. Set Display, Turn Display Off After setting to "Never".

    When completed, click Apply and then Done.

Computer Power Management Settings on Windows XP

1. From the Start menu, choose Settings, then Control Panel.
2. Choose Power Options, the Power Options Properties window will open.
3. On the Power Schemes tab, set all power scheme settings to **Never**.
4. On the Hibernate tab, deselect the **Enable Hibernation** checkbox.
5. Return to the Control Panel window, and select **Display**; the Display Properties window will open.
6. On the Screen Saver tab, select **None**.
7. Return to the Control Panel window, and select System, the System Properties window will open.
8. On the Hardware tab, click on the Device Manager button to open the Device Manager file tree. Click on the "+" button to expand the Universal Serial Bus Controllers list.
9. Scroll down and highlight the first listed USB Root Hub. Right-click and select the Properties option to open the USB Root Hub Properties window.
10. Select the Power Management tab, and deselect the Allow the Computer to Turn Off This Device to Save Power checkbox.
11. Repeat steps 9–10 for each individual USB root hub listed.

1.5 Calibrating the Instrument

Before using the real-time PCR detection system for the first time, mask alignment, background calibration, persistent well factor collection, and pure dye calibration (for MyiQ2 and iQ5 systems only) must be performed. See section 7, Calibrating the Instrument, for detailed instructions on calibration.

1.6 Compatibility with Earlier Versions of the iQ5 Optical System Software

Data Files

Protocol, plate setup, and data files generated or saved with version 3.0 or 3.1 of the iQ Real-Time Detection System software and earlier versions of the iQ5 software are recognized by version 2.1 of the iQ5 software. These .tmo, .pts, and .opd files can be opened from within iQ5 software for analysis.

To open or import files generated by older versions of the iQ Real-Tme Detection System software (version 2.3b and earlier), you must open the file in version 3.0 or 3.1 of the iQ software first and then save the file again.

Calibration Files

Earlier versions of the iQ5 Optical System software (earlier than version 2.1) have volume-dependent calibration files for both persistent well factors and pure dye calibration collection. In version 2.1, there is no volume dependency for these calibration files. If a computer is upgraded from an earlier version of the iQ5 Optical System software to version 2.1, existing calibration files will be upgraded to remove calibration volume dependency.

For example, **Persistent_Plates_Film_25ul_IQ2Emulator_IQ2Emulator.xml** will be upgraded and renamed as **Persistent_Plates_Film_IQ2Emulator_IQ2Emulator.xml.**

In all cases, the original calibration files with the volume dependency will be backed up to the backup folder after upgrading.

Section 2. Quick Guides

This section contains quick guides for the following topics:

The fundamental steps of running a real-time PCR experiment on the MyiQ2, MyiQ, or iQ5 Real-Time Detection System are the following:

1. Select, Edit, or Create a Protocol.
2. Select, Edit, or Create a Plate Setup.
3. Click Run
4. Select the appropriate Well Factor option and begin the run
5. Analyze the data

The quick guides below outline key procedures from each step. These quick guides are also located in the online help of the iQ5 Optical System software.

For more complete information regarding the procedures and features of the iQ5 Optical System software, refer to the relevant sections of this user manual.

2.1 Protocol Quick Guide

Within the Workshop module click the Protocol button.

Selecting a Protocol

1. Navigate to your desired protocol file using the file tree browser.
2. Click the protocol name once. The selected protocol appears in the Selected Protocol pane.
   NOTE: The iQ5 software has a number of sample protocol files which may be used.

Editing or Creating a Protocol

Edit the selected protocol by clicking **Edit** in the Selected Protocol window, or 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 pane opens the Protocol Editor.

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

1. Edit the protocol by performing one or more of the following five tasks:
    - **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 a colon (:), then type 10 (that is, as the time appears in the spreadsheet). Alternatively, 10 seconds can be entered as 0.10.
    - **Set Data Acquisition Step:** 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.
    - **Insert a cycle:** Insert a cycle by clicking in the Insert column within the cycle row. Cycles have a blue background. The iQ5 software inserts the new cycle below the current cycle
    - **Insert a step:** Insert a step by clicking in the Insert column within a step row. Steps have a white background. The iQ5 software inserts the new step below the current cycle. 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 the iQ5 software will insert when you insert a cycle
    - **Delete a cycle:** Delete a cycle by clicking in the Delete column within a cycle row. Cycles are indicated with a blue background.
    - **Delete a step:** Delete a step by clicking in the Delete column within a step row. Steps are indicated with a white background
2. Save the protocol by clicking **Save & Exit Protocol Editing**. Enter the name of the protocol in the **Save As** dialog box, and then click **Save**.

2.2 Plate Setup Quick Guide

Within the Workshop module:

1. Open the Plate Setup Editor Window using one of the following methods:
    - Click **Create New** in the plate setup display pane to enter the Plate Setup Editor

      or
    - Click **Plate** and select the desired plate setup file from the file tree directory. Double-click the file name to go directly to the Plate Setup Editor

      or
    - Click **Plate** and select the desired plate setup file from the file tree directory. Click the file name to open the plate setup in the bottom right section of the Workshop

window. Click **Edit** to open the plate setup in the Plate Setup Editor

or

- Click **Data File** and select the desired data setup file from the file tree directory. Click the file name to open the plate setup associated with the data file in the bottom right section 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.
6. For most experiments, leave the **Whole Plate Loading** box checked. With Whole Plate Loading, changes made to any fluorophore within a well are extended to the other fluorophores within the well and the replicate group. If you are editing a plate, the Whole Plate Loading checkbox may be unavailable because it is not appropriate based on the current definition of the plate.
7. Click a sample type icon.
8. Select the type of replicate loading desired.
9. Click a fluorophore.
10. Click or drag across the plate to define wells with the selected fluorophore and sample type.
11. Continue defining the remaining wells that will contain the first fluorophore by changing to any other sample type icons required.

    To calculate standard concentrations automatically, click **Dilution Series** and enter the upper or lower concentrations, and units of the standards, set the dilution factor, and click **Apply Dilution Series**.
12. Repeat steps 7–11 for any additional dye layers/fluorophores as required. Remember that if the Whole Plate Loading box is checked, changes made in standard concentrations will be applied to all dye layers for that well and extended to all replicates in the group.

    NOTE: To delete a previously defined well, click the Delete All icon, and then click the well. To delete the selected fluorophore from a previously defined well, click the Delete Fluorophore icon, and then click the well.
13. Click **Save & Exit Plate Editing**.

2.3 Running a Real-Time Experiment Quick Guide

After you click Run in the Workshop module, the iQ5 software opens the Initiate Run tab within the Run-Time Central module.

2.3.1 Beginning a Run

To begin a run:

1. Insert the experimental plate into the iCycler reaction module.

2. From the Workshop Setup window, select your desired Plate (.pts) and Protocol (.tmo) file individually, or your desired Run Set (.run).
3. Click **Run**.
4. Check that the desired Protocol and Plate Setup are displayed in the bottom half of the Initiate Run screen.
5. Select the type of well factors to use by selecting either:
   - Use Persistent Well Factors

     or
   - Collect Well Factors from Experimental Plate
6. Click **Begin Run**.
7. Name the file with a unique name in the **Save Optical Data File** dialog box.
8. Click **OK**.

2.3.2 Monitoring the Run

When the real-time PCR detection system begins the run, the iQ5 software 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. You can choose between displaying the data in the Data Analysis module or returning to the Workshop module.

Click **Yes** to proceed to the Data Analysis module.

2.4 Data Analysis Quick Guides

When the iQ5 software opens, the Data Analysis module is grayed out and inactive. To analyze a data file, click the Data File tab of the Workshop module, select the data file, and then click Analyze.

The Data Analysis module consists of six tabs:

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

2.4.1 PCR Quant Tab Quick Guide

Use the PCR Quant tab to set the analysis conditions for the data file including setting the PCR baseline, setting the threshold and determining which wells to exclude or include in the experiment.

To analyze a data file:

1. Click the Data File tab in the Workshop module.

2. Select a data file from the file tree browser, and then click Analyze. The file opens in the PCR Quant tab within the Data Analysis module.
3. Select or deselect wells to be included in the analysis by selecting **Analyze Wells**. Select or deselect wells to be displayed by selecting **Display Wells**.
4. The iQ5 software automatically chooses the data analysis conditions including baselines and thresholds. 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 threshold by clicking and dragging the green threshold line.
6. Make any manual adjustments to the baseline by right-clicking on the amplification traces plot to access the baseline threshold parameters popup window and editing User Defined options.

   NOTE: You can revert to software auto calculated threshold and baseline by right-clicking on the amplification traces plot to access the baseline threshold parameters popup window and selecting **Auto Calculated**.

2.4.2 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 End Point tab in the Data Analysis module for an existing data file

Initiating an End Point Run

1. Insert the experimental plate into the iCycler reaction module.
2. From the Workshop Setup Window Select or Create the Plate Setup from the Plate Setup tab.
3. Click Run End Point.
4. Check that the desired Protocol and Plate Setup are displayed in the bottom half of the Initiate Run screen.
5. 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.
6. Name the file with a unique name in the Save Optical Data File dialog box, and then click Save.

End Point Analysis of an Newly Completed End Point Run

1. Once the real-time PCR detection system completes the run, the End Point tab is displayed.
2. Make selections for the following parameters:
   - Method — Use Negatives to differentiate samples that do not amplify the target sequence from those that do amplify the target sequence.
   - End Point Tolerance and Tolerance Parameter.

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

End Point Analysis of an Existing Data File

1. Within the Workshop module click **Data** and select your desired data file using the file tree browser.
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 Analyze Wells. 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.

2.4.3 Allelic Discrimination Quick Guide – For Multiplex Data Only

The Allelic Discrimination feature of the Data Analysis module is available post-run and offers flexibility for analyzing allelic discrimination data from multiplex PCR experiments. You can display samples on a scatter plot based on threshold cycle or relative fluorescence units (RFU) values at any PCR cycle. You can have the iQ5 software automatically make allele calls, or you may manually make the allele calls.

To analyze an allelic discrimination file:

1. Within the Workshop module, click Data, and select your desired data file using the file tree browser
2. Click Analyze.
3. The file opens in the PCR Quant tab of the Data Analysis module. The iQ5 software automatically chooses the Data Analysis conditions. To manually adjust these conditions, refer to the PCR Quant Tab.
4. You can select or deselect wells that will be included in the analysis by selecting the Analyze Wells checkbox.
5. Click the Allelic Disc tab in the Data Analysis module.
6. Select the Fluorophores that represent Allele1 and Allele2 in the Assign Fluorophores area.

7. Choose between the Threshold Cycle and RFU display modes in the Display Mode area to display the allelic discrimination data.
   - Threshold Cycle displays the distribution of samples on the scatter plot based on the threshold cycle (C_T). Samples that do not cross threshold will be assigned the C_T value of the last cycle run in the experiment.
   - RFU displays the distribution of samples on the scatter plot based on the RFU generated by each sample at the last PCR cycle number. Click the drop-down list next to the Select Cycle box to generate the scatter plot based on an RFU from a different cycle of the PCR experiment.
8. Click Automatic Call or Manual Call. Automatic Call is the default parameter.

   In Automatic Call: threshold bars are positioned automatically in one of two ways:
   - Based on distribution of the control wells, when at least three wells have been assigned to Control 1 and three to Control 2
   - At 90 percent of the C_T range or 10 percent of the RFU range on each axis if no controls are named

   Manual Call is the alternative analysis mode. Adjustments may be made either in the scatter plot or in the data spreadsheet:
   - Scatter plot: Make a selection in the Allele Call box. Then click and drag the cursor over the corresponding sample(s) in the scatter plot. The iQ5 software reassigns the samples to the allele call selected by the radio button, and updates the data spreadsheet accordingly
   - Data spreadsheet: Click in the Call cell of the spreadsheet. A menu appears that lets you select an allele call for that sample. Once you select an allele call, the scatter plot reflects the change

     NOTE: You may click and drag threshold bars directly on the plot to adjust Automatic Call assignment.
9. Click **Reports** in the menu to obtain customized reports for the allelic discrimination data.

2.4.4 Gene Expression Quick Guide

The iQ5 software can present expression data normalized to one or more reference genes, or, for data normalized before PCR, as a relative quantity.

Calculating Relative Quantity (dC_T, ΔC_T)

To calculate Relative Quantity (dC_T):

In the PCR Quant screen (with a .opd data file open), assess Threshold and Baseline information for the data file and make changes if necessary.

1. Click the **Gene Expr** tab.
2. Make any changes to Gene and Condition (for example, Sample and Treatment) assignments in the Gene Expression Plate interface.
   - Expand the Gene Expression Plate Interface view by clicking on the "+" button to make well identification and selection easier. Highlight the wells in the gene expression plate interface you wish to edit

- Change the gene assignments in these wells by typing your desired name into the gene pull-down men, then click enter to apply the name to the selected wells
- Change the condition assignments in these wells by typing your desired name into the condition pull-down menu, then click enter to apply the name to the selected wells

  NOTE: The Gene and Condition Names have a character entry limit of 15 characters
- Minimize the Gene Expression Plate interface by clicking on the "-" button to return to the standard view of the Gene Expr tab window

3. Click **Relative Quantity (dCt)**.
4. Click **Recalculate** to see your results.
5. Relative Quantity results are graphed. The Data Table spreadsheet is accessed by clicking **Data Table**. The Data Table spreadsheet lists the Condition and Gene name, calculated expression values and C_T values. Right-click to print or export this data to Excel.

   NOTE: Click the Settings tab, then select Gene List to enter a specific user-defined gene reaction efficiency.

   NOTE: Click the Settings tab, then select Condition List to select a particular sample as a control sample.

If you need to compare these data to results obtained in other .opd files, you will need to enable this file for Multi-file Gene Expression analysis, also called a Gene Study. To enable your file for Gene Study:

1. Click the Enable for Gene Study button.
2. Go to the File menu to save your file.

Calculating Normalized Expression (ddC_T, $\Delta\Delta C_T$)

To calculate Normalized Expression (ddC_T):

1. In the PCR Quant screen (with a .opd data file open), assess Threshold and Baseline information for the data file and make changes if necessary.
2. Click the **Gene Expr** tab.
3. Make any changes to Gene and Condition (for example, Sample and Treatment) assignments in the Gene Expression Plate interface.
   - Expand the Gene Expression Plate Interface view by clicking on the "+" button to make well identification and selection easier. Highlight the wells in the gene expression plate interface you wish to edit
   - Change the gene assignments in these wells by typing your desired name into the gene pull-down men, then click enter to apply the name to the selected wells
   - Change the condition assignments in these wells by typing your desired name into the condition pull-down menu, then click enter to apply the name to the selected wells

     NOTE: The Gene and Condition Names have a character entry limit of 15 characters

- Minimize the Gene Expression Plate interface by clicking on the "–" button to return to the standard view of the Gene Expr tab window

4. Click the **Settings** tab, then select **Gene List** to set your desired reference gene(s).
5. Click **Normalized Expression (ddCt)**.
6. Click Recalculate to see your results.
7. Normalized expression results are graphed. The Data Table spreadsheet is accessed by clicking Data Table. The Data Table spreadsheet lists the Condition and Gene name, calculated expression values and C_T values. Right-click to print or export this data to Excel.

   NOTE: Use the **Settings** tab, then select **Gene List** to enter a specific user-defined gene reaction efficiency. Use the **Settings** tab, then select **Condition List** to select a particular sample as a control sample.

If you need to compare these data to results obtained in other .opd files, you will need to enable this file for Multi-file Gene Expression analysis, also called a Gene Study. To enable your file for Gene Study:

1. Click the Enable for Gene Study button.
2. Go to the File menu to save your file.

Gene Study (Multiple File Gene Expression) Quick Guide

To create a Gene Study (for analysis of multiple .opd files):

1. For each .opd file you wish to combine to a Gene Study.
   a. In the PCR Quant screen (with a .opd data file open), assess Threshold and Baseline information for the data file and make changes if necessary.
   b. Click the **Gene Expr** tab.
   c. Make sure all files to be included in the Gene Study have the Enable for Gene Study button active.
   d. Save each individual file.
2. From the menu toolbar select File/New/Gene Study.
3. In the Gene Expression Study Manager select Add .opd's.
4. Select the files that you wish to add to the Gene Study. More than one file can be selected.
5. Once the files are added to the Gene Expression Study Manger, select OK.
6. Make any changes to Gene and Condition (for example, Sample and Treatment) assignments in the Gene Expression Plate interface.
   - Expand the Gene Expression Plate interface view by clicking on the "+" button to make well identification and selection easier. Highlight the wells in the gene expression plate interface you wish to edit
   - Change the gene assignments in these wells by typing your desired name into the gene pull-down men, then click enter to apply the name to the selected wells

- Change the condition assignments in these wells by typing your desired name into the condition pull-down menu, then click enter to apply the name to the selected wells

  NOTE: The Gene and Condition Names have a character entry limit of 15 characters.

- Minimize the Gene Expression Plate interface by clicking on the "–" button to return to the standard view of the Gene Expr tab window

7. Select either **Normalized expression (ddCt)** or **Relative quantity (dCt)**.

   NOTE: For Normalized Expression analysis you must first assign reference gene(s). Click the Settings tab, then select Gene List spreadsheet to set your desired reference gene(s).

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

9. Normalized Expression results are graphed. The Data Table spreadsheet is accessed by clicking **Data Table**. The Data Table spreadsheet lists the Condition and Gene name, calculated expression values and C_T values. Right-click on the spreadsheet to print or export this data to Excel.

   NOTE: Click the Settings tab, then select Gene List to enter a specific user-defined gene reaction efficiency.

   NOTE: Use the Settings tab, then select Condition List to select a particular sample as a control sample.

2.4.5 Post-Run Plate Setup Editing Quick Guide

For post-run editing of the Plate Setup saved within a data file:

1. From the Workshop module:

   - 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

   - 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 section 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 will open. In this modified window, you cannot add fluorophores to or remove fluorophores from the fluorophore list. Nor may you add a previously defined fluorophore or remove a previously defined fluorophore from a well.

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. For most experiments the Whole Plate Loading box will be checked. With Whole Plate Loading, changes made to any fluorophore within a well are extended to the other fluorophores within the well and within the replicate group. If you are editing a plate, the Whole Plate Loading checkbox may be unavailable because it is not appropriate based on the current definition of the plate.

7. Click a fluorophore.

8. Click a sample type icon.
9. Select the type of replicate loading desired.
10. Click or drag across the plate to define wells with the selected fluorophore and sample type.
11. Continue defining the remaining wells that will contain the first fluorophore by changing to other sample type icons as appropriate.

    To calculate standard concentrations automatically, click Dilution Series and enter the upper or lower concentrations and units of the standards, set the dilution factor, and click Apply Dilution Series.
12. Repeat steps 7–11 for any additional dye layers/fluorophores as required. Remember that if the Whole Plate Loading box is checked, changes made in standard concentrations will be applied to all dye layers for that well and extended to all replicates in the group.
13. Click Apply Plate Changes to make the changes. To see the effect on analysis, go to one of the other Data Analysis windows.

    NOTE: To delete a previously defined well, click the Delete All icon, and then click the well. To delete the selected fluorophore from a previously defined well, click the Delete Fluorophore icon, and then click the well.

    NOTE: Use the **Next #** checkbox to enter a particular number to assign to the next standard or sample you define.

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

Section 3. Introduction to the iQ5 Optical System Software (Version 2.1)

The iQ5 software is divided into five 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 an orange background, whereas unselected modules have a gray background. Each module is subdivided into windows that perform a specific function for that module.

The five modules in the iQ5 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 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, Allelic Discrimination, 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 MyiQ2, MyiQ or iQ5 systems must be calibrated. These simple and easily-performed calibration routines are accessed in the Calibration module. There are calibration routines for Pure Dyes, Mask Alignment, Background, and Well Factors collection.

User Profile Module: This module can be used by the Administrator to add new users and to set users access/restrictions to various functions of the iQ5 software.

All users can use this module to set their personal preferences for the iQ5 software.

Section 4. Workshop Module

This section contains information on the following topics:

The Workshop module consists of the **Setup** and **Plate Summary** tabs. For detailed information on the information contained in the Plate Summary tab, refer to section 4.2.10

4.1 Setup Tab

The Setup tab window (Figure 4.1) consists of four sections.

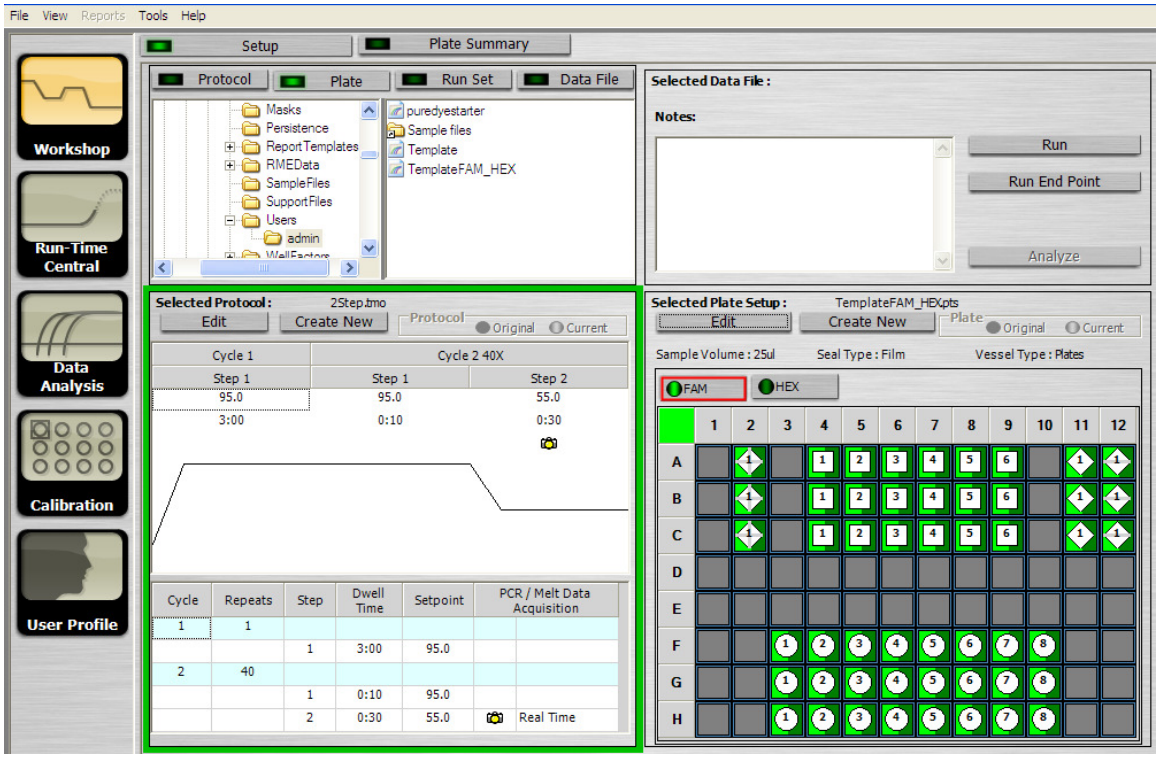


Fig. 4.1. The Setup tab.

4.1.1 File Browser Section

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.

The files displayed in the list depend upon the selected Setup window tab. With the Setup tab active, the displayed Setup window contains four additional tabs:

- **Protocol:** Used to select, edit, or create a protocol for running a real-time experiment. If the Protocol tab is selected, the iQ5 software displays protocol files, file extension .tmo. The selected protocol appears in the Selected Protocol window
- **Plate:** Used to select, edit, or create a plate setup for running a real-time experiment. If the Plate tab is selected, the iQ5 software displays plate setup files, file extension .pts. The selected plate appears in the Selected Plate Setup window
- **Run Set:** Consists of a linked protocol and plate setup, useful when you repeat the same experiment on a regular basis. If the Run Set tab is selected, the iQ5 software displays run set files with the extension .run. The selected run set, which is a linked protocol and plate setup, is displayed in the Selected Protocol and Selected Plate Setup windows
- **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 or Current (last saved) plate setup. If the Data File tab is selected, the iQ5 software displays data files created from previous experimental runs, which have the file extension .opd. The iQ5 software 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. The iQ5 software displays the protocol and plate setup used in creating this data file in the Selected Protocol and Selected Plate Setup windows, respectively

4.1.2 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 a data file is selected, the iQ5 software displays the protocol used to create the data file and the data file name. The selected protocol is displayed both graphically and in spreadsheet format.

4.1.3 Selected Plate Setup Area

Located in the bottom right of the Setup window, the Selected Plate Setup window (Figure 4.2) 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 the iQ5 software.

When you select the Data File tab and a data file, the iQ5 software 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. 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.

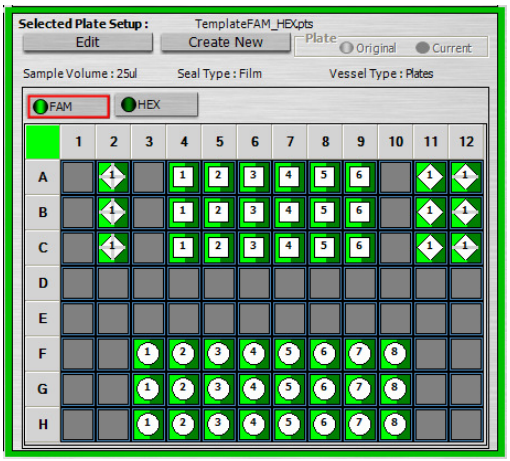


Fig. 4.2. The Selected Plate Setup Area.

4.1.4 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.
- **Begin 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.

4.2 Plate Setup

The MyiQ2, MyiQ, and iQ5 Real-Time PCR Detection systems only display and analyze data from wells defined in plate setup as containing sample and at least one fluorophore. In the Plate Setup Editor window, you specify the type of sample and the fluorophores present in each well.

The Plate Setup Editor window is accessed from the main Workshop module window by clicking on either Edit or Create New in the Selected Plate Setup window (see Figure 4.1), or by double-clicking on an existing plate setup file from the file browser folder tree.

4.2.1 Plate Setup Editor Window

The Plate Setup Editor window (Figure 4.3) 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 fluorophore for any individual well.

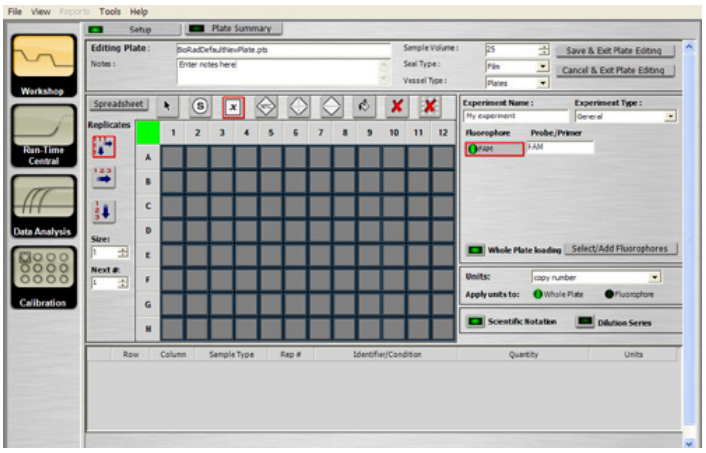


Fig. 4.3. The Plate Setup Editor Window.

Exiting the Plate Setup Editor
You can return to the Workshop at any time by pressing Cancel & Exit Plate Editing. Any current changes made since the file was last saved will be lost.

To save changes before returning to the home screen, click Save & Exit Plate Editing 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 earlier versions of the iQ5, iQ, or MyiQ software can be opened iQ5 Optical System software version 2.1, but before they can be saved, you must assign a vessel type and sealing mechanism if not previously defined.

4.2.2 Well Definition Icons

Fig. 4.4. The Well Definition toolbar.

There are nine icon running across the top of the representation of the experimental plate (Figure 4.4). 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** for selecting, but not altering, wells.

Defines **Standards**

Defines **Unknowns**

Defines **No-Template Controls**

Defines **Positive Controls**

Paint Bucket icon: When the Paint Bucket icon is active and you click a well in the plate setup, all wells in that replicate group will be assigned the currently selected fluorophore.

Delete Fluorophore icon: When the Delete Fluorophore icon is active and you click in a well in the plate setup, the currently selected fluorophore is removed from all wells in that replicate group. It does not change the definition of the sample type in that well.

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

4.2.3 Editing or Creating a New Plate Setup

1. Open the Plate Setup Editor Window within the Workshop module by using one of the following methods:
   - Click Create New in the plate setup display pane to enter the Plate Setup Editor

     or
   - Click Plate. Select the desired plate setup file from the file tree directory. Double-click the file name to go directly to the Plate Setup Editor

     or
   - Click Plate. Select the desired plate setup file from the file tree directory. Click the file name to open the plate setup in the bottom right section of the Workshop window. Click Edit to open the plate setup in the Plate Setup Editor

     or
   - Click Data File. Select the desired data setup file from the file tree directory. Click the file name to open the plate setup associated with the data file in the bottom right section 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.
6. For most experiments leave the Whole Plate Loading box checked.
   - With Whole Plate Loading, changes made to any fluorophore within a well are extended to the other fluorophores within the well and within the replicate group.
   - When Whole Plate Loading is deselected, no assumption is made about the sample type in the second and subsequent fluorophores. The sample type can vary from fluorophore to fluorophore and if there are standards you can define individual dilution series for individual fluorophores.
7. Click a sample type icon.
8. Select the type of replicate loading desired.

9. Click a fluorophore.
10. Click or drag across the plate to define wells with the selected fluorophore and sample type.
11. Continue defining the remaining wells that will contain the first fluorophore by changing to any other sample type icons required.

    To calculate standard concentrations automatically, click Dilution Series and enter the upper or lower concentrations and units of the standards, set the dilution factor, and click Apply Dilution Series.
12. Repeat steps 7–11 for any additional dye layers/fluorophores as required. Remember that if the Whole Plate Loading box is checked, changes made in standard concentrations will be applied to all dye layers for that well and extended to all replicates in the group.

    NOTE: To delete a previously defined well, click the Delete All icon, and then click the well. To delete the selected fluorophore from a previously defined well, click the Delete Fluorophore icon, and then click the well.
13. Click Save & Exit Plate Editing. The extension .pts will be added automatically

    NOTE: Selecting or creating plate setup files that contain fluorophores not compatible with the connected instrument will result in a warning message displayed at the bottom of the screen (Figure 4.5). This message indicates that while the plate setup can be viewed and edited, the plate setup contains fluorophores that cannot be used to collect new data using this plate setup.

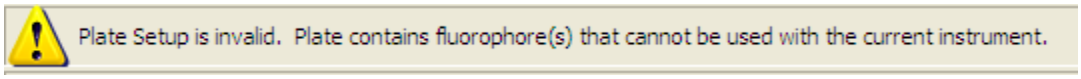


Fig. 4.5. Invalid Plate Setup Error.

4.2.4 Fluorophore Selection

When using the MyiQ2, MyiQ, and iQ5 systems, you may specify as many as five different fluorophores on a single plate provided they are read through the filters available on the respective systems. Refer to section 9.2.1 for information about system filter specifications and recommended fluorophores.

With the iQ5 software, as many as five different sets of wells can be read with the same filter pair (that is, in the same fluorophore) as shown in Figure 4.6. For example, you may have five different experiments on the same plate, all using SYBR® Green, and each experiment will be treated independently.

NOTE: The software cannot distinguish between fluorophores read by the same filter pair in the same well; therefore, in all cases, such dyes have to be in separate wells. For example, you may not put FAM and SYBR® Green in the same well.

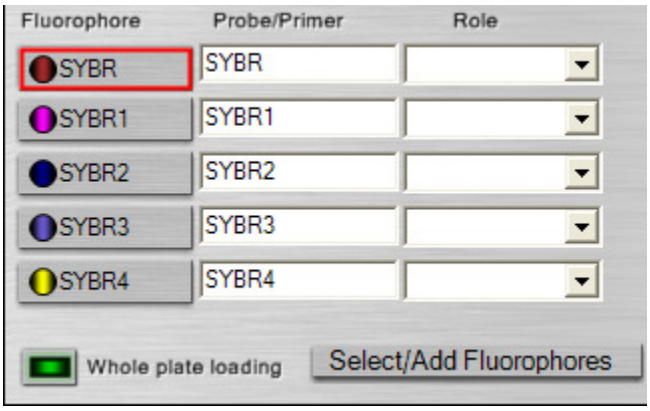


Fig. 4.6. List of Five Sets of Wells.

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

To change the default color associated with a fluorophore, click the color box on the row with the fluorophore name and choose a new color as shown below in Figure 4.7.

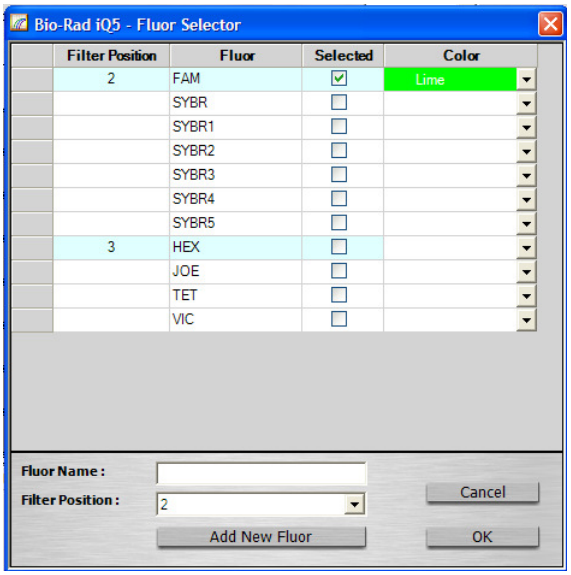


Fig. 4.7. The Fluor Selector Window for the MyiQ2 Real-Time PCR Detection System.

The iQ5 Optical System software automatically recognizes which instrument is detected and displays only the fluorophores appropriate to that system for selection. Figure 4.7 illustrates the fluor selector window when connected to a MyiQ2 instrument.

You may add new dyes to the list at any time by clicking Add New Fluor. When you add a new dye, you must specify the filter to be used to monitor that fluorophore and perform a pure dye calibration for that fluorophore.

4.2.5 Whole Plate Loading

There are two pieces of information required for each well: sample type and fluorophore(s) to be monitored. There are two ways of providing that information to the plate setup editor.

When Whole Plate Loading is selected, you specify the sample type and fluorophores separately.

- In Whole Plate Loading, changes made to any fluorophore within a well are extended to the other fluorophores within the well upon pressing Enter or clicking in another cell of the spreadsheet. These changes are then extended to every other well within the replicate group. This is the most efficient way of defining a plate setup for a multicolor experiment when all wells have the same sample type at each dye layer (monitored fluorophore). For example, you might choose to monitor four fluorophores in well A1, and in each dye layer the sample type is Unknown. Note that if you choose Whole Plate Loading, it is assumed that standards in each dye layer are at the same concentration, that is, the FAM standard might be at 500 copies and so, by definition, the HEX standard is also at 500 copies.
- When the sample types vary within a well, or in the special case of standards, when the concentration of standard varies with the dye layer, deselect Whole Plate Loading. When Whole Plate Loading is deselected, you specify the type of sample and fluorophore simultaneously, one dye layer at a time.
- When Whole Plate Loading is toggled off, changes made to one fluorophore are considered unique to the fluorophore and are not automatically extended to the other fluorophores in the well. However, these changes are extended in the same fluorophore to every other well within the replicate group.

4.2.6 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 identical (that is, a single replicate). With this button active, if you:

- Click an unused 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 assigned the identical replicate number
- Drag across a selection, the sample type for every member within the selection is assigned the identical replicate number

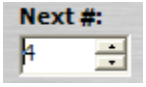
The Horizontal and Vertical Replication buttons, together with the number in the Size box, specify the direction and number of replicates to be automatically defined.

With the horizontal direction (row) 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, and each of the five wells will contain an identical replicate number. If there are not five wells left in the row, the software 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, and 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 (column) 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, and 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.

4.2.7 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. This is to properly associate the appropriate calibration files unique for each vessel and sealing combination with the data file. 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.

Fig. 4.8. The Seal and Vessel Type Dialog Box.

4.2.8 Defining a Dilution Series

The iQ5 software can automatically calculate the concentrations of each member of a dilution series, given the dilution factor and the concentration of either the most or least concentrated sample (Figure 4.9).

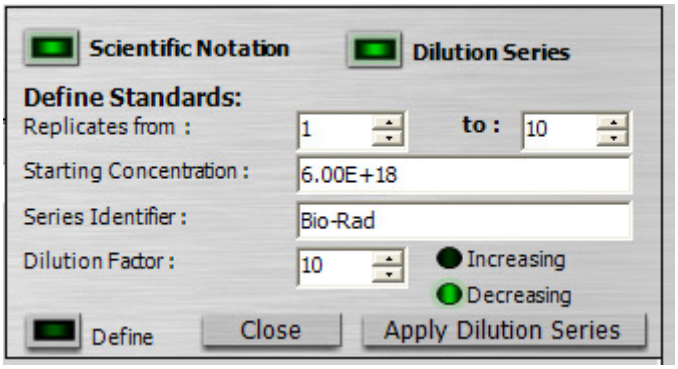


Fig. 4.9. The Dilution Series Dialog Box.

1. Access the dilution series box by clicking on Dilution Series.
2. 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.
3. Enter the starting concentration, the concentration of the lowest-numbered standard to be defined. The Scientific Notation button controls only how the data are displayed in the spreadsheet.
4. Enter a series identifier for the standard if desired. It will be shown in the spreadsheet and in the reports.
5. Enter the fold dilution factor. The default value is 10.
6. Indicate whether the concentrations decrease or increase as the standard replicates increase in number. The default setting is Decreasing.
7. Click Apply Dilution Series, and all standard concentrations will be automatically calculated.

Adding Additional Standards

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

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

4.2.9 Plate Setup Editor Spreadsheets

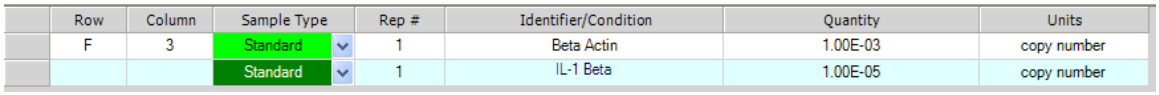
There are two different spreadsheets available from the Plate Setup Editor window: the Well Identifier and the Plate Setup Editor Spreadsheets.

Well Identifier Spreadsheet

The Well Identifier spreadsheet (Figure 4.10) 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 applied to all members of a replicate group. It is also possible to change the replicate group assignment of a well, but doing so 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.

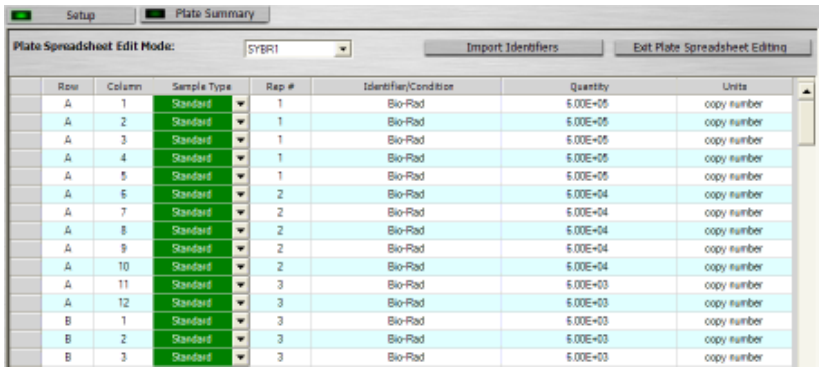


	Row	Column	Sample Type	Rep #	Identifier/Condition	Quantity	Units
	F	3	Standard	1	Beta Actin	1.00E-03	copy number
			Standard	1	IL-1 Beta	1.00E-05	copy number

Fig. 4.10. The Well Identifier Spreadsheet.

Plate Setup Editor Spreadsheet

The Plate Setup Editor spreadsheet can be opened by clicking the spreadsheet button in the Plate Setup Editor window. This spreadsheet displays information about all wells simultaneously, one fluorophore at a time. Fluorophore selection is made at the top of the spreadsheet.



Setup | Plate Summary

Plate Spreadsheet Edit Mode: SYBR1 | Import Identifiers | Exit Plate Spreadsheet Editing

Row	Column	Sample Type	Rep #	Identifier/Condition	Quantity	Units
A	1	Standard	1	Bio-Rad	6.00E+05	copy number
A	2	Standard	1	Bio-Rad	6.00E+05	copy number
A	3	Standard	1	Bio-Rad	6.00E+05	copy number
A	4	Standard	1	Bio-Rad	6.00E+05	copy number
A	5	Standard	1	Bio-Rad	6.00E+05	copy number
A	6	Standard	2	Bio-Rad	6.00E+04	copy number
A	7	Standard	2	Bio-Rad	6.00E+04	copy number
A	8	Standard	2	Bio-Rad	6.00E+04	copy number
A	9	Standard	2	Bio-Rad	6.00E+04	copy number
A	10	Standard	2	Bio-Rad	6.00E+04	copy number
A	11	Standard	3	Bio-Rad	6.00E+03	copy number
A	12	Standard	3	Bio-Rad	6.00E+03	copy number
B	1	Standard	3	Bio-Rad	6.00E+03	copy number
B	2	Standard	3	Bio-Rad	6.00E+03	copy number
B	3	Standard	3	Bio-Rad	6.00E+03	copy number

Fig. 4.11 The Plate Editor Spreadsheet.

Within this spreadsheet you may change the sample type, identifier, and 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.

This single-dye layer spreadsheet has another feature that differentiates it from the single-well spreadsheet. From this spreadsheet you may Import Well Identifiers from an external comma separated values (CSV) file.

Importing Well Identifiers

From the Plate Editor spreadsheet you may import well/sample identifiers from an external CSV file. The number of identifiers must match the number of wells 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 in the sample files folder and then save the file in the CSV format.

4.2.10 Plate Summary

The Plate Summary window shows well definition one fluorophore at a time and provides a more detailed view of the plate including standard quantities and identifiers, as shown in Figure 4.12. 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.

The Plate Summary window can be accessed by:

- Clicking the Plate Summary tab from the Plate Editor window
- Selecting a Plate Setup, Run Set, or Data File, and clicking the Plate Summary tab from the Workshop module main window. If the Data File tab is selected, the plate shown in the Plate Summary window depends on whether Original or Current is clicked 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

FAM | HEX

		1	2	3	4	5	6	7	8	9	10	11	12
A	SampleType		Pos Ctrl-1		Unkn-1	Unkn-2	Unkn-3	Unkn-4	Unkn-5	Unkn-6		NTC-1	Neg Ctrl-1
	copy number		N/A		N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A
	Identifier												-RT
B	SampleType		Pos Ctrl-1		Unkn-1	Unkn-2	Unkn-3	Unkn-4	Unkn-5	Unkn-6		NTC-1	Neg Ctrl-1
	copy number		N/A		N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A
	Identifier												-RT
C	SampleType		Pos Ctrl-1		Unkn-1	Unkn-2	Unkn-3	Unkn-4	Unkn-5	Unkn-6		NTC-1	Neg Ctrl-1
	copy number		N/A		N/A	N/A	N/A	N/A	N/A	N/A		N/A	N/A
	Identifier												-RT
D	SampleType												
	Concentration												
	Identifier												
E	SampleType												
	Concentration												
	Identifier												
F	SampleType			Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8		
	copy number			1.00e-08	1.00e-09	1.00e-10	1.00e-11	1.00e-12	1.00e-13	1.00e-14	1.00e-15		
	Identifier												
G	SampleType			Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8		
	copy number			1.00e-08	1.00e-09	1.00e-10	1.00e-11	1.00e-12	1.00e-13	1.00e-14	1.00e-15		
	Identifier												
H	SampleType			Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8		
	copy number			1.00e-08	1.00e-09	1.00e-10	1.00e-11	1.00e-12	1.00e-13	1.00e-14	1.00e-15		
	Identifier												

Fig.4.12. The Plate Summary Tab.

The spreadsheet displays data one fluorophore at a time. Use the fluorophore buttons to display the plate summary for a specific dye layer. To print the selected plate setup display, click **Print**. To copy and paste the entire plate setup, click **Copy to Clipboard**.

4.3 Protocol

Protocol files direct the operation of the iCycler thermal cycler and also specify when optical data will be collected during the thermal cycling run. iQ5 software protocol files are stored with the extension .tmo.

- A protocol is made up of as many as 9 cycles
- 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.

Temperature and Dwell Time Ranges

- Temperatures between 4.0 and 100.0°C may be entered for any set point temperature
- Finite dwell times may be as short as 1 second (00:01) or as long as 99 minutes and 59 seconds (99:59)

Minimum Dwell Times for Data Collection

- 1 fluorophore = 10 sec
- 2 fluorophores = 20 sec
- 3 fluorophores = 30sec
- 4 fluorophores = 40 sec

Bio-Rad recommends using a slightly higher dwell time than the minimum values, so that more data points are collected at each repeat.

4.3.1 Selecting a Protocol

1. With the Protocol tab active, use the browser to locate the folder where the protocol is located, and then click on the protocol name to select it.
2. The selected protocol appears in the Selected Protocol pane (Figure 4.13).
3. The iQ5 software has a number of sample protocol files you can use.

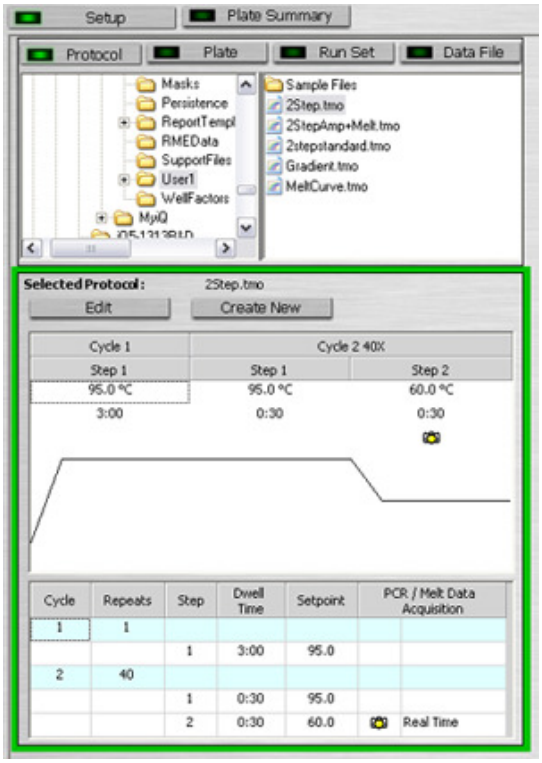


Fig. 4.13. The Protocol Selected Protocol Pane.

4.3.2 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:

- **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
- **Data Acquisition:** Click in the spreadsheet in the Data Acquisition column, and select real-time data collection for any step. Choose Melt Curve if data from a melt curve is required

  NOTE: Ensure that every thermal protocol has at least one data acquisition step.
- **Inserting Cycles and Steps:** To insert a cycle, click in the Insert column on the Cycle row. Cycles are indicated with a blue background. To insert a step, click in the Insert column on a Step row. Steps are indicated with a white background.
- **Deleting Cycles and Steps:** To delete a cycle, click in the Delete column on the Cycle row. Cycles are indicated with a blue background. To delete a step, click in the Delete column on a Step row. Steps are indicated with a white background.
- **Saving the Protocol:** To save a protocol, click **Save & Exit Protocol Editing**. Enter the name of the protocol in the **Save As dialog** box and click **Save** again

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

4.3.3 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 Protocol Options include:

- Gradient (see section 4.3.4 for details)
- Infinite Hold
- Ramping
- Temperature Change
- Time Change
- Cycle Description
- 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 checkbox for the step that you want to maintain at a constant temperature and enter the desired temperature in the Setpoint cell of the spreadsheet.

Ramping

The ramp rate is the speed with which the iCycler thermal cycler changes temperatures between the steps of a cycle, or between cycles. The default condition is for the iCycler thermal cycler to adjust temperatures at the maximum ramp rate. The iCycler thermal cycler allows you to change temperatures at a fixed rate less than the maximum.

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

To adjust the ramp rate:

Click Ramping in the Show Options box. A new column titled Ramp Rate will appear in the spreadsheet.

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 rates 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 (for example, –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 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 (for example, –0:05).
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.

A cycle description or step process may be entered in the spreadsheet in the following manner:

1. Click Cycle Description or Step Process in the Show Options box.
2. Click the cell of the spreadsheet for the cycle or step you wish to name and either choose one of the listed names from the pull-down menu or enter one of your own.

4.3.4 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 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.

NOTE: A gradient cannot be applied to any step, which also has a melt curve.

Programming a Thermal Gradient

To program a thermal gradient:

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 (Figure 4.14).
2. Click the Gradient checkbox in the spreadsheet for the desired step.

Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition	Gradient	Range
1	1						
		1	3:00	95.0		☐	
2	40						
		1	0:10	95.0		☐	
		2	0:30	55.0	Real Time	☑	10.0

Fig. 4.14. The Protocol Editing Table.

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 table (Figure 4.15) will update with the temperatures at each row.
4. You can change the range in the spreadsheet or make a direct entry of the range in the gradient display. Press Enter and the display will update with the new calculated temperature for each row.

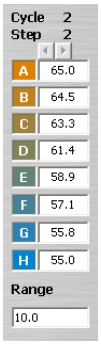


Fig. 4.15. Gradient Display Table.

Programming a Specific Temperature for a Specific Row

To program a specific temperature in any single row:

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. Enter the desired temperature into a specific single 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.

   NOTE: You cannot specify the exact temperature on more than one row at a time.

4.3.5 Melt Curve/Peak

Melt Curve/Peak analysis is a dynamic tool used to measure the melting temperature (T_m) of double-stranded DNA (dsDNA) 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. 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 dsDNA complexes.

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 (4–100°C).
4. Enter an appropriate dwell time for data collection under the Dwell Time column. Dwell times for the melt curve will vary based on the number of fluorophores in the experiment. To enter 10 seconds, type 0 followed by a colon (:), then type 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 select Melt Curve from the pull-down menu. A green camera will appear in the Data Acquisition cell, and two additional columns entitled Temperature Change and End Temperature will be added to the spreadsheet. By default, the temperature change is set at 0.5°C and the end temperature is at 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.

Cycle	Repeats	Step	Dwell Time	Setpoint	PCR / Melt Data Acquisition	Temperature Change	End Temperature
1	1						
		1	1:00	95.0			
2	1						
		1	1:00	55.0			
3	81						
		1	0:10	55.0	Melt Curve	0.5	95.0

Fig. 4.16. Melt Curve Protocol Editing Table.

6. Anneal curves can be also created by entering the temperature change as a negative value (for example, –0.5).
7. Save the protocol by entering a file name and then clicking Save & Exit Protocol Editing.

   NOTE: 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.

4.3.6 Sample Protocol Files

There are several sample protocol files that can be used as editable templates for custom protocol design, including:

- **2Step.tmo:** This protocol can be used for most single fluorophore or SYBR® Green I real-time PCR experiments
- **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
- **Gradient.tmo:** This protocol can be used to determine the most optimal temperature to perform real-time PCR experiments

4.4 Run Set

A Run Set is a linked pairing of a Plate Setup and Protocol files. Run Set files generated by the iQ5 software are stored with the extension .run. Run sets are useful for experiments that are repeated on a frequent basis.

When the Run Set tab is selected, run set files are shown in the file browser.

The protocol defined in the run set is shown in the Selected Protocol pane and the plate setup defined in the run set is shown in the Selected Plate Setup pane (Figure 4.17).

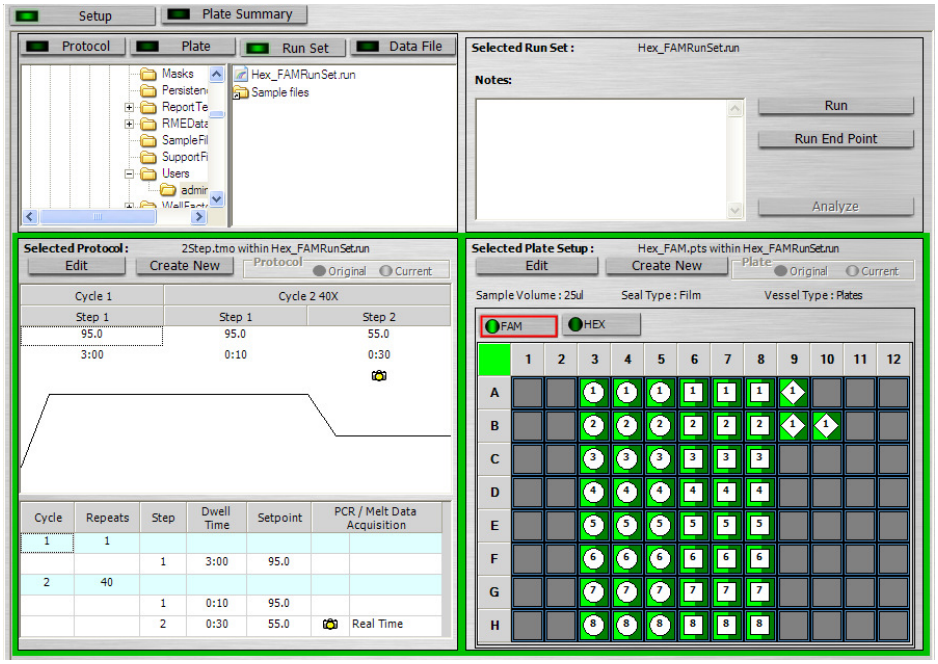


Fig. 4.17. The Run Set Selection Window.

4.4.1 Selecting a Run Set

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

4.4.2 Creating a Run Set

To create a run set:

1. Select the plate setup and protocol that are to be linked.
2. The plate setup and protocol can be stand-alone files (plate setup files have the extension .pts while protocol files have the extension .tmo) or they can be already together in a data file (with the extension .opd). A data file has two plate setups: Original (the Plate Setup the data file was created with) and Current (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.
3. Once you have chosen the protocol and plate setup files, from the File toolbar menu, select New, Run Set and enter a name for your new run set.

4.5 Data File

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

4.5.1 Selecting a Data File

1. Click the Data File button.
2. Use the file browser below the Data File button to locate the folder that contains the data file, and then click on the 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 or 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.

4.5.2 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 click Analyze (or double-click the file name).

NOTE: Data files collected using an iQ5 Real-Time PCR Detection System can be opened when the computer running iQ5 software (version 2.1) is connected to a MyiQ2 or MyiQ Real-Time Detection System. A warning message will display at the bottom of the screen indicating that while the data can be viewed and analyzed, the plate setup contains fluorophores that cannot be used to collect new data using this plate setup. Similarly, opening a data file collected using a MyiQ2 system while a MyiQ system is connected to the software will result in the same message (Figure 4.18).

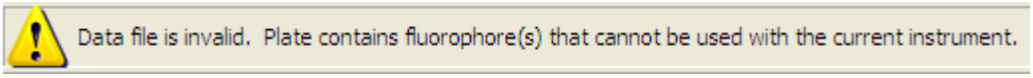


Fig. 4.18. Invalid Data File Error.

4.5.3 Opening a Pure Dye Calibration file

Opening a pure dye calibration file will not only show the pure dye fluorescent data in the PCR Quant screen but will also write this calibration file to the appropriate calibration folder. This will overwrite a calibration file of the same reaction vessel, seal, optical camera and thermal cycler, and all subsequent experimental data will be collected and analyzed with the new calibration file.

4.5.4 Applying Alternate Pure Dye Calibrations (Alternate RME)

Optical data is saved to the data file together with the appropriate calibration files. For the MyiQ2 and iQ5 Real-Time systems, it is possible to overwrite the pure dye calibration files stored within the .opd data file. This feature protects you from losing valuable experimental data if, for example, the calibrations were conducted incorrectly or a run was completed with expired well factors.

To apply an alternate pure dye calibration RME file to a data file;

1. Select the data file in the file browser.
2. Select Apply Alternate RMEs from the Tools menu.
3. Use the Select RME File Name dialog box to navigate to, then open the RME calibration file.

4. After the RME calibration file has been opened a Save Optical Data File dialog box will appear. The existing pure dye calibration will be overwritten.

NOTE: It is recommended to save the optical data file with a different name as this will ensure that the original file, with the original calibration data, is always maintained for reference.

4.5.5 Applying Alternate Background or Well Factors

If well factors have been collected incorrectly, or data has been collected with expired well factors, you may apply an alternate well factor calibration to a data file.

When selecting an alternate well factors file to apply use Persistent Well Factor files only.

To apply an alternate Well Factor file to a dataset:

1. Select the data file in the file browser.
2. Select Apply Alternate Well Factors from the Tools menu.
3. Use the Select Well Factors File Name dialog box to navigate to, then open the desired Well Factors calibration file
4. 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.

The same approach can be used to apply alternate Background Factors.

NOTE: 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 well factor calibration data, is maintained for reference.

NOTE: Do not use the well factor file containing dynamic well factors (identified by the name Dynamic_.....), 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 factor.

Section 5. Run-Time Central Module

This section contains information of the following topics:

The Run-Time Central module is entered automatically after clicking Run or Run End Point from the Workshop Setup window (Figure 5.1). There are three tabs within the Run-Time Central module: Initiate Run, Show Plate and Monitor Run.

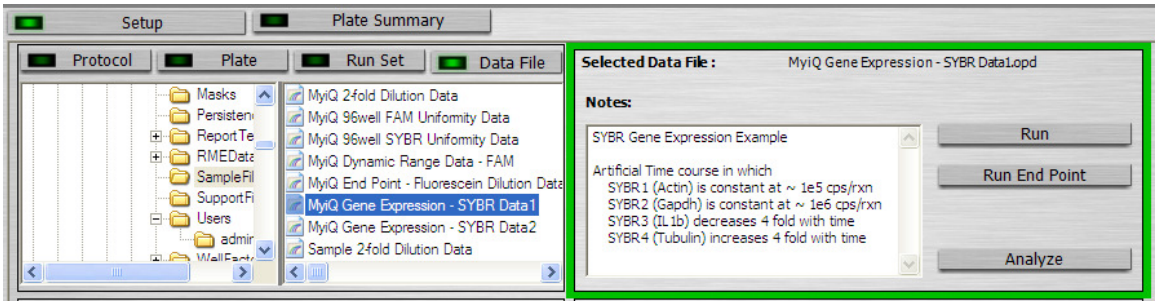


Fig. 5.1. Accessing the Run-Time Central Module.

5.1 Initiate Run Window: Run Selected

Use the Initiate Run window (Figure 5.2) 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. Record details of the experiment in the Notes box. These notes will be incorporated into the experimental file.

To begin a run, select your desired well factor collection method and click Begin Run. The Save dialog box opens. Type a unique name for the optical data file. The iQ5 software saves data automatically during the experiment.

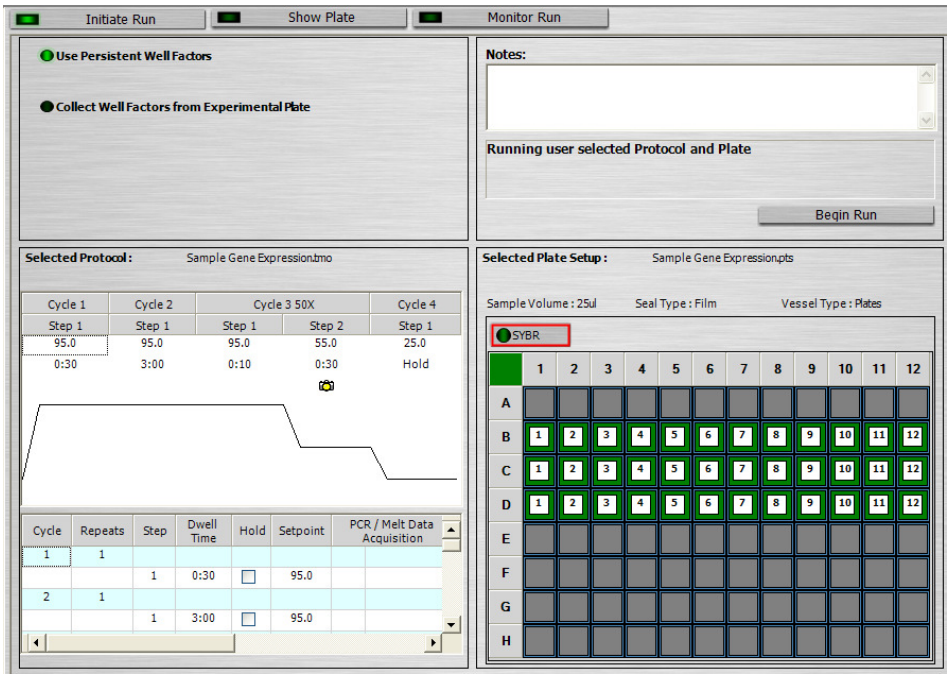


Fig. 5.2. The Initiate Run Window.

5.2 Well Factors

Well Factors are used to compensate for any system or pipeting 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 best source of well factors, for correcting non-uniformity, are dynamic well factors collected from the actual experimental plate. However, in order to collect well factors from the experimental plate, the plate must meet certain requirements and be cycled for approximately 5 extra minutes.

5.2.1 Dynamic Well Factors

In order to collect dynamic well factors one of the following must be true:

- All wells must have the same combination of fluorophores AND the same concentration of fluorophores
- 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 Begin Run is clicked and the file name saved. When collecting dynamic well factors, the plate is held at 95°C for 2.5 minutes prior to the first cycle with a setpoint of 90.0°C or higher.

5.2.2 Persistent Well Factors

When setting up a plate setup to run the experiment specific reaction vessel and sealing methods are chosen. This plate setup will not be able to be used to run an experiment unless persistent well factor and background calibration files with matching vessel and sealing type have already been collected.

Persistent well factors are stored in the Well Factors folder in the iQ5 Folder. Refer to section 7 for information on the calibration and generation of persistent well factors.

In general, persistent well factors can be used for approximately three months, but should be re-generated anytime something pertinent to the optical system is changed such as the optical filters or the camera lamp. A warning reminder to re-calibrate to generate new persistent well factors will occur when Persistent Well Factors are older than 90 days.

5.3 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:

- Use Bio-Rad iQ™ SYBR® Green supermix which contains fluorescein, enabling dynamic well factors to be collected directly from the experimental plate
- Use persistent well factors
- Spike reaction mixes that do not come premixed with fluorescein with fluorescein to a final concentration of 10 nM. 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

5.3.1 Spiking Real-Time PCR Experiments Using DNA binding Dyes With Fluorescein

The 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.

Prepare a 1 mM solution of fluorescein by making 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 $MgCl_2$). Then mix 1 part of the 1 mM fluorescein with 990 µl of master mix to yield a final concentration of 10 nM fluorescein. Once well factors are collected from the experimental plate, they are written to the .opd file and the software continues to execute the programmed protocol.

5.4 Initiate Run Window: Run End Point Selected

Initiate an End Point run by selecting a Plate Setup, then click Run End Point. The software transfers to the Initiate Run window in the Run-Time Central module. An End Point run uses a predefined 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 in Figure 5.3. Click Enter. Changes entered into the Setpoint box appear in the Selected Protocol region of the Initiate Run window.

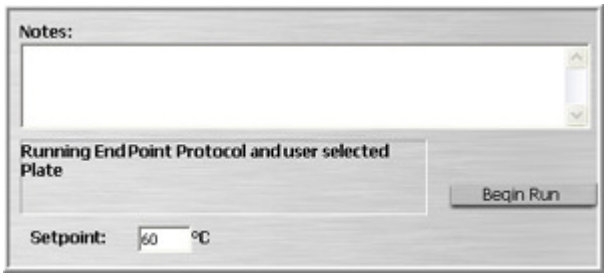


Fig. 5.3. The End Point Setpoint Box.

To begin a run, click Begin Run. The Save dialog box opens. Type a name for the optical data file. The iQ5 software saves data automatically during the experiment.

5.5 Show Plate Window

The Show Plate window (Figure 5.4) can be used to visualize samples loaded into the reaction block of the system. Use the Show Plate window to verify the position and orientation of samples prior to starting a run in the MyiQ2, MyiQ or iQ5 instruments.

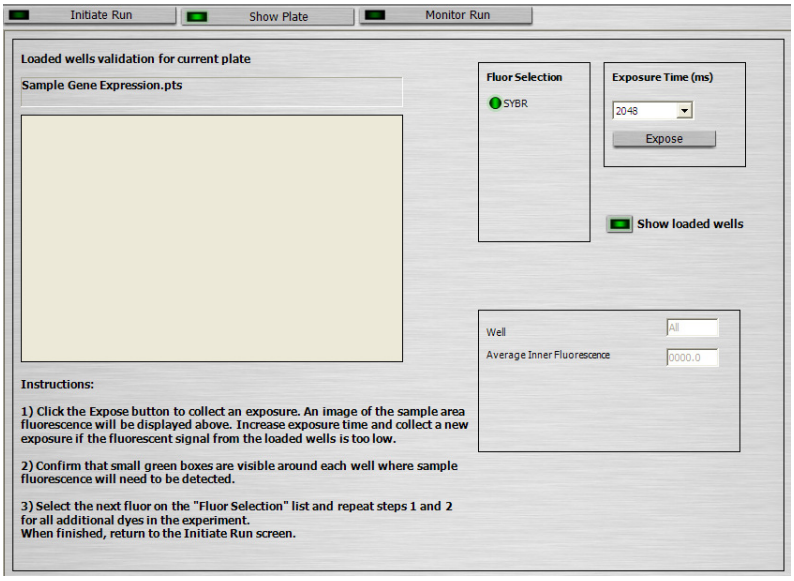


Fig. 5.4. The Show Plate Window.

The following steps describe typical use of the Show Plate window:

1. Collect an exposure from the camera by clicking on **Expose**. An image of the fluorescence from the sample block area will be displayed.
   NOTE: If the image displayed is too bright or too dim, it may be necessary to adjust the exposure time using the pull-down box in the right hand corner of the Show Plate window.
2. Confirm that the small green boxes are visible around each well where sample fluorescence will need to be detected. If necessary, open the instrument and correct sample positions to match the plate setup for sample detection.

3. If a multiplex assay is being performed, repeat steps 1 & 2 for all fluors present in Fluor Selection list.
4. When finished, return to the Initiate Run screen.

5.6 Monitor Run Window

Open the Monitor Run window by clicking the Monitor Run tab. The window appears as shown in Figure 5.5. Traces are displayed in real-time and run progress is monitored in the top left hand section of the window.

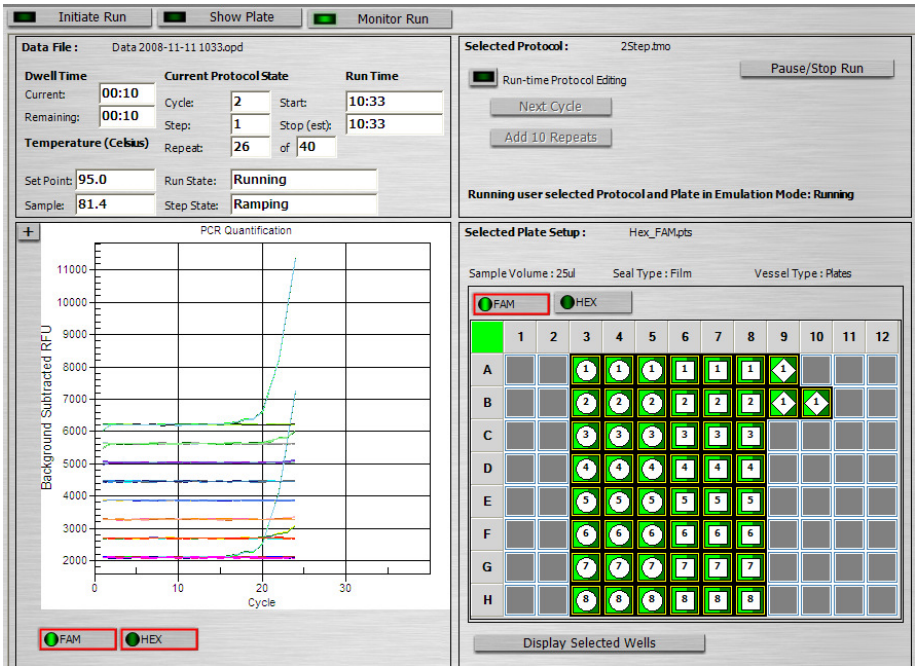


Fig. 5.5. The Monitor Run Window.

5.7 Run-Time Protocol Editing

During a run, you can access real-time protocol editing options by selecting Run-time Protocol Editing as shown below.

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

- **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

- **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. Modifications to the protocol are updated on the protocol displayed on the iCycler base module
- **Pause/Stop/Resume Run:** The **Pause/Stop Run** allows you to pause a thermal cycling protocol. If you click Pause/Stop Run when the iCycler thermal cycler is at a setpoint temperature, the instrument will hold at the setpoint temperature and stop counting down the dwell time. If you click Pause/Stop Run when the iCycler cycler is ramping to the temperature, the instrument 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 6. Data Analysis Module

This section contains information on the following topics:

The Data Analysis module is where data is presented and analyzed. When the iQ5 software opens, the Data Analysis module is gray out and inactive. To analyze a data file, open the file in the Data File tab of the Workshop module by selecting the file and then clicking Analyze.

The Data Analysis module consists of six tabs:

- **PCR Quant**: The PCR Quant tab is used to set the analysis conditions for the data file. The analysis conditions include setting the PCR baseline and threshold, and the wells to be excluded or included in the experiment. The analysis conditions should be set before using the Gene Expression, End Point, or Allelic Discrimination 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, and allelic discrimination
- **End Point :** The End Point tab 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
- **Allelic Disc:** The Allelic Disc tab is useful for assigning genotypes to unknown samples by making comparisons to known genotypes. It can be used to distinguish among homozygous wild types, homozygous mutants, and heterozygous individuals based either on threshold cycle or on RFU. Allelic Discrimination analysis can only be performed on datasets that contain multiplexed PCR data

- **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 circumstances, for example, temporally, geographically, or developmentally different points
- **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 remains with the data file so that you may revert to it at any time

6.1 PCR Quant Tab

The PCR Quant tab is the tab that is first displayed after opening a data file that contains amplification data (Figure 6.1). 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, End Point, or Allelic Discrimination 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.

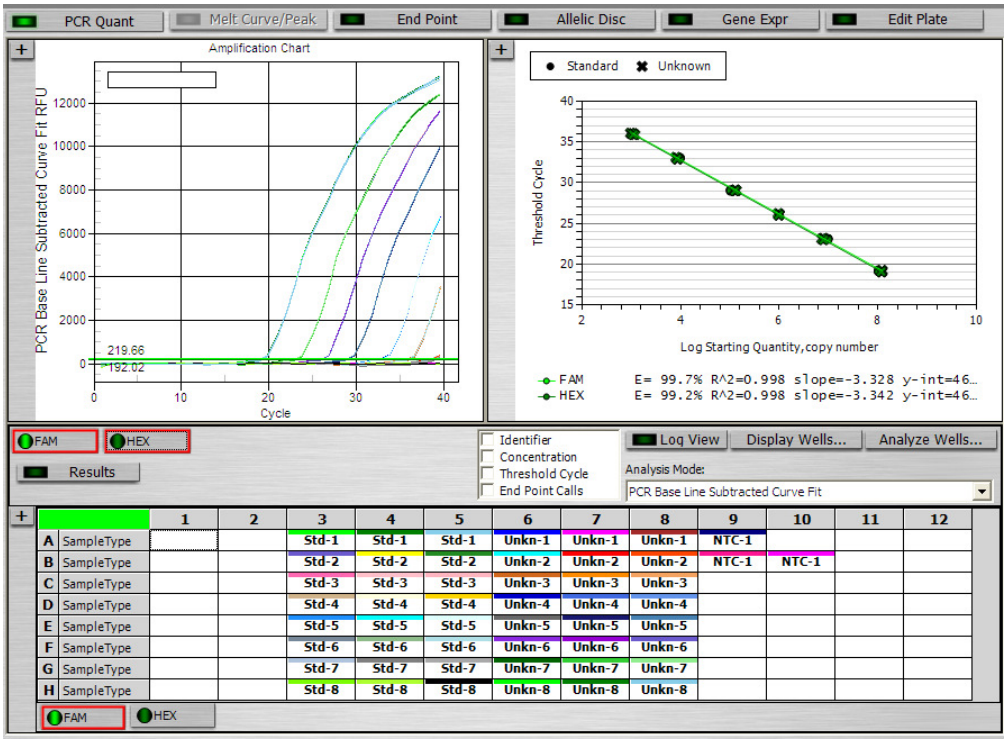


Fig. 6.1. The PCR Quant Tab.

The PCR Quant tab consists of three sections:

- Amplification chart
- Standard Curve chart
- Results section

6.1.1 Customizing the PCR Quant Display

You can customize the size of the sections in the PCR Quant tab in a number of ways. In the upper corner of each section is a **+** (plus) button that enlarges the section when clicked. The enlarged section has a **–** (minus) button that reduces the section when clicked. You can move divider bars between each section by clicking and dragging on the divider bar to resize panes to a specific size.

6.2 Amplification Chart

The amplification chart (Figure 6.2) 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.

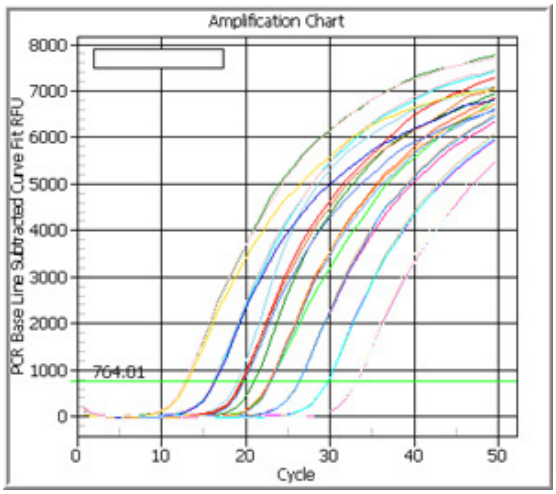


Fig. 6.2. The Amplification Chart.

6.2.1 Fluorophore Selector Buttons

Selecting Fluorophores to Display

You can use the fluorophore selector buttons, which are located under the amplification chart, to display which fluorophores appear in the amplification and standard curve charts. Selecting a single fluorophore 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.

Use the fluorophore selector buttons to select and deselect which fluorophores to display. De-selecting a fluorophore removes it from the display, but not from the analysis, selected fluorophore buttons are displayed with a red border around their perimeter. De-selected fluorophores are displayed without this red border. In Figure 6.3, the FAM fluorophore has been selected, and HEX has been de-selected.

Fig. 6.3. Fluorophore Selector Buttons.

6.2.2 Select Analysis Mode

You can select from three options in the Analysis Mode drop-down list box (Figure 6.4).

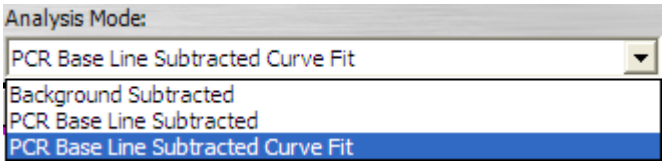


Fig. 6.4. The Analysis Mode Drop-Down List.

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, Allelic Disc, 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. The iQ5 software determines each PCR baseline subtracted trace by fitting the best straight line through the recorded fluorescence of each well during the baseline cycles. The iQ5 software then subtracts the best fit data from the background subtracted data at each cycle to generate the PCR baseline subtracted trace. By default, the software automatically chooses the beginning and end baseline cycles. You can override this default and manually give each trace a beginning and ending baseline cycle. User specified settings for PCR baseline subtraction may also be specified in the User Preferences module
- **PCR Base Line Subtracted Curve Fit:** The iQ5 software 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 the C_T is left invariant for all traces

6.2.3 Log View Button

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

6.2.4 Selecting and Viewing Traces

- **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 the fluorophore selector buttons from the amplification chart, traces from other fluorophores in the selected well also appear
- **Viewing all traces:** To restore a view of all traces right-click on the amplification chart and select Show All Traces
- **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

Analyze Wells

Click **Analyze Wells** on the PCR Quant screen to select the wells that you wish to include in data analysis. The Select Wells to Analyze floating window appears as shown in Figure 6.5.

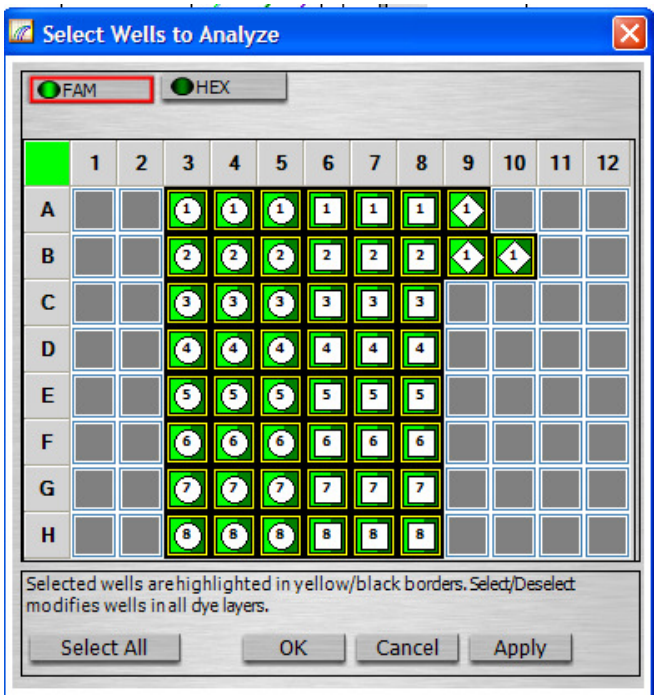


Fig. 6.5. The Select Wells to Analyze Window.

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

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 (C_T) location, thus affecting all subsequent data analyses that depend on C_T values. This would include standard curve calculations and quantification of unknowns, gene expression, and allelic discrimination 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 Wells to Analyze floating window appears. A set of fluorophore selector buttons appears at the top of this window. When you click a fluorophore button, the sample type present in that well for that fluorophore is colored.
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. 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 uppermost 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 Wells to Analyze window to remain open after the iQ5 software 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, the iQ5 software 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 Wells to Analyze dialog box.

Display Wells

Click the Display Wells button on the PCR Quant screen to select the wells that you wish to include in the data display.

After you click Display Wells, the Select Analyzed Wells for Display floating window appears, as shown below in Figure 6.6.

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

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

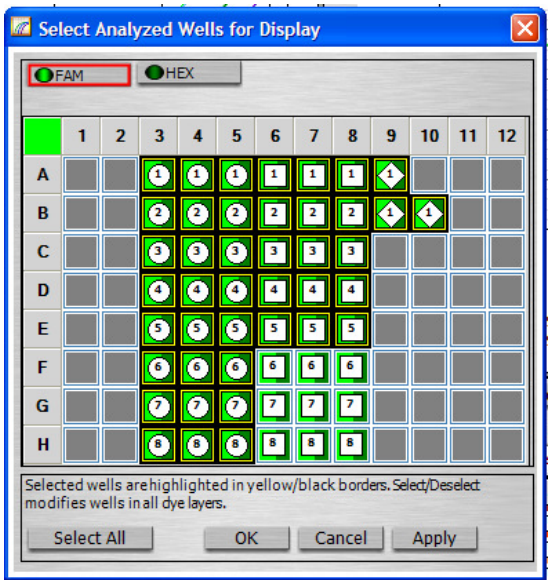


Fig. 6.6. The Select Analyzed Wells for Display Window.

To select the wells to include in data display:

1. In the PCR Quant tab, click **Display Wells**. The Select Analyzed Wells for Display window appears. Only those wells that are included in the analysis appear in the window.
2. Select and de-select wells using the same methods described in the analyze wells section on page 51.
3. After you determine which wells to include in analysis, click **Apply** if you want the Select Analyzed Wells for Display window to remain open after the iQ5 software re-analyzes the wells. If you are satisfied with your selection, click **OK** to close the window and update the wells to include in the analysis. If you click **Cancel**, the iQ5 software closes the window and discards all changes.

The Select Analyzed Wells for Display window displays traces for fluorophores with parameters per the selected fluorophore selector buttons on the amplification chart. 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.

6.3 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 fluorophore selector buttons on the amplification chart determine which fluorophores appear in the standard curve chart shown below in Figure 6.7.

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 section.

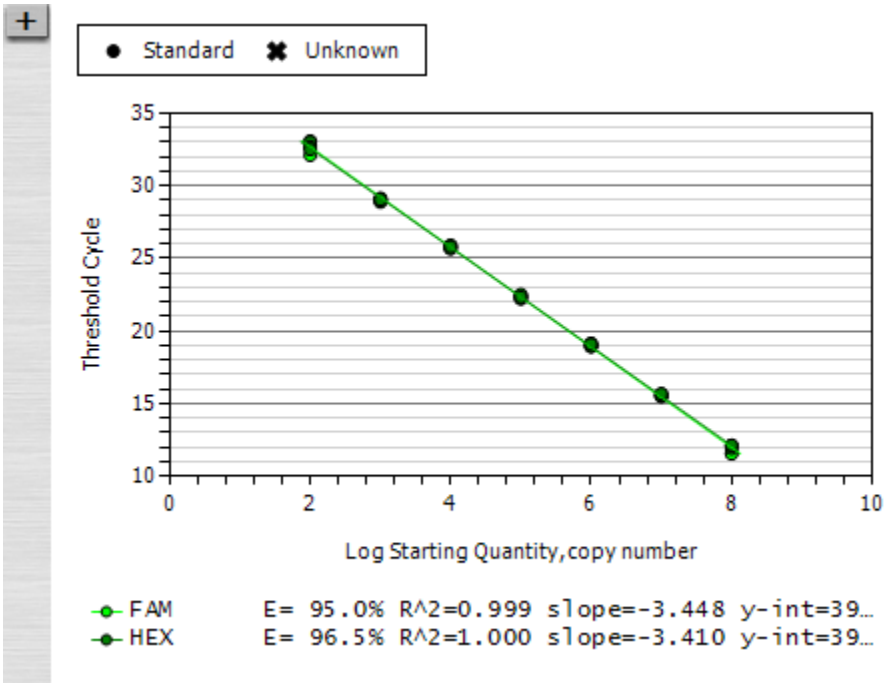


Fig. 6.7. The Standard Curve Chart.

6.3.1 Standard Curve Chart Menu

Right-clicking on the standard curve chart opens a menu (Figure 6.8).

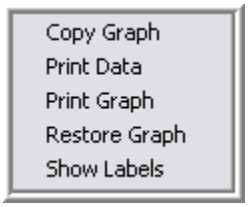


Fig. 6.8. The Standard Curve Chart Menu.

This menu includes the following options:

- **Copy Graph:** Copy Graph copies the standard curve chart to the clipboard. To copy the entire graph, enlarge the chart by clicking the + button in the upper left corner of the standard curve Chart. Then click Copy Graph in the menu

- **Print Data:** Print Data prints the Standard Curve/C_T Results spreadsheet to your specified printer in Windows
- **Print Graph:** Print Graph prints the graph to your specified printer in Windows
- **Restore Graph:** Restore Graph is active only after you zoom in on the chart. Clicking Restore Graph returns the standard curve chart to its un-zoomed state
- **Show Labels:** Show Labels labels standards and unknowns with the well name as shown in Figure 6.9

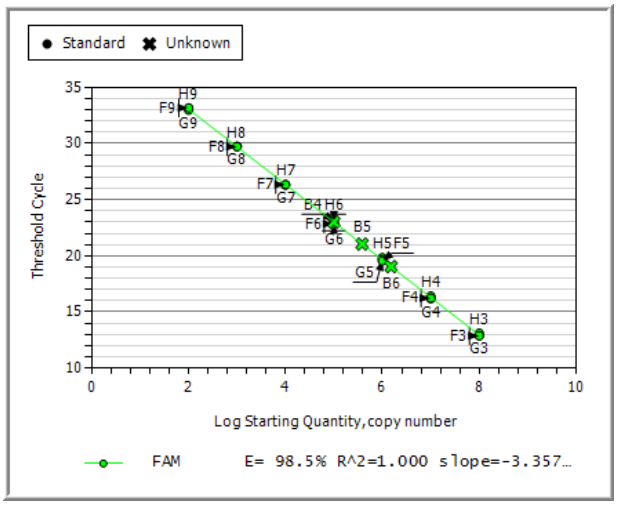


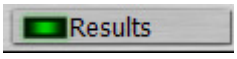
Fig. 6.9. The Standard Curve Chart with Labels.

6.4 Results Section

The Results workbook consists of three spreadsheets:

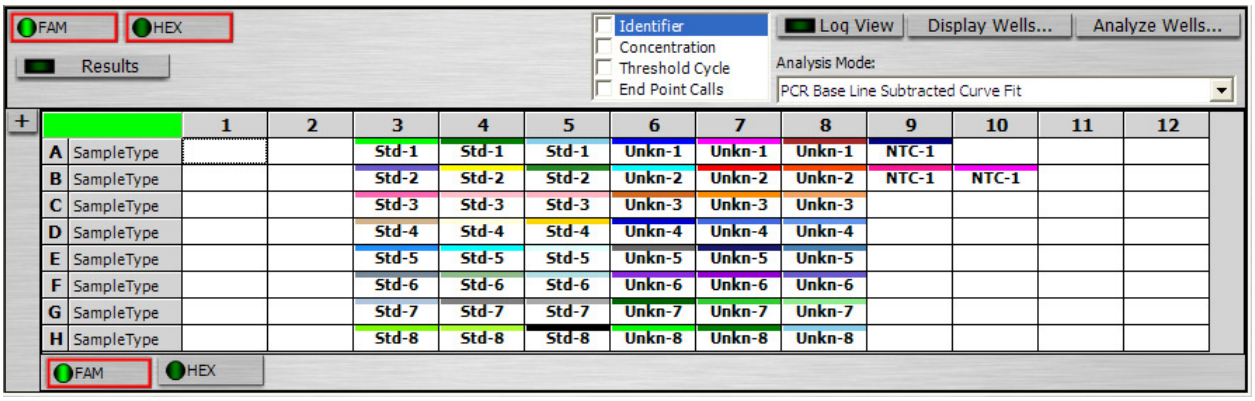
- Plate spreadsheet
- Standard Curve/C_T Results spreadsheet
- Amplification Data (RFU) spreadsheet

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



6.4.1 Plate Spreadsheet

The Plate spreadsheet (Figure 6.10) displays data for each well in a grid fashion that represents the plate setup used in the experiment. The spreadsheet is viewable at the bottom of the PCR Quant screen and can be expanded to full screen by clicking on the **+** button in the top left hand corner of the spreadsheet.



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

FAM HEX

Fig. 6.10. The Plate Spreadsheet.

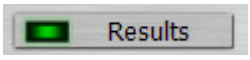
The spreadsheet displays only one fluorophore at a time. You can use the Plate spreadsheet fluorophore selector buttons found under the Plate spreadsheet to determine which fluorophore is displayed in the spreadsheet. Each well has a colored bar at the top of the cell, which is the color used in the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

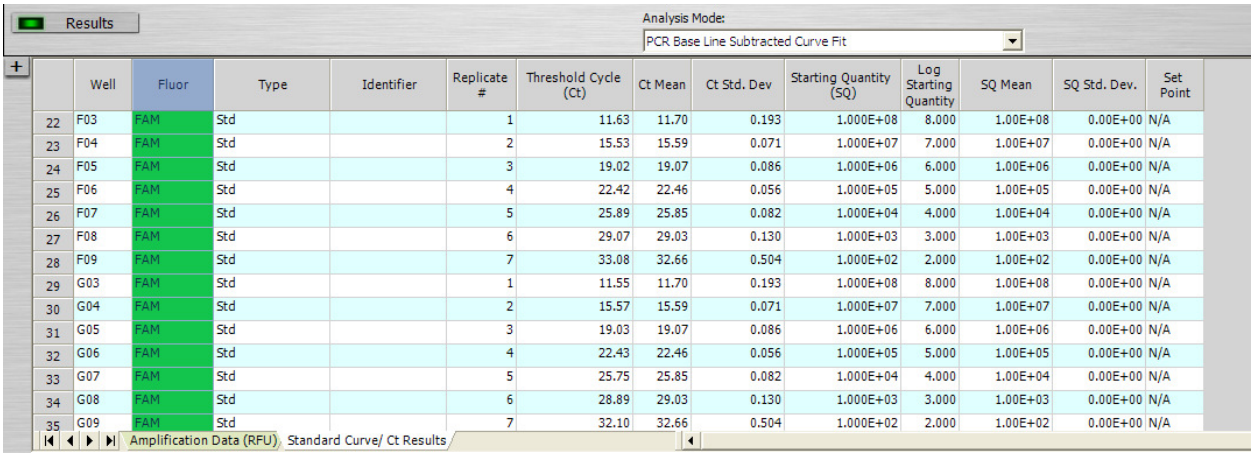
Above the spreadsheet is a set of checkboxes you can use to display the Identifier, Concentration, Threshold Cycle, and End Point Calls in the Plate spreadsheet.

6.4.2 Standard Curve/C_T Results Spreadsheet

The Standard Curve/C_T Results spreadsheet displays the well, fluorophore, sample type, identifier, replicate number, threshold cycle, starting quantity, and statistics for replicate groups.

Access the Standard Curve/C_T Results spreadsheet by clicking Results on the PCR Quant screen. By default the Standard Curve/C_T Results tab will open displaying the spreadsheet results (Figure 6.11).





	Well	Fluor	Type	Identifier	Replicate #	Threshold Cycle (Ct)	Ct Mean	Ct Std. Dev	Starting Quantity (SQ)	Log Starting Quantity	SQ Mean	SQ Std. Dev.	Set Point
22	F03	FAM	Std		1	11.63	11.70	0.193	1.000E+08	8.000	1.00E+08	0.00E+00	N/A
23	F04	FAM	Std		2	15.53	15.59	0.071	1.000E+07	7.000	1.00E+07	0.00E+00	N/A
24	F05	FAM	Std		3	19.02	19.07	0.086	1.000E+06	6.000	1.00E+06	0.00E+00	N/A
25	F06	FAM	Std		4	22.42	22.46	0.056	1.000E+05	5.000	1.00E+05	0.00E+00	N/A
26	F07	FAM	Std		5	25.89	25.85	0.082	1.000E+04	4.000	1.00E+04	0.00E+00	N/A
27	F08	FAM	Std		6	29.07	29.03	0.130	1.000E+03	3.000	1.00E+03	0.00E+00	N/A
28	F09	FAM	Std		7	33.08	32.66	0.504	1.000E+02	2.000	1.00E+02	0.00E+00	N/A
29	G03	FAM	Std		1	11.55	11.70	0.193	1.000E+08	8.000	1.00E+08	0.00E+00	N/A
30	G04	FAM	Std		2	15.57	15.59	0.071	1.000E+07	7.000	1.00E+07	0.00E+00	N/A
31	G05	FAM	Std		3	19.03	19.07	0.086	1.000E+06	6.000	1.00E+06	0.00E+00	N/A
32	G06	FAM	Std		4	22.43	22.46	0.056	1.000E+05	5.000	1.00E+05	0.00E+00	N/A
33	G07	FAM	Std		5	25.75	25.85	0.082	1.000E+04	4.000	1.00E+04	0.00E+00	N/A
34	G08	FAM	Std		6	28.89	29.03	0.130	1.000E+03	3.000	1.00E+03	0.00E+00	N/A
35	G09	FAM	Std		7	32.10	32.66	0.504	1.000E+02	2.000	1.00E+02	0.00E+00	N/A

Amplification Data (RFU) Standard Curve/ Ct Results

Fig. 6.11. Standard Curve/C_T Results Spreadsheet.

Columns can be sorted by clicking on the column headings and reordered by clicking and dragging to move columns. Data can be exported by right-clicking on the spreadsheet and selecting Export to Excel. Data can be printed by right-clicking on the spreadsheet and selecting Print. None of the data in this spreadsheet may be edited.

6.4.3 Amplification Data (RFU) Spreadsheet

You can view the individual RFU readings for each amplification trace at every cycle in the Amplification Data (RFU) spreadsheet. Access the Standard Curve/C_T Results spreadsheet by clicking Results on the PCR Quant screen and selecting the Amplification Data (RFU) tab at the bottom of the spreadsheet view (see Figure 6.11).

The spreadsheet can display data for only one fluorophore. 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.

6.5 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 (Figure 6.12).

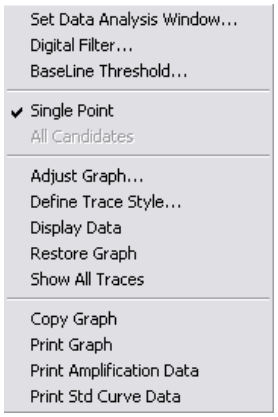


Fig. 6.12. The Amplification Plot Menu.

6.5.1 Data Analysis Options

There are three additional data analysis options in the amplification plot menu:

- Set Data Analysis Window
- Digital Filter
- BaseLine Threshold

Set Data Analysis Window

After you click Set Data Analysis Window in the Amplification plot menu, the Set Data Analysis Window appears as shown in Figure 6.13. The number of data points collected per cycle depends on the exposure 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.

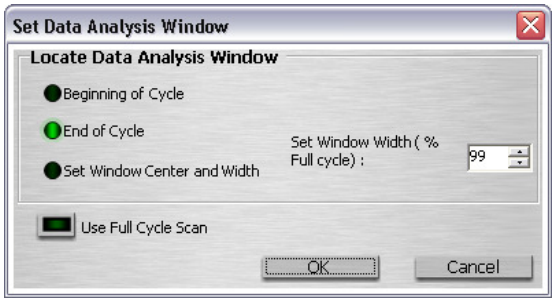


Fig. 6.13. The Set Data Analysis Window.

To set the Data Analysis Window:

1. Right-click on the PCR amplification plot, and click Set Data Analysis Window in the menu.
2. Click either the Beginning of Cycle or End of Cycle option 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 the data collected with a window in the middle of the cycle, select Set Window Center and Width. 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 Set Window Width (% 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 (Figure 6.14). 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. 6.14. The Set Digital Filters Window.

The weighted mean is determined by the equation:

$$\mathbf{O_i = (R_i + c*M)/(1 + c)}$$

Where:

- O_i is the filtered value for a given 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 boxcar 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 rolling boxcar filters only apply within a cycle. A global filter that smoothes data from cycle to cycle is also available by clicking Enable Global Filter. The global filter operates on the trace for a given well 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.

Base Line Threshold Settings

In the amplification chart, the iQ5 software uses the baselines for each individual trace and the threshold that is set for the fluorophore to set conditions to determine the threshold cycle. Baseline cycles and threshold are only calculated by the software when in PCR Base Line Subtracted or PCR Baseline Subtracted Curve Fit mode. You can override the automatic conditions set for baseline and threshold in the Base Line Threshold window.

1. Ensure that only one fluorophore from the Amp Chart Fluorophore Selector buttons is selected
2. Right-click on the amplification chart
3. Click Base Line Threshold. After you click Base Line Threshold in the amplification plot menu, the Base Line Threshold Parameter window appears and displays the fluorophore of the traces to be adjusted.
4. Select **User Defined**. In Figure 6.15, the Base Line Threshold Parameter window shows the FAM traces are to be adjusted.

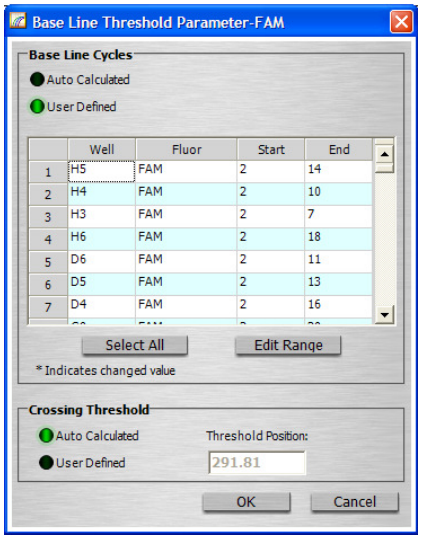


Fig. 6.15. The Base Line Threshold Parameter Window.

Start and Ending Cycle Mode

To change the baseline range for all wells, click **Select All**. Then click **Edit Range** to enter the start and end cycle for baseline calculation (Figure 6.16).

To change the baseline for individual wells, click on a well and then click **Edit Range** to enter the start and end cycle for baseline calculation.

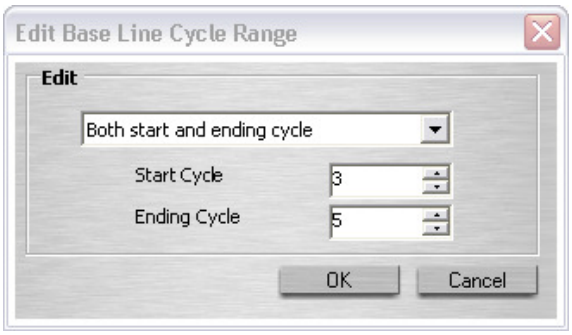


Fig. 6.16. The Both Start and Ending Cycle Mode.

Start Cycle Only Mode

By default both the start and ending cycle for baseline calculation are altered. If you select 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 (Figure 6.17). Select individual wells or all wells as described above.

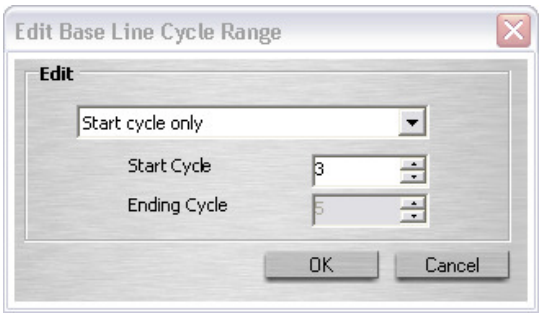


Fig. 6.17. The Start Cycle Only Mode.

Ending Cycle Only Mode

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 (Figure 6.18). Select individual wells or all wells as described above.

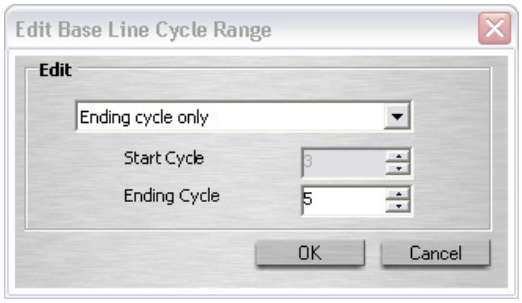


Fig. 6.18. The Ending Cycle Only Mode.

Setting the Threshold Manually

By default, the iQ5 software automatically calculates the threshold. You can override auto-calculation in one of two ways:

- On the amplification chart, move the cursor over the displayed threshold line until the cursor icon becomes a hand. Left-click on the displayed threshold line and drag the threshold to the desired position
- From the Base Line Threshold Parameter dialog box, select User Defined in the Crossing Threshold section at the bottom of the dialog box. Enter the desired value for the threshold position, and then click OK

6.5.2 Amplification Plot Data Display Options

There are seven additional data analysis options in the amplification plot menu:

- Single Point
- All Candidates
- Adjust Graph
- Define Trace Style
- Display Data
- Restore Graph
- Show All Traces

Single Point

The **Single Point** mode of data presentation, in which the iQ5 software averages all data collected at a particular step and then plots the average, is the default mode. For example, if the iQ5 software 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 **All Candidates** mode, the iQ5 software plots every single data point that is collected. You cannot use automated data analysis features when the data are presented 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

Rescale or change the amplification plot from linear to log, or vice versa, by selecting **Adjust Graph**. The Chart Axes Range Definition window appears (Figure 6.19).

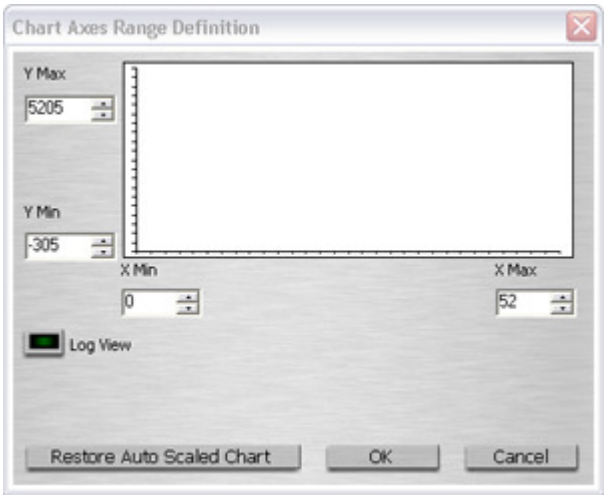


Fig. 6.19. The Chart Axes Range Definition Window.

Enter the maximum and minimum values for the x-and y-axes 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.

Change the display to a semi-logarithmic view by clicking Log View. Click Log View again 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 **Log View** in the PCR Quant tab.

Define Trace Style Option

You can customize the display by selecting Define Trace Style option. The Define Trace Style window appears (Figure 6.20).

Change the trace color and symbol, which are used to display data points, on a well-by-well basis or in groups of sample types. You can preview all changes before you apply them by clicking **Preview**.

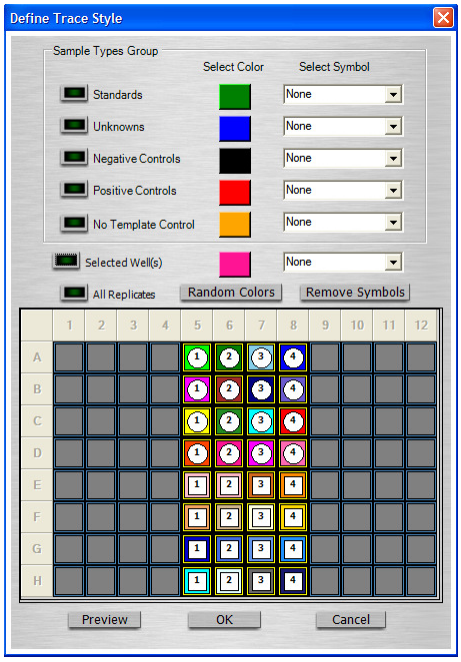


Fig. 6.20. The Define Trace Style Window.

To modify a trace style:

1. Select the Sample Types Group to be modified (for example, Standards). (Note that green is the default color that is automatically assigned to the Standards group when this button is active.)
2. To specify a different color for a given sample type group on the plate, click on the colored button in the Select Color column.
3. Select the symbol type (the default style is "None").
4. Click Preview to see your changes, and if those changes are satisfactory, click OK.

To choose on a well–by-well basis, click the Selected Well(s) button, select a color and symbol type, and then select the desired 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 All Replicates to apply the selected color to all selected replicates of the wells.

To reset original trace style settings, click Random Colors and Remove Symbols, then click OK.

Display Data Option

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/C_T Results tab. You can perform the same function by clicking Results

Restore Graph Option

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

Show All Traces Option

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.

6.5.3 Data Export Options

The amplification plot menu offers four additional options for printing and exporting graphs or data.

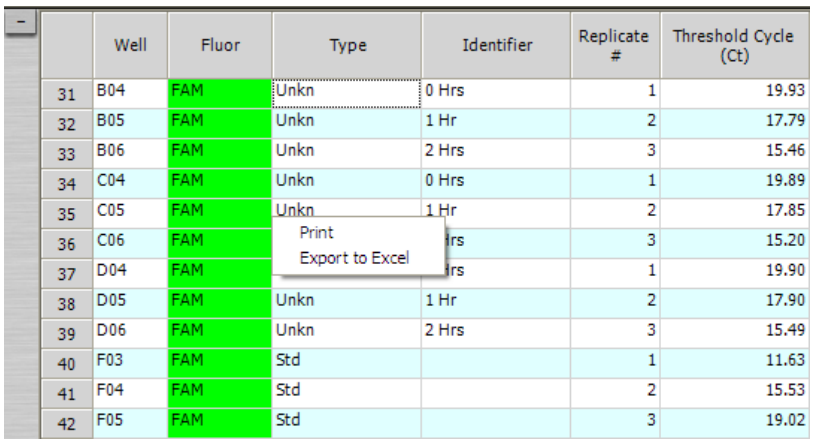
- **Copy Graph:** Click Copy Graph to copy the amplification plot into the clipboard to import into other programs
- **Print Graph:** Click Print Graph to print the graph to your default Windows printer
- **Print Amplification Data:** The Print Amplification Data option is only available when the iQ5 software 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 the iQ5 software displays the Standard Curve/C_T 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/C_T Results spreadsheet, the printed spreadsheet will reflect that change.

6.5.4 Exporting Results to Microsoft Excel

Exporting Data from the Results Table

1. Make sure the Results button is activated. If not, click **Results**.
2. Right-click on the spreadsheet. A shortcut menu appears (Figure 6.21).
3. Select **Export to Excel**.
4. Enter a name and file destination for the Excel file generated with your data export.



	Well	Fluor	Type	Identifier	Replicate #	Threshold Cycle (Ct)
31	B04	FAM	Unkn	0 Hrs	1	19.93
32	B05	FAM	Unkn	1 Hr	2	17.79
33	B06	FAM	Unkn	2 Hrs	3	15.46
34	C04	FAM	Unkn	0 Hrs	1	19.89
35	C05	FAM	Unkn	1 Hr	2	17.85
36	C06	FAM		rs	3	15.20
37	D04	FAM		rs	1	19.90
38	D05	FAM	Unkn	1 Hr	2	17.90
39	D06	FAM	Unkn	2 Hrs	3	15.49
40	F03	FAM	Std		1	11.63
41	F04	FAM	Std		2	15.53
42	F05	FAM	Std		3	19.02

Fig. 6.21. Exporting PCR Quant Data Tables to Microsoft Excel.

The Export to Excel command is useful for exporting exact values from the spreadsheet. When the Export to Excel command is selected from the menu, an Export to Excel file save box is displayed. Choose a location where the Excel file is to be saved and click Save. The iQ5 software automatically exports the selected data into a protected workbook.

- The protected workbook generated by the iQ5 software contains the text values of what is represented on the spreadsheet. For example, checkboxes from the software application are replaced by "True" or "False" text in Excel.
- The numeric values contained in the protected workbook are exact values from the software application that include several non-significant figures beyond the decimal point. This is important to note when considering whether to transfer iQ5 data spreadsheets by a copy and paste command or the Export to Excel command. With the copy and paste command only the significant digits displayed in the iQ5 software interface are transferred to Excel.

6.5.5 Printing Results

The Print command on the Results table menu will print the displayed spreadsheet (Amplification Data or Standard Curve/C_T Results). When selected, a Print Preview box is opened which contains an illustration of the spreadsheet as it will appear once printed. Clicking the printer icon opens the Windows print dialog box. Click OK to complete the printing task.

6.6 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 in Figure 6.22.

The vertical temperature bar may be dragged to any position on the plot. The temperature bar on the melt peak plot moves in sync to the same relative position on the melt curve plot and *vice versa*. 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.

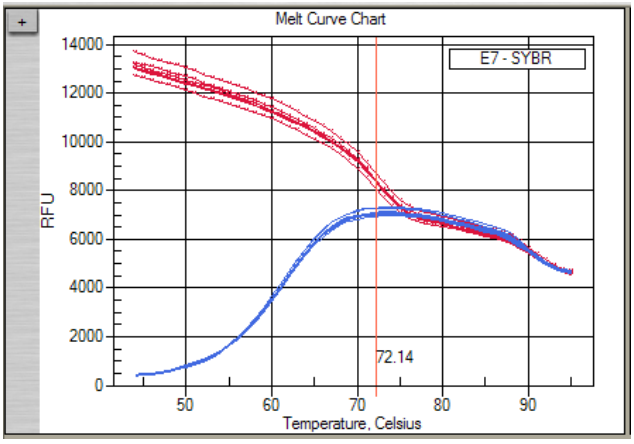


Fig. 6.22. Melt Curve Chart.

The data in the melt peak chart are derived from the melt curve chart (Figure 6.23).

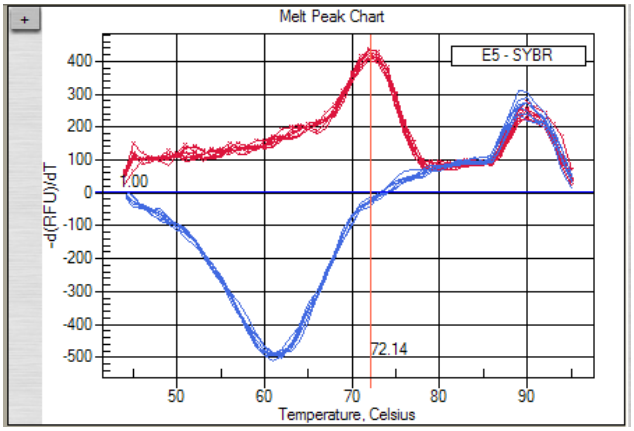


Fig. 6.23. Melt Peak Chart.

The data in the melt peak chart show the negative rate of change in fluorescence with changing temperature, that is:

$$-d(RFU)/dT$$

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 minimum height relative to the distance between the threshold bar and the height of the highest peak. Therefore, if the threshold bar is dragged downward, increasing 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 up 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.

6.6.1 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 peak plot are also carried out on the melt curve plot. Those features are accessed by a right click on the chart, which brings up the menu shown in Figure 6.24.

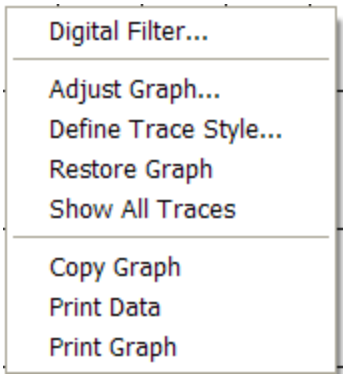
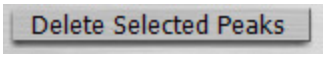


Fig. 6.24. Melt Curve/Peak Chart Context Menu.

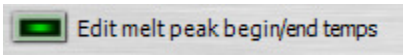
6.6.2 Delete Selected Peaks

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



6.6.3 Edit Melt Peak Begin/End Temperature

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



After you click this option button, the currently active peak displays alone 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, the iQ5 software tracks the movement on the melt curve chart and updates the movement in the spreadsheet. When the Edit melt peak begin/end temps option is activated, the Display Wells and Analyze Wells dialog boxes are unavailable.

In Figure 6.25, the peak begin temperature is 59°C, the peak end temperature is 79°C, and the threshold is 1.

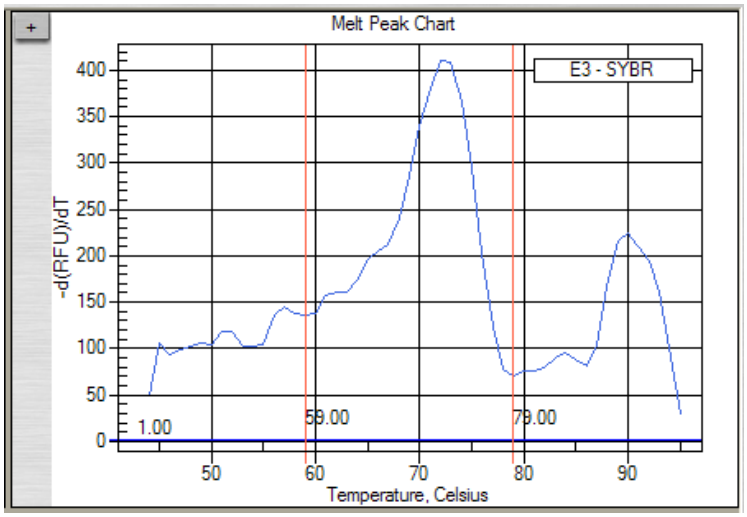


Fig. 6.25. A Melt Peak Chart.

6.6.4 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 and the Restore Defaults function (Figure 6.26).

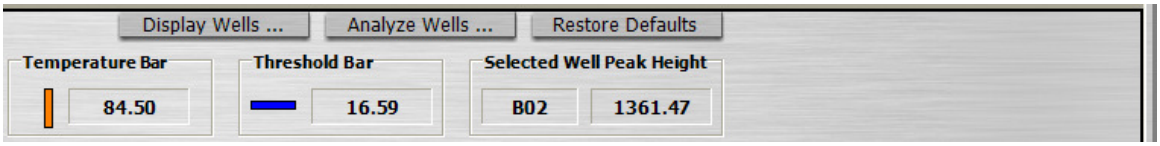


Fig. 6.26. The Control Area.

The position of the temperature bar, threshold bar, and 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 is active, the most-recently modified temperature (begin or end) is displayed in the Temperature Bar field.

6.6.5 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 (Figure 6.27). 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.

	Identifier	Peak ID	Melt Temp.	Peak Height	Begin Temp	End Temp	Area	Area Fraction %	Threshold Crossing Begin Temp	Threshold Crossing End Temp	Threshold Crossing Area	Threshold Crossing Area Fraction %	Edited Begin Temp	Edited End Temp
E03		E03.0	72.00	410.10	59.00	79.00	4636.64	76.27	59.00	79.00	4636.64	78.85	59.00	79.00
		E03.1	90.00	224.31	86.00	95.00	1442.91	23.73	86.00	93.00	1243.71	21.15	86.00	95.00
E04		E04.0	72.00	413.50	60.00	79.00	4522.79	72.28	60.00	79.00	4522.79	74.77	60.00	79.00
		E04.1	90.00	232.34	83.00	95.00	1734.30	27.72	83.00	93.00	1525.87	25.23	83.00	95.00
E05		E05.0	72.00	402.60	66.00	79.00	3500.89	70.24	66.00	79.00	3500.89	72.63	66.00	79.00
		E05.1	90.00	247.08	86.00	95.00	1483.48	29.76	86.00	93.00	1319.17	27.37	86.00	95.00
E06		E06.0	72.00	420.38	56.00	80.00	5206.20	74.91	56.00	80.00	5206.20	77.34	56.00	80.00
		E06.1	90.00	252.15	84.00	95.00	1743.86	25.09	84.00	93.00	1525.55	22.66	84.00	95.00

Melt Peaks | RFU | -d(RFU)/dT

Fig. 6.27. The Melt Peaks Spreadsheet.

The spreadsheet displays the following information for each peak:

- **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 Figure 6.27, 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. The iQ5 software makes this calculation using default begin and end temperatures and the default position of the threshold bar, so data are 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

6.7 Allelic Discrimination Module (For Multiplex Data Only)

The Allelic Discrimination module is useful for assigning genotypes to unknown samples by making comparisons to known genotypes. It can be used to distinguish among homozygous wild types, homozygous mutants and heterozygous individuals based either on the C_T or RFU value. RFU data may be chosen from any cycle in the experiment. The assignments can be made automatically if controls are specified, or you can make the assignments manually.

NOTE: The Allelic Discrimination module is not available with working with single color data files or any data file generated by the MyiQ Real-Time PCR Detection System.

6.7.1 Allelic Discrimination Plot

The Allelic Discrimination plot shows either RFU (Figure 6.28A) or C_T (Figure 6.28B) data from two different fluorophores at the same time. Choose which plot to view using the RFU or Threshold Cycle radio button in the Display Mode box.

In Automatic Call mode, two bars, one vertical and one horizontal, divide the plot into four sections: one for each homozygous state, one for the heterozygous state and a non-reactive section. The positions of these bars may be adjusted in the Automatic Call mode. The bars do not appear in Manual Call mode.

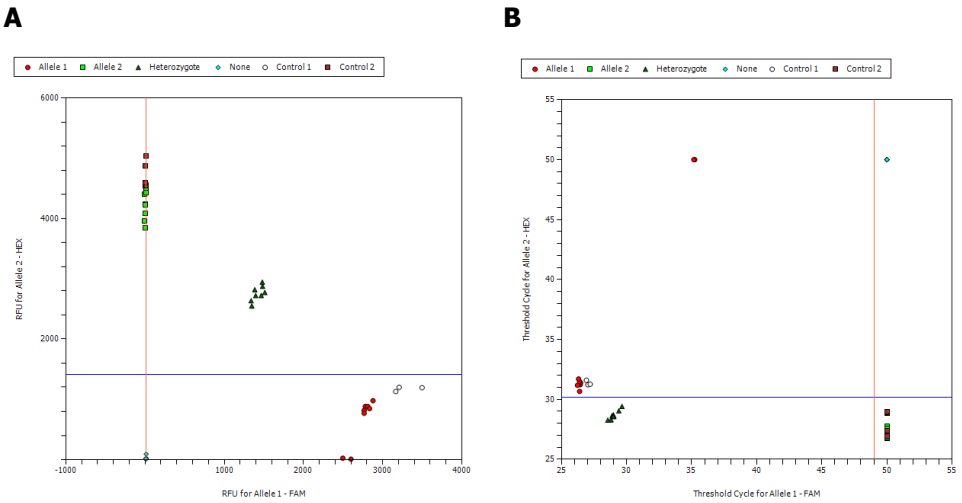


Fig. 6.28. Allelic Discrimination Plot; RFU View (A) and Threshold Cycle View (b).

Genotype assignments for unknown samples are determined by plotting the RFU for one fluorophore (allele 1 on the x-axis) against the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination plot.

- If the unknown RFU values are greater than the horizontal and vertical bars, then the genotype is heterozygous
- If the unknown RFU values are greater than the horizontal bar and less than the vertical bar, the genotype is homozygous for allele 2 (allele 2 RFU is plotted on the y-axis)
- If the unknown RFU values are less than the horizontal bar and greater than the vertical bar, the genotype is homozygous for allele 1 (allele 1 RFU is plotted on the x-axis)
- If the unknown RFU values are less than the horizontal and vertical bars, then no genotype can be assigned

Genotype assignments for unknown samples may also be determined by plotting the threshold cycle for one fluorophore (allele 1 on the x-axis) against the threshold cycle for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination plot.

- If the unknown threshold cycle values are greater than the horizontal and vertical bars, then the genotype is "none", or not assigned. Samples not crossing threshold during the protocol are placed in this quadrant
- If the unknown threshold cycle values are greater than the horizontal bar and less than the vertical bar, the genotype is homozygous for allele 1 (allele 1 threshold cycle is plotted on the x-axis)
- If the unknown threshold cycle values are less than the horizontal bar and greater than the vertical bar, the genotype is homozygous for allele 2 (allele 2 threshold cycle is plotted on the y-axis)
- If the unknown threshold cycle values are less than the horizontal and vertical bars, then the genotype is heterozygous

In Manual Call mode, the plot displays the RFU or threshold cycle data only. The threshold bars do not appear, and modifications to calls are made manually by using the drop-down menu in the Call column of the data spreadsheet. Alternatively, modifications to calls are made by choosing the call type from the radio buttons in the Allele Call box and then clicking and dragging to select desired samples in the graph.

You may zoom in on the allelic discrimination plot by first selecting Zoom Enabled from the plot menu, then left-clicking and dragging over the area you wish to enlarge. Zoom out by choosing Restore Graph from the context menu.

6.7.2 Allelic Discrimination Plot Menu

Open the allelic discrimination plot menu by right-clicking on the allelic discrimination plot (Figure 6.29).

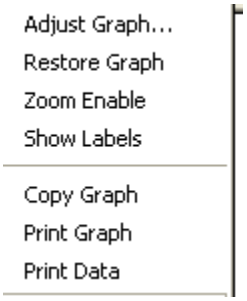
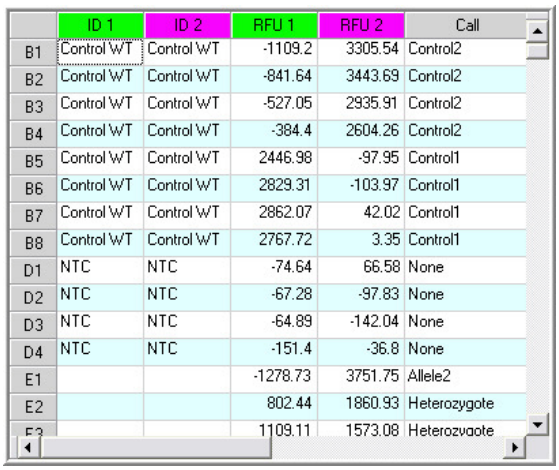


Fig. 6.29. The Allelic Discrimination Plot Menu.

- **Adjust Graph:** This feature allows you to change the way that the plot is presented. The maximum and minimum values for either axis may be entered directly into the text boxes. Alternatively the up and down arrows can be used to set the maximum and minimum values
- **Restore Graph:** Use this to redraw the graph after zooming
- **Zoom Enable:** Use this to enlarge the desired plot area
- **Show Labels:** Labels each sample on the Allelic Discrimination plot with its well name
- **Copy Graph:** This will copy the displayed Allelic Discrimination plot to the clipboard for import into other programs
- **Print Graph:** This prints only the graph
- **Print Data:** This prints the Allelic Discrimination spreadsheet data

6.7.3 Allelic Data Spreadsheet

A six-column spreadsheet is displayed in the window (Figure 6.30). The first column is the well number, and the second and third are the identifier entered at plate setup for the two fluorophores. The fourth and fifth column present the RFU or threshold cycle data for both fluorophores, and the last column is the genotype call made by either the software or the user.



	ID 1	ID 2	RFU 1	RFU 2	Call
B1	Control WT	Control WT	-1109.2	3305.54	Control2
B2	Control WT	Control WT	-841.64	3443.69	Control2
B3	Control WT	Control WT	-527.05	2935.91	Control2
B4	Control WT	Control WT	-384.4	2604.26	Control2
B5	Control WT	Control WT	2446.98	-97.95	Control1
B6	Control WT	Control WT	2829.31	-103.97	Control1
B7	Control WT	Control WT	2862.07	42.02	Control1
B8	Control WT	Control WT	2767.72	3.35	Control1
D1	NTC	NTC	-74.64	66.58	None
D2	NTC	NTC	-67.28	-97.83	None
D3	NTC	NTC	-64.89	-142.04	None
D4	NTC	NTC	-151.4	-36.8	None
E1			-1278.73	3751.75	Allele2
E2			802.44	1860.93	Heterozygote
E3			1109.11	1573.08	Heterozygote

Fig. 6.30. The Allelic Data Spreadsheet.

- In Automatic Call mode, the iQ5 software creates the assignments based on the positions of the vertical and horizontal bars
- In Manual Call mode, the Call column becomes editable through a menu. This can be very useful if you want to change the definition of a sample from an unknown to a positive control, for example
- The data in the spreadsheet may be copied to the clipboard for export to another program by clicking in the top left corner of the spreadsheet and typing CTRL+C

6.7.4 Automatic/Manual Call

Select the type of analysis by clicking Automatic Call or Manual Call (Figure 6.31).

Fig. 6.31. The Automatic and Manual Call Options.

Automatic Call is the default option.

- Choose Automatic Call for the software to make genotype assignments for every unknown based on the positions of the vertical and horizontal bars and presents those assignments in the data spreadsheet. If at least three positive (homozygous) controls are specified for each fluorophore, the positions of the bars are based on the mean and standard deviation of the threshold cycles or RFU values of the controls. If insufficient numbers of controls are specified, then the position of each bar is determined by the range of threshold cycles or RFU values in the selected fluorophores. The positions of the bars may be manually adjusted by dragging them. Click Recalculate after any change in the position of the vertical and horizontal bars and the software will make new genotype assignments based on the adjusted positions.
- Choose Manual Call to make the genotype assignments directly into the data spreadsheet or on the plot. You can use the manual call feature to change the definitions of one or

more wells on the plot or in the spreadsheet and then return to Automatic Call and the software will position the vertical and horizontal bars based on the new definitions, and then make genotype assignments.

6.7.5 Manual Calls

To make manual calls on the plot:

1. Click Manual Call. A new box appears in the window (Figure 6.32).

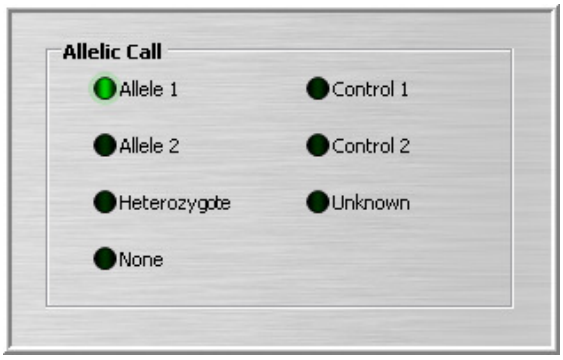


Fig. 6.32. The Manual Call Specification Options Box.

2. Select one of the allelic call types by clicking its radio button.
3. On the plot, drag the cursor around the well or wells to be assigned the call. When the mouse button is released, the new assignment will be made on both the plot and the data spreadsheet.

To make manual calls in the data spreadsheet:

1. Click Manual Call.
2. Click the cell for the desired well in the Call column of the data spreadsheet. Select the new call from the pull-down menu.
3. Note that "unknown" may only be selected in Manual Call mode.

6.7.6 Display Mode

Choose the aspect of the data that will be analyzed by clicking either **Threshold Cycle** or **RFU** (Figure 6.33).

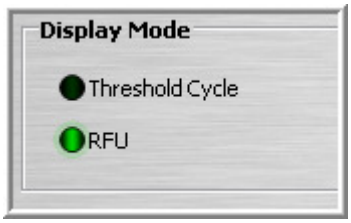


Fig. 6.33. The Display Mode Option Buttons.

When you click RFU, the Select Cycle option (Figure 6.34) appears so you can analyze RFU data from any cycle. Change the cycle by entering a new value in the Select Cycle drop-down list.

Fig. 6.34. The Select Cycle Drop-Down List Box.

6.7.7 Vertical Threshold

The Vertical Threshold box (Figure 6.35) displays the current position of the vertical bar. This box is not editable. The positions of the vertical bar along with the position of the horizontal bar determine the genotype assignment for unknown samples.

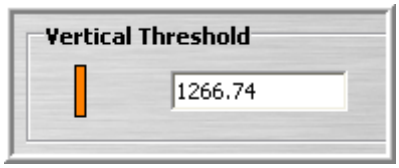


Fig. 6.35. The Vertical Threshold Box.

6.7.8 Horizontal Threshold

The Horizontal Threshold box (Figure 6.36) displays the current position of the horizontal bar. This box is not editable. The position of the horizontal bar along with the position of the vertical bar determines the genotype assignment of unknown samples.

Fig. 6.36. The Horizontal Threshold Box.

6.7.9 Normalize Data

When the RFU option button is selected, the Normalize Data option (Figure 6.37) appears as shown below if the plate setup contains a no template control (NTC) sample type for both Allele 1 and Allele 2.

Fig. 6.37. The Normalize Data Button.

The RFU data may be normalized and displayed on a plot that ranges from 0 to 1 on both axes. This display is sometimes a very effective presentation of RFU data. The RFU data is normalized to the NTC value as a linear combination of Allele 1 and Allele 2 specific signals. The iQ5 software uses the following formula initially introduced by Livak et al. (1995):

$$Normalized\ A_1 = \frac{A_1}{A_1 + A_2 + \bar{x}\left(NTC_{A1 + A2}\right)}$$

Where, A_1 represents Allele 1
A_2 represents Allele 2
$\bar{x}$ represents the mean
NTC_{A1+A2} represents the sum of NTC for Allele 1 and Allele 2

Reference: Livak JL, Marmaro J and Todd JA, Towards fully automated genome-wide polymorphism screening, *Nature Genetics*, 9, 341-342 (1995)

6.7.10 Restore Default

At any time, all modifications to the allelic discrimination data can be reversed by clicking **Restore Default**. The iQ5 software will reload the original x-axis allelic 1 fluorophore and y-axis allelic 2 fluorophore data, original display mode and Show Labels setting that were originally saved in the .opd file and will also set the Automatic Call method to recalculate the data, plot the chart, and display the results in the allelic discrimination data spreadsheet.

6.8 End Point Analysis

The End Point analysis module is a convenient method of analyzing final relative fluorescence unit (RFU) values.

End point analysis can be performed in two ways:

- Selecting the End Point 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

Newly collected End Point data is displayed immediately following an End Point run. Any existing .opd data file can also be viewed in the end point module 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. Although any file with amplification data may be analyzed post-run in both the PCR Quant and End Point tabs, an End Point 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.

6.8.1 The End Point Analysis Settings

The End Point tab is comprised of several sections, each one described in detail below. The Display Wells and Analyze Wells buttons have the same functions as described in section 6.2.4.

Method Box: End Point Analysis 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 or 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 or 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. The definition of the RFU range is dependent on the method chosen
- For the negative method, the range is the highest RFU value minus the negative control average.
- For the positive 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.
- **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.

End Cycles to Average

End Cycles to Average is the number of cycles from the last cycle, which 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 the iQ5 software to analyze non-End Point Only data in the End Point tab, at least 6 repeats of 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 end point analysis of PCR quantification data, 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 Quant 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 dependent on the method chosen
    - If the Positives method is chosen, the Range is the Positive Controls Average RFU minus the lowest RFU.
    - If the Negatives method is chosen, the Range is the highest RFU minus the Negative Controls average RFU.
    - If the Positives & Negatives method is chosen, the Range is the Positive 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 Positives 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.

6.8.2 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 **Recalculate** is clicked. 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 plate setup. You may click on the Sample Type column header to sort the table by sample type
- **End RFUs:** Lists the absolute RFU averages for each well, as calculated from the end cycles, which are specified in the End Cycles to Average field. You may click on the End RFUs column header to sort the table by end RFUs

- **Define Controls:** Lists any positive or negative controls defined in the original plate setup file. You may also add new controls or edit existing controls in this column by clicking on the drop-down menu on the right side. The options are (+) Positive, (–) Negative, or blank. Alternatively, you may type the letter 'p' or the plus sign (+) to select a positive control, and you may also type the letter "n" or the minus sign (–) to select a negative control. The controls specified in this column are used in the end point analysis calculations that assign positive or negative values to the unknowns. 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 Recalculate has been clicked. Unknowns Call may be (+) Positive, (–) Negative, or blank. You may click on the Unknowns Call column header to sort the table by Unknowns Call
- **Unknowns Ranking:** Lists the rank into which each unknown falls. An unknowns ranking depends on the total number of ranks and the end RFUs value. You may click on the Unknowns Ranking column header to sort the table by Unknowns Ranking
- **Identifier:** Lists the identifier for every well, as defined in the plate setup. You may click on the Identifier column header to sort the table by identifier

6.8.3 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 any of these error conditions is present when you click Recalculate.

Once 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 analysis spreadsheet.

6.9 Gene Expression Analysis

The Gene Expression (Gene Expr) tab is used 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.

Gene expression analysis is the most common real-time PCR 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 how to use the gene expression module.

Without stringently quantified controls, you cannot use this part of the iQ5 software to evaluate target concentrations of particular sequences relative to each other. The iQ5 software can only evaluate relative differences of a sequence between a group of samples.

The gene expression analysis screen has flexible tools for the determination of the fold induction of one gene relative to another gene, or relative to itself under different circumstances; that is, temporally, geographically, or developmentally different points. Additionally, within the gene expression analysis screen, it is possible to create a Gene Study from multiple plates of data collected at different times.

The gene expression analysis screen (Figure 6.38) consists of four main areas:

- **The graphical data view section:** Displays graphed data and graph settings
- **The Settings and Data Table options:** Located to the right of the graph section. Used respectively to specify analysis settings and access the data spreadsheet
- **The gene expression plate interface section:** Located at the bottom of the screen. A spreadsheet view of the plate setup used to modify and edit gene and condition names
- **Analysis method radio buttons:** Located above the gene expression plate interface section. Used to select between normalized and relative quantity gene expression analysis methods

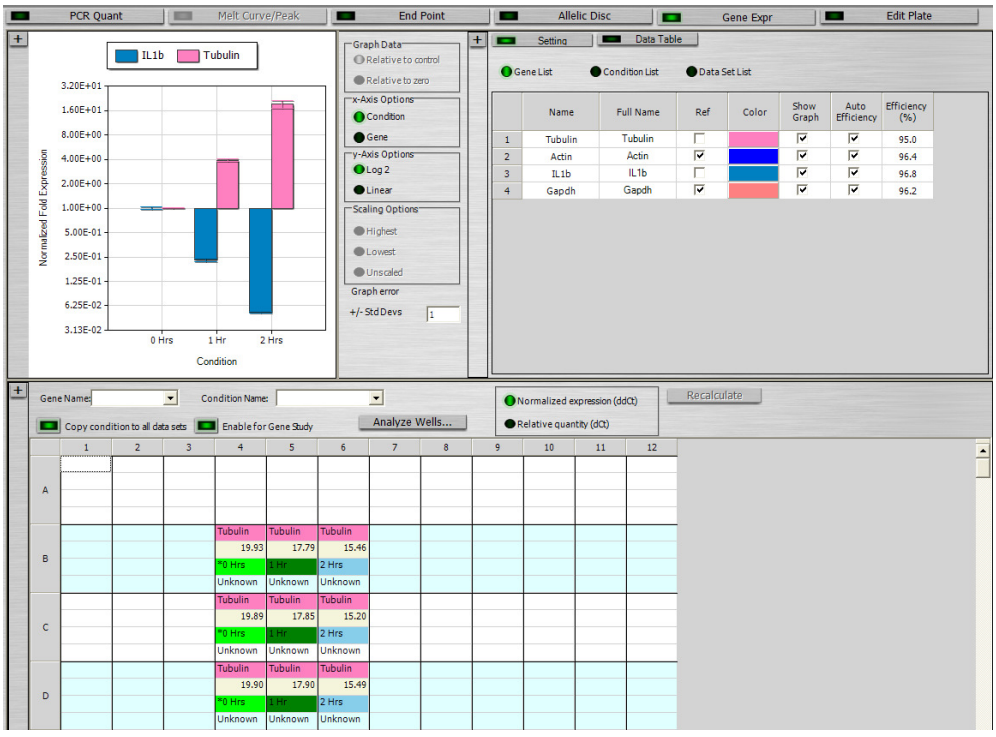


Fig. 6.38. The Gene Expression Analysis Tab Window.

Basic Workflow Steps for Gene Expression Analysis

1. In the PCR Quant screen with a .opd data file open, assess threshold and baseline information for the data file and make changes if necessary.
2. Click the Gene Expr tab.
3. Make any changes to the Gene and Condition (for example, Sample and Treatment) assignments in the gene expression plate interface.
4. Set reference genes if required, and choose an analysis method:
   - Normalized Expression (ddC_T) is the default; or
   - Relative Quantity (dC_T)
6. **OPTIONAL:** Assign attributes (sample color, show graph, etc.) and user-defined reaction efficiencies in the Gene List.
7. **OPTIONAL:** Assign attributes (sample color, show graph, etc.) and set control samples in the Condition List.
8. Click Recalculate to see your results.

If you want to compare these data to results obtained in other .opd files, you will need to enable this file for Multi-file Gene Expression analysis, also called a Gene Study. To enable your file for Gene Study:

1. Click Enable for Gene Study.
2. Go to the File menu to save your file.

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.

6.9.1 Normalized Gene Expression (ddC_T, $\Delta\Delta C_T$)

Rather than using some other method to normalize data, you may use the measured expression level of one or more reference genes as a normalization factor. Reference genes should be genes which are not regulated in the biological system being studied.

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, normalized expression calculations will account for loading differences or variations in cell number represented in each of your samples. This value is sometimes referred to as ddC_T or $\Delta\Delta C_T$ because of the equation initially introduced by Livak et al. (1995) to evaluate normalized expression. The software uses a modification of this equation.

Normalized expression can be viewed as normalized target sequence quantity if your assay has included controls for this purpose.

To calculate Normalized Expression (ddC_T):

1. In the PCR Quant screen with a .opd data file open, assess Threshold and Baseline information for the data file and make changes if necessary.
2. Click the **Gene Expr** tab.

3. Make any changes to Gene and Condition (for example, Sample and Treatment) assignments in the gene expression plate interface.
    - Expand the gene expression plate interface view by clicking on the "+" button to make well identification and selection easier. Highlight the wells in the gene expression plate interface you wish to edit
    - Change the gene assignments in these wells by typing your desired name into the gene pull-down men, then click enter to apply the name to the selected wells
    - Change the condition assignments in these wells by typing your desired name into the condition pull-down menu, then click enter to apply the name to the selected wells

    NOTE: The Gene and Condition Names have a character entry limit of 15 characters. Minimize the gene expression plate interface by clicking on the "–" button to return to the standard view of the Gene Expr tab window.
4. Click the **Settings** tab, then select **Gene List** to set your desired reference gene(s).
5. Click **Normalized expression (ddCt).**
6. Click **Recalculate** to see your results.
7. Normalized expression results are graphed. The Data Table spreadsheet is accessed by clicking **Data Table**. The Data Table spreadsheet lists the Condition and Gene name, calculated expression values and C_T values. Right-click to print or export this data to Excel.

    NOTE: Use the **Settings** tab, then click Gene List to enter a specific user-defined gene reaction efficiency. Use the Settings tab, then click Condition List to select a particular sample as a control sample.

6.9.2 Relative Quantity (dC_T, 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 dC_T or ΔC_T because of the equation used to calculate relative quantity.

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:

- 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 to obtain normalized data. The data are normalized by experimental design

  or;
- Any variance in the amount of biological sample loaded will be normalized in post-PCR analysis by some method. For example, a researcher might choose to 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:
    - Mass of nucleic acid loaded for each sample. Rel Quant/ng RNA represented in each sample

- Number of cells from which nucleic acid was isolated. Rel Quant/number of cells represented in each sample
- Mass of tissue from which nucleic acid was isolated. Rel Quant/mass of tissue represented in each sample

To calculate Relative Quantity (dC_T):

1. In the PCR Quant screen with a .opd data file open, assess threshold and baseline information for the data file and make changes if necessary.
2. Click the **Gene Expr** tab.
3. Make any changes to Gene and Condition (for example, Sample and Treatment) assignments in the gene expression plate interface.
   - Expand the gene expression plate interface view by clicking on the "+" button to make well identification and selection easier. Highlight the wells in the gene expression plate interface you wish to edit.
   - Change the gene assignments in these wells by typing your desired name into the gene pull-down men, then click enter to apply the name to the selected wells.
   - Change the condition assignments in these wells by typing your desired name into the condition pull-down menu, then click enter to apply the name to the selected wells.

     NOTE: The Gene and Condition Names have a character entry limit of 15 characters
   - Minimize the gene expression plate interface by clicking on the "-" button to return to the standard view of the Gene Expr tab window.
4. Click **Relative Quantity (dCt)**.
5. Click **Recalculate** to see your results.
6. Relative quantity results are graphed. The Data Table spreadsheet is accessed by clicking **Data Table**. The Data Table spreadsheet lists the Condition and Gene names, and calculated expression values and C_T values. Right click to print or export this data to Excel.

   NOTE: Use the **Settings** tab, then click **Gene List** to enter a specific user-defined gene reaction efficiency.

   NOTE: Use the **Settings** tab, then click **Condition List** to select a particular sample as a control sample.

6.9.3 Specifying Gene and Condition Labels for Gene Expression Analysis

Use the gene expression plate interface to edit gene names (for example actin, GAPDH), and experimental conditions names (for example, treatment types, time course points, etc.). Use the Settings option to select which of these genes and/or conditions to use for normalization and set other analysis and display parameters.

Using the Gene Expression Plate Interface to Edit Gene and Condition Names

Open a .opd Real-Time PCR Data file.

1. In the PCR Quant screen, assess threshold and baseline for the data file and then make changes if necessary.
2. Click the Gene Expr tab.
3. Highlight and select the wells to be edited in the gene expression plate interface.
   NOTE: Expanding the gene expression plate interface view by clicking on the "+" button makes the entire plate area visible for easier well identification and selection.
4. Change the gene and condition assignments in these wells by typing in the gene and condition pull-down menus, then click Enter to apply the name to the selected wells. Entered names will then be added to the pull-down menu, and become available for selection from this menu.
   NOTE: The Gene and Condition Names have a character entry limit of 15 characters.

   In Figure 6.39, wells B4, C4, D4 are selected, and are being changed from condition name 1 Hr to 1.5 Hrs. The new condition name, 1.5 Hrs, was typed in the Condition Name box.

In addition to the gene expression plate interface, gene and condition names can also be edited post-run using Edit Plate. Opening this window when analyzing collected data by clicking on the Edit Plate tab at the top of the screen will open a modified version of this window. In this modified window you can edit sample type, replicates, standard concentrations and primer/probe-gene names. For further details on post-run edit plate functionality, refer to section 6.11.

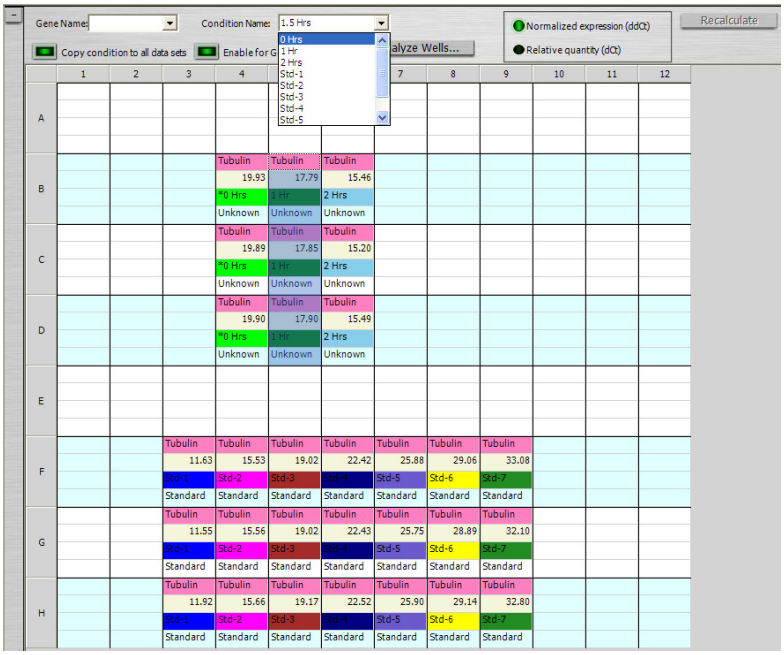


Fig. 6.39. Editing Gene and Condition Names in Gene Expression Plate Interface.

Copying Conditions to All Data Sets

The **Copy conditions to all data sets** button should be activated if you want all fluorophores or data sets to have the same Condition Name(s) assigned to selected groups of wells. This is a useful function if:

- You have a multiplex experiment. In this case all sample types in the varying fluorophores must be the same

  or;

- You are importing multiple .opd (data files) into a Gene Study

Make sure the **Copy conditions to all data sets** button is highlighted (active) when you assign condition (sample) names as shown in Figure 6.40. Active is the default state for this button.

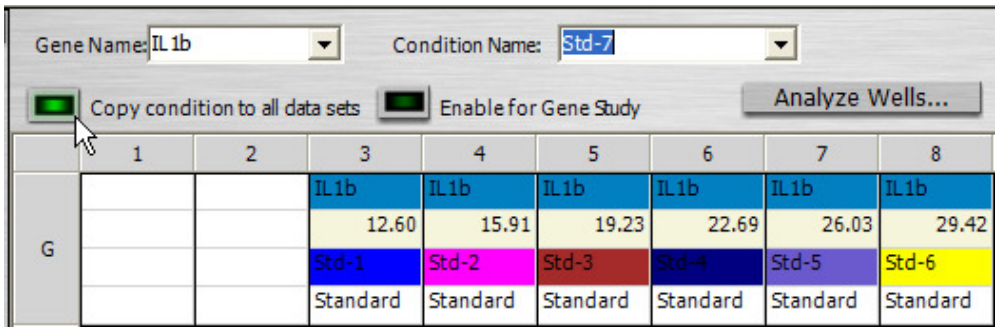


Fig. 6.40. Copying Gene and Condition Settings to all Fluorophores.

Gene Expression Plate Interface Context Menu

The plate interface menu is displayed by right-clicking on the plate interface. When this action is performed, a menu is displayed with the following items: Copy Gene Name, Copy Condition Name, Paste Gene/Condition Name, Show Sample Type, Print and Export to Excel (Figure 6.41).

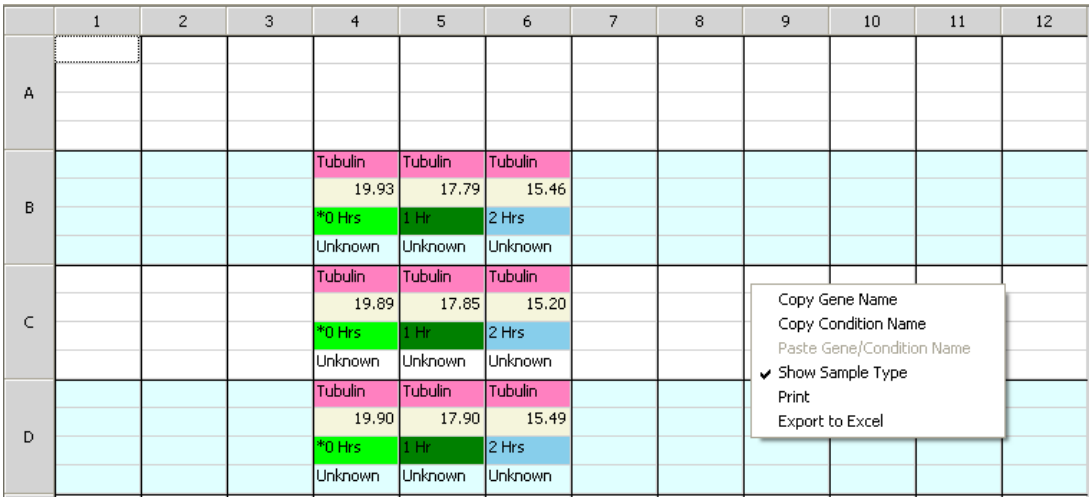


Fig. 6.41. The Context Menu of the Gene Expression Plate Interface.

- **Copy Gene Name** and **Copy Condition Name:** These two commands are useful shortcuts for copying and pasting gene and condition names across many different samples within a single .opd or Gene Study.

1. Activate the copy command by first selecting the wells to be copied from the plate interface.
2. Right-click on the plate interface to reveal the Copy Gene or Copy Condition options, and select the desired option.
3. Once the Copy Gene or Copy Condition option has been selected, the Paste Gene/Condition Name text from the menu becomes active (no longer grayed out).
4. Return to the plate interface to select the destination wells for the copied identification information.
5. Right-click on the plate interface to reveal and select the Paste Gene/Condition Name option.

- **Show Sample Type:** The Show Sample Type option is selected by default. This selection is illustrated by a check mark shown before the text in the menu. Clicking on Show Sample Type toggles the display of the Sample Type in the plate interface. Choosing to remove the sample type information from the plate interface simply allows visibility of a greater number of wells in the plate interface grid.
- **Print:** The Print command, on the plate interface menu, will print the displayed plate interface.
- **Export to Excel:** The Export to Excel command is useful for exporting exact values from the plate interface. When the Export to Excel command is selected from the menu, the iQ5 software exports the selected data into a protected workbook that is automatically open by Microsoft Excel. The protected workbook contains all of the formatting and fluorophore information separated into worksheets.

  NOTE: The numeric values contained in the protected workbook are exact values from the software application that include several non-significant figures beyond the decimal point. This is important to note when considering whether to transfer spreadsheets by a copy and paste command or the Export to Excel command. With the copy and paste command, only the significant digits displayed in the iQ5 software interface are transferred to Excel.

6.9.4 Setting Analysis Parameters for Gene Expression Analysis Using the Settings Tab

The Settings tab is used to assign gene and conditions parameters and efficiency values to be used in the calculations for gene expression analysis. There are three sections of the Settings options window:

- Gene List
- Condition List
- Data Set List

6.9.4.1 Gene List: Assigning Reference Genes and Target-Specific Reaction Efficiency

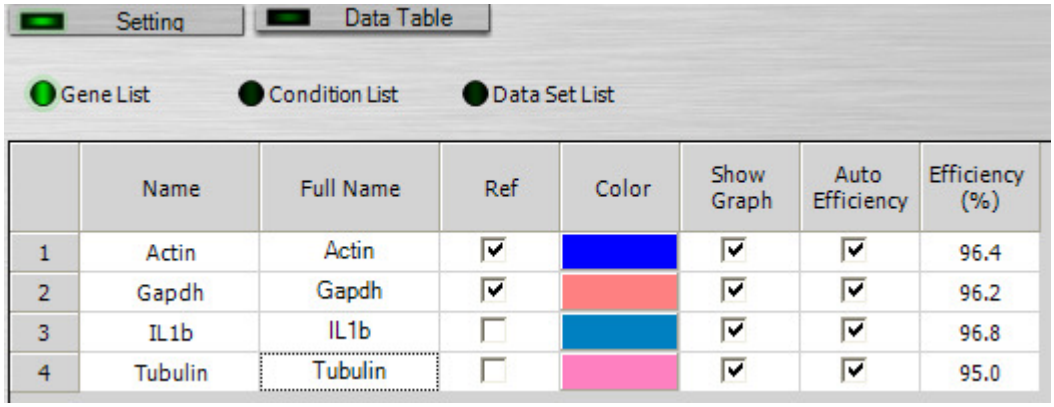
When the Settings button is active and Gene List is selected, the genes being analyzed in the experiment are listed along with their full name. This Full Name column is editable so that longer, more descriptive names may be entered if your Gene Name entry was abbreviated.

NOTE: Gene Name entries have a character entry limit of 15 characters.

To access the gene list:

1. Make sure the Settings button is activated. If not, click Settings. See the example in Figure 6.42.
2. Click Gene List.

The list of genes appears in the spreadsheet directly below the Gene List option button as shown in Figure 6.42.



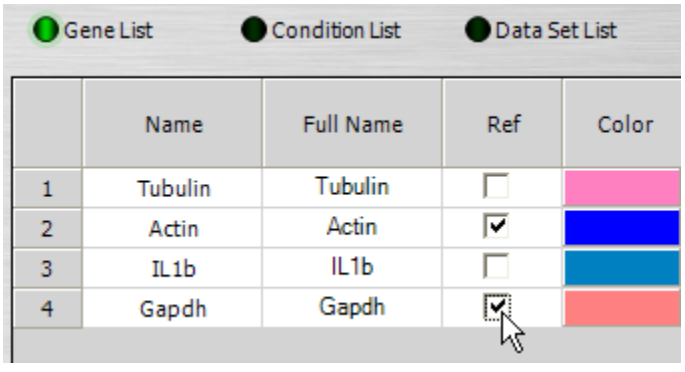
	Name	Full Name	Ref	Color	Show Graph	Auto Efficiency	Efficiency (%)
1	Actin	Actin	☑		☑	☑	96.4
2	Gapdh	Gapdh	☑		☑	☑	96.2
3	IL1b	IL1b	☐		☑	☑	96.8
4	Tubulin	Tubulin	☐		☑	☑	95.0

Fig. 6.42. Selecting the Gene List Option.

Assigning a Reference Gene

To assign a reference gene:

1. With the Settings button active, select Gene List.
2. Select the checkbox in the Ref column within the control condition(s) to which you want to assign a reference gene. You can assign the role of reference gene to as many genes as you wish. In Figure 6.43, Actin and Gapdh have been selected as reference genes.



	Name	Full Name	Ref	Color
1	Tubulin	Tubulin	☐	
2	Actin	Actin	☑	
3	IL1b	IL1b	☐	
4	Gapdh	Gapdh	☑	

Fig. 6.43. Assigning a Reference Gene.

Reaction Efficiency and Gene Expression Analysis

Efficiency describes how much of your sequence of interest is being produced with each cycle. An efficiency value of 100% 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 gene (primer/probe) pairs will give you more accurate results when using the mathematical modules used in the Gene Expr tab.

You set the efficiency for each of your genes in the Gene List. The default value is 100%. So if no standards were included on the plate, by default an efficiency value of 100% will be reported. This efficiency value is fully editable to allow entry of other than a default 100% value established from previous experiments.

If a standard curve was included on the plate, the software will automatically calculate the efficiency for that gene and report it in the Efficiency (%) column. To use Auto Efficiency, you must have standards in your experiment that result in valid standard curves in the PCR Quant tab. The iQ5 software only requires two standards at different concentrations, though it is recommended to have at least four samples in triplicate across a relevant dynamic range.

6.9.4.2 Assigning and Naming Control Samples Using the Condition List

When the Settings button is active and Condition List is selected, the conditions being analyzed in the experiment are listed along with their full name. This Full Name column is editable so that longer more descriptive names may be entered if your Condition Name entry was abbreviated.

NOTE: Condition Name entries have a character entry limit of 15 characters.

The Condition List option represents particular tests or treatments being evaluated for the purposes of your experiment. 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 on a graph.

You can view conditions as samples in the Condition List spreadsheet as shown in Figure 6.44.

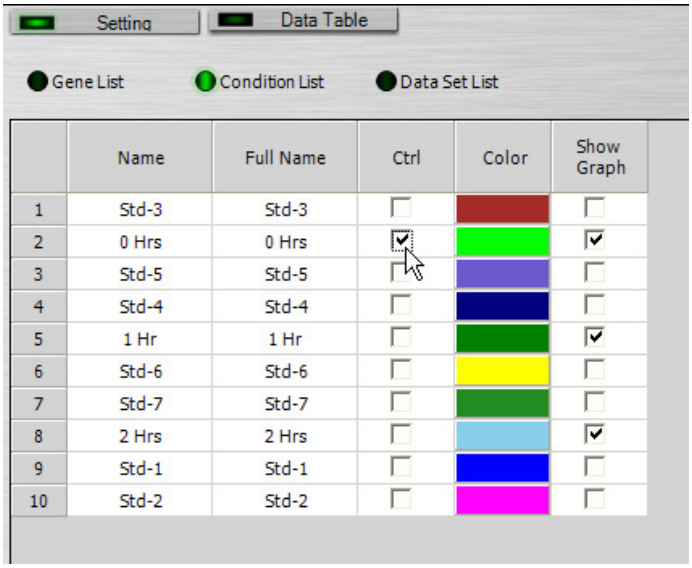


Fig. 6.44. The Condition List.

Setting Control Conditions

You can assign one condition as the control condition by selecting a checkbox next to the sample in the Condition List as shown in Figure 6.44.

The iQ5 software 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 1 for all genes.

To access the condition list:

1. Make sure the Settings radio button is activated.
2. Click Condition List option.
3. Select the checkbox(es) in the Ctrl column within the control condition(s) to which you want to assign a control condition.

6.9.4.3 Data Set List

The Data Set List displays pertinent information about the data being analyzed in a given Gene Study or .opd file. The information contained within the cells of the Data Set List is for display only, and cannot be modified.

Each row of the Data Set List is linked to a single data tab of the gene expression plate interface, located at the bottom of the Gene Expr window. Selecting a row from the spreadsheet automatically toggles to its representative dye layer data tab in the plate interface. Selecting a dye layer data tab results in toggling to its representative row in the Data Set List spreadsheet.

Information displayed in the Data Set List includes:

- **Name**: Displays the fluorophore name for a given set of collected data. A number is placed before the abbreviated fluorophore name. The assigned number helps to distinguish between data from the same fluorophore when multiple files are used (as in a Gene Study)
- **Full Name**: This cell contains the original name of the opd file as well as the original location of the opd when it was created
- **Created Date**: This cell displays the date and time the .opd file was created

The Name and Full Name column contents can be alphabetically sorted by clicking on the title header of each cell. This feature is helpful when long list of fluorophores are created as with multiple files (see Gene Study).

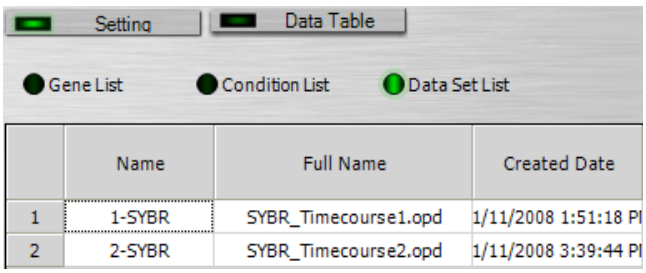


Fig. 6.45. Accessing and viewing the Data Set List.

6.9.5 Applying an Analysis Method

1. Choose an analysis method by clicking one of the option buttons:
   - Normalized expression (ddCt) is the default and requires you to first choose a reference gene)

   or

   - Relative quantity (dCt)
2. Click on **Recalculate** to apply the new settings.

   NOTE: 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 (Figure 6,46).

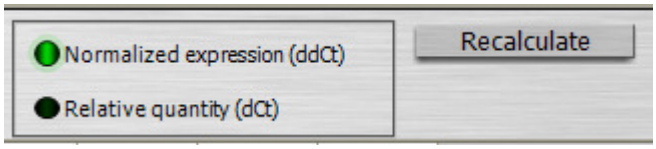
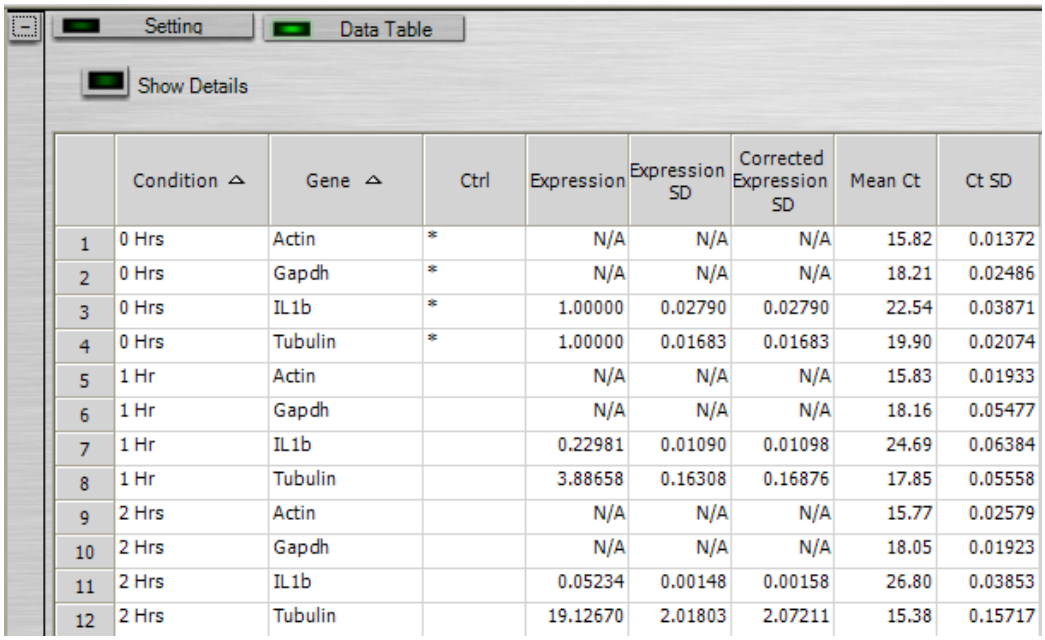


Fig. 6.46. Applying an Analysis Method.

6.9.6 Data Table for Gene Expression Analysis

The Data Table spreadsheet for gene expression analysis is accessed by clicking Data Table. The Data Table spreadsheet lists the Condition and Gene names, and calculated expression and C_T values (Figure 6.47). Columns can be resized and re-ordered by clicking and dragging. Any condition being used as a control is indicated by an asterisk in the Ctrl column. Columns can be sorted by Condition or Gene name by clicking on the sort triangle icon next to the column heading.



	Condition	Gene	Ctrl	Expression	Expression SD	Corrected Expression SD	Mean Ct	Ct SD
1	0 Hrs	Actin	*	N/A	N/A	N/A	15.82	0.01372
2	0 Hrs	Gapdh	*	N/A	N/A	N/A	18.21	0.02486
3	0 Hrs	IL1b	*	1.00000	0.02790	0.02790	22.54	0.03871
4	0 Hrs	Tubulin	*	1.00000	0.01683	0.01683	19.90	0.02074
5	1 Hr	Actin		N/A	N/A	N/A	15.83	0.01933
6	1 Hr	Gapdh		N/A	N/A	N/A	18.16	0.05477
7	1 Hr	IL1b		0.22981	0.01090	0.01098	24.69	0.06384
8	1 Hr	Tubulin		3.88658	0.16308	0.16876	17.85	0.05558
9	2 Hrs	Actin		N/A	N/A	N/A	15.77	0.02579
10	2 Hrs	Gapdh		N/A	N/A	N/A	18.05	0.01923
11	2 Hrs	IL1b		0.05234	0.00148	0.00158	26.80	0.03853
12	2 Hrs	Tubulin		19.12670	2.01803	2.07211	15.38	0.15717

Fig. 6.47 Gene Expression Analysis Data Table.

Corrected Values Calculations

The efficiency value (E) used in gene expression calculations has an associated error. If a standard curve was generated as part of the real-time PCR assay, this error can be calculated and used to adjust the error associated with the following standard deviation values:

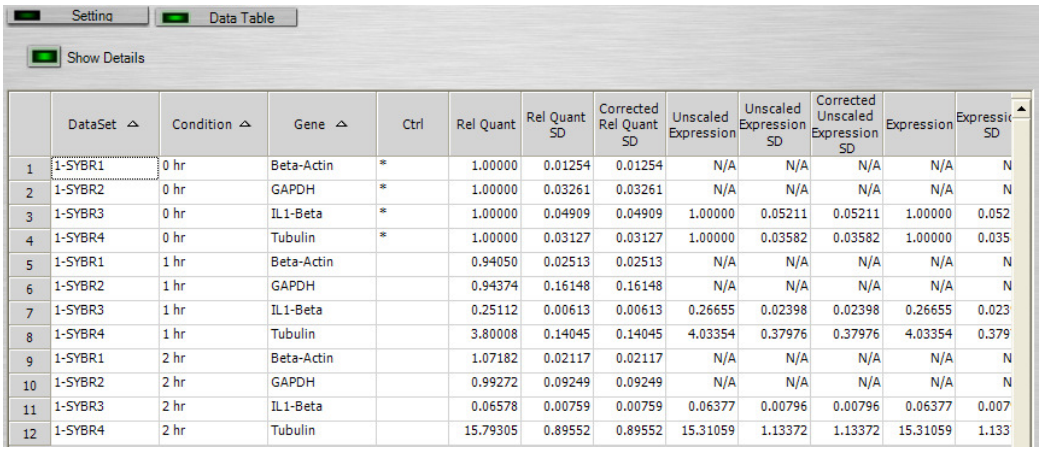
- Rel Quant SD
- Unscaled Expression SD
- Expression SD

Corrected Values for all gene expression results are displayed on the Data Table spreadsheet when **Show Details** is selected (Figure 6.48). When Show Details is active, three new columns appear in the Data Table spreadsheet. The new columns display the error correction propagated from the standard curve data for:

- Relative Quantitation Standard Deviation
- Unscaled Expression Standard Deviation
- Expression Standard Deviation

The new columns are named:

- Corrected Rel Quant SD
- Corrected Unscaled Expression SD
- Corrected Expression SD



	DataSet	Condition	Gene	Ctrl	Rel Quant	Rel Quant SD	Corrected Rel Quant SD	Unscaled Expression	Unscaled Expression SD	Corrected Unscaled Expression SD	Expression	Expressi SD
1	1-SYBR1	0 hr	Beta-Actin	*	1.00000	0.01254	0.01254	N/A	N/A	N/A	N/A	N
2	1-SYBR2	0 hr	GAPDH	*	1.00000	0.03261	0.03261	N/A	N/A	N/A	N/A	N
3	1-SYBR3	0 hr	IL1-Beta	*	1.00000	0.04909	0.04909	1.00000	0.05211	0.05211	1.00000	0.052
4	1-SYBR4	0 hr	Tubulin	*	1.00000	0.03127	0.03127	1.00000	0.03582	0.03582	1.00000	0.035
5	1-SYBR1	1 hr	Beta-Actin		0.94050	0.02513	0.02513	N/A	N/A	N/A	N/A	N
6	1-SYBR2	1 hr	GAPDH		0.94374	0.16148	0.16148	N/A	N/A	N/A	N/A	N
7	1-SYBR3	1 hr	IL1-Beta		0.25112	0.00613	0.00613	0.26655	0.02398	0.02398	0.26655	0.023
8	1-SYBR4	1 hr	Tubulin		3.80008	0.14045	0.14045	4.03354	0.37976	0.37976	4.03354	0.379
9	1-SYBR1	2 hr	Beta-Actin		1.07182	0.02117	0.02117	N/A	N/A	N/A	N/A	N
10	1-SYBR2	2 hr	GAPDH		0.99272	0.09249	0.09249	N/A	N/A	N/A	N/A	N
11	1-SYBR3	2 hr	IL1-Beta		0.06578	0.00759	0.00759	0.06377	0.00796	0.00796	0.06377	0.007
12	1-SYBR4	2 hr	Tubulin		15.79305	0.89552	0.89552	15.31059	1.13372	1.13372	15.31059	1.133

Fig. 6.48. The Gene Expression analysis Data Table – Show Details view.

A difference between corrected and non-corrected values will only be seen if a standard curve was created as part of the real-time PCR assay. The equations used in determining the error propagation are the Standard Error, Standard Error for Normalized Expression, and the Standard Error for the Normalized Gene of Interest (GOI).

The equation for Standard Error is shown below:

$$SE = \frac{SD}{\sqrt{n}}$$

Where n = number of measurements

The Standard Error for Normalized Expression equation is shown below:

$$SE\ NF_n = NF_n \times \sqrt{\left(\frac{SE\ \mathrm{Re}l\ Quant_{\ sample\ (\mathrm{Re}f\ 1)}}{n \times \mathrm{Re}l\ Quant_{\ sample\ (\mathrm{Re}f\ 1)}}\right)^2 + \left(\frac{SE\ \mathrm{Re}l\ Quant_{\ sample\ (\mathrm{Re}f\ 2)}}{n \times \mathrm{Re}l\ Quant_{\ sample\ (\mathrm{Re}f\ 2)}}\right)^2 + \cdots + \left(\frac{SE\ \mathrm{Re}l\ Quant_{\ sample\ (\mathrm{Re}f\ n)}}{n \times \mathrm{Re}l\ Quant_{\ sample\ (\mathrm{Re}f\ n)}}\right)^2}$$

Where *NF* = Normalization Factor

The Standard Error for Normalized GOI equation is the following:

$$SE\quad GOI_{norm} = GOI_{norm} \times \sqrt{\left(\frac{SE\quad NF_n}{NF_n}\right)^2 + \left(\frac{SE\quad GOI}{GOI}\right)^2}$$

Where GOI_{norm} = the Normalized Gene of Interest

Exporting Data from the Data Table

1. Make sure the **Data Table** button is activated. If not, click **Data Table**.
2. Right-click on the spreadsheet. A shortcut menu appears (Figure 6.49).
3. Select **Export to Excel**.
4. Enter a name and file destination for the Excel file generated with your data export.

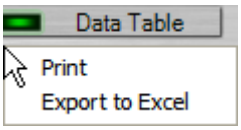


Fig. 6.49. Exporting Data Table Spreadsheets to Microsoft Excel.

Printing Data

The **Print** command on the spreadsheet menu will print the displayed spreadsheet (Gene List, Condition List, Data Set List, or Data Table). When selected, a **Print Preview** box is opened, which contains an illustration of the spreadsheet as it will appear once printed. Clicking the printer icon opens the Windows Print dialog box. Click **OK** to complete the printing task.

The Export to Excel command on the spreadsheet menu is useful for exporting exact values from the spreadsheet. When the Export to Excel command is selected from the menu, an Export to Excel file save box is displayed. Choose a location of where the Excel file is to be saved and click Save. The iQ5 software automatically exports the selected data into a protected workbook.

The protected workbook generated by the iQ5 software contains the text values of what is represented on the spreadsheet. For example, checkboxes from the software application are replaced by "True" or "False" text in Excel.

The numeric values contained in the protected workbook are exact values from the software application that include several non-significant figures beyond the decimal point. This is important to note when considering whether to transfer data by a copy and paste command or the Export to Excel command. With the copy and paste command only the significant digits displayed in the iQ5 software interface are transferred to Excel.

6.9.7 Graphing Options for Expression Data

Graph Expression Relative to Control or Relative to Zero

These options, shown in Figure 6.50, allow you to present data with bars originating at 1 (relative to control) or at zero (relative to zero). If you assign a control in your dataset, selecting the option to graph data relative to control allows you to quickly visualize upregulation and downregulation. The values graphed are exactly the same.

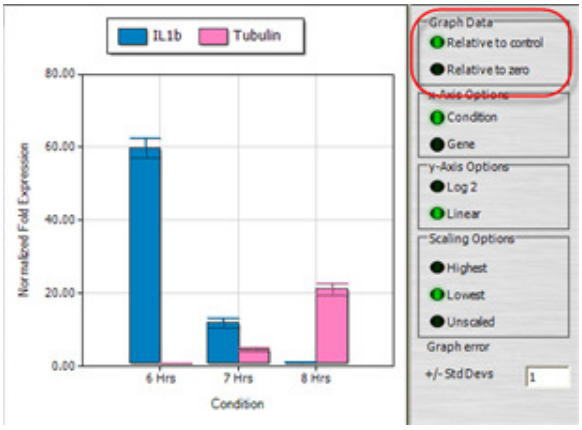


Fig. 6.50. Graphing Options for Displaying Gene Expression Data.

y-Axis Options

This option allows you to display the graph with the y-axis in log2 or linear scale as shown in Figure 6.51. The log2 scale is useful when you evaluate samples across a large dynamic range.

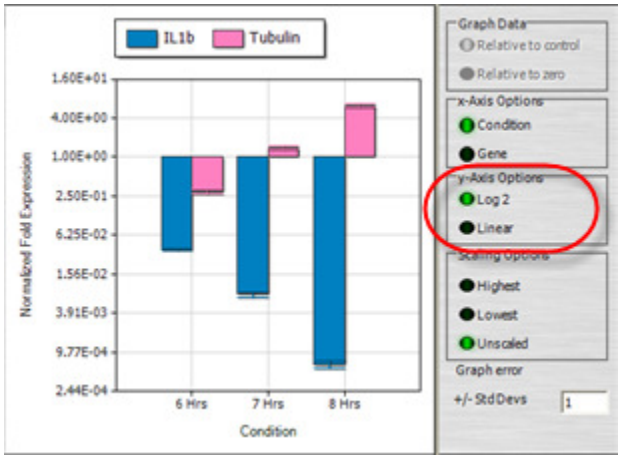


Fig. 6.51. Options for Displaying the y-axis on the Gene Expression Chart.

Scaling Options

These options, shown in Figure 6.52, 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.

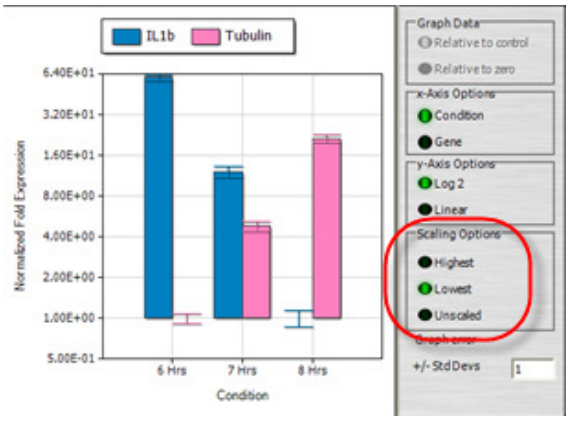


Fig. 6.52. Scaling options for Normalized Gene Expression.

- **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.

x-Axis Options

You may graph either genes or conditions on the x-axis by changing these options (Figure 6.53).

Fig. 6.53. Selecting x-axis Grouping Options.

Graph Error Options

The default presentation for the error bars is +/– one standard deviation (Figure 6.54). You can change the multiplier to get +/– 2 or 3 standard deviations.

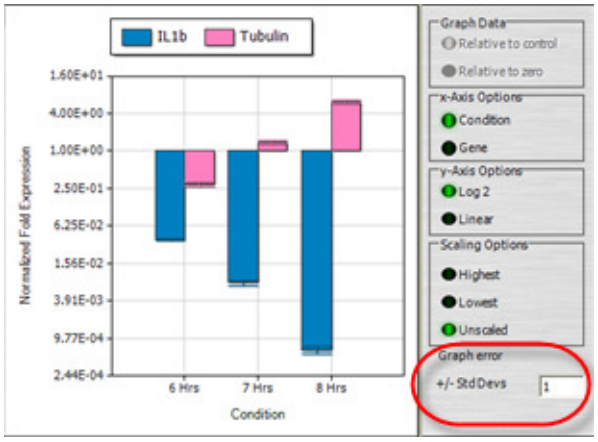


Fig. 6.54. Selecting Error Bar options for Gene Expression Analysis.

Gene Expression Module Context Menu

Right-clicking on the gene expression chart will reveal the context menu shown in Figure 6.55.

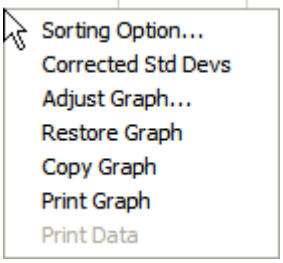


Fig. 6.55. The Gene Expression Chart Context Menu.

Sorting Option

Sorting Option (Figure 6.56) allows you to sort of the Gene and Condition Names on the chart display. When the Sorting Option is selected, a Sort Options menu box is displayed. The menu box is divided into three sections: Groups, Options, and the Group member list. Use the **Groups** radio buttons to view a list of Gene Names or Condition Names configured in your data set.

With the **Options** radio buttons, you can elect to organize the names within the Group member list alphabetically, or to rearrange these items manually

- **Alphabetic order:** The default selection for Groups is Gene Names and the default selection for Options is Alphabetical order. The arrows buttons to the right of the group member list can be used to toggle between an alphabetical sort of the group members in ascending or descending order
- **Manual order:** To rearrange Gene or Condition Names manually, select the radio button for Manual order. Select the group member that you would like to re-order and use the

arrow buttons to move the member to the desired position within the list of names. More than one Gene or Condition Name can be selected (highlighted) by clicking on the desired names. Note that when the Manual order radio button is active, a total of four arrow buttons are displayed. The outermost arrow boxes move the selected items to the top or bottom of the list, while the innermost arrow buttons allow for stepwise movement of the Gene or Condition Name

- When finished sorting, click OK to view the changes to the chart

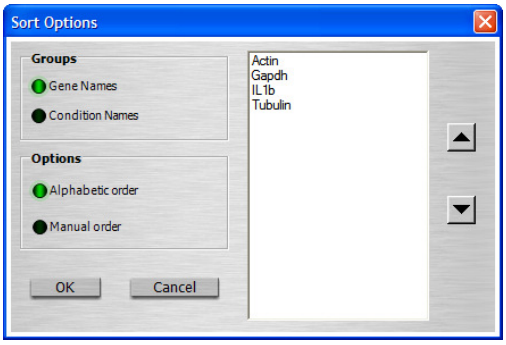


Fig. 6.56. The Sort Options Dialog Box.

Corrected Std Devs

The Corrected Std Devs selection enables the display of error correction propagated from the standard curve data. This option should only be used if a standard curve was created as part of the gene expression analysis. The resulting error correction is displayed as a property of the individual error bars on the chart. The exact error value can be displayed by mousing over the error bars to reveal the tool tip, or by displaying the Data Table and then selecting Show Details. When Corrected Std Devs is active, a check mark is displayed next to its name in the gene expression chart context menu.

Gene Expression Chart Tool Tips

Tool Tips are visible on the gene expression chart when the mouse cursor is positioned on one of the chart data bars or error bars.

When the mouse cursor is positioned on the chart data bars, the resulting Tool Tip will display the data label (that is, Gene or Condition Name, depending on which x-axis mode is selected) along with the calculated Expression value in parenthesis.

When the mouse cursor is positioned on the chart error bars, the resulting Tool Tip will display the Condition Error or Gene Error, depending on which x-axis mode is selected. The error value displayed is the Unscaled Expression standard deviation. However, the Tool Tip will display the Corrected Unscaled Expression standard deviation if Corrected Std Devs is selected from the Gene Expression context menu.

Copy Graph/Export a Graph

To export a graph:

1. Place the mouse pointer over the graph.
2. Right-click on the graph. A shortcut menu appears.

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.

6.9.8 Normalized Expression Calculations

The normalized expression calculation is stated below. To see how the iQ5 software calculates relative quantities, go to relative quantity calculations.

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

Normalized Expression when a Control is Chosen

When a control is chosen, the iQ5 software uses the equation listed above to calculate normalized expression. If a control is assigned, relative quantities for all genes within the control sample are equal to 1. This results in "normalized" expression of 1 for the control, and relative quantities for all other conditions will be presented relative to this normalized control sample. According to the calculations performed by the iQ5 software, normalized expression is equivalent to unscaled normalized expression analysis when a control is chosen.

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.

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

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.

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

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

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

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.

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

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

6.9.9 Relative Quantity Calculations

Relative Quantity Calculations with No Controls Identified

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

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

Where E = Efficiency of primer (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 with the lowest average C_T for gene x

$C_{T\ (sample)}$ = Average C_T for the sample

Relative Quantity When a Control is Assigned

With a control assigned relative quantity (dC_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 (primer/probe) set

this efficiency is calculated as follows (% Efficiency * 0.01 + 1)
where 100% = 2

$C_{T\ (Control)}$ = 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 Dr. Jo Vandesompele on the geNorm web site (http://medgen.ugent.be/~jvdesomp/genorm/). In the example on the geNorm Web site, the results are not scaled the control until 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 Relative Quantity = SD C_T sample * Relative Quantity sample * Ln(E(Gene x))

Where:

SD Relative Quantity = Standard Deviation of the Relative Quantity

SD C_T sample = Standard Deviation of the C_T of the sample

Relative Quantity sample = Relative Quantity of sample

E = Efficiency of primer (primer/probe) set

this efficiency is calculated as follows (% Efficiency * 0.01 + 1)
where 100% = 2

6.9.10 Gene Expression - Frequently Asked Questions

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 µg 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 of using less RNA and not the result of some biological response.

How does normalized expression calculated by this software compare to the value calculated using the ddC_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 ddC_T equation. The standard deviations will be larger since the error propagation outlined in the initial publication is inappropriate.

How does normalized expression as calculated by this software compare to the model introduced by Dr. M. Pfaffl, et al.
If you only evaluate one reference gene and one gene of interest you will get exactly the same results using the iQ5 software 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?
The iQ5 software uses the models outline on the geNorm web site (http://medgen.ugent.be/~jvdesomp/genorm/) and gives you the same results.

Why would I have to assign gene names in the Gene Expr 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 Expr tab to assign these relationships.

Can I customize my gene and condition names?
Yes. Both the gene and condition pull-down menus will accept any text you type into them. If your names are very long, you can edit the name field in the gene and condition lists. The long name will only be displayed in the legend above the graph. The name is displayed on the x-axis.

How do I determine reaction efficiencies?
Typically the efficiency for each primer (or 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.

6.10 Gene Study: Multi File Gene Expression Analysis

When conducting gene expression experiments, it may become necessary to run more than a single plate in order to analyze all required samples. Similarly, the experimental goal may be to analyze samples/conditions over a fixed time course. In both cases, it is essential to perform gene expression analysis on data generated in different data sets. To accommodate this need, a Gene Study file can be created in the iQ5 software.

6.10.1 The Gene Study File

A Gene Study file is a specialized file consisting of data and sample information imported from multiple .opd files. The imported data is grouped into a single study, which can be edited (files added or removed) and further analyzed at the users discretion. Gene Study files are assigned a file extension of .gxd by the iQ5 software.

A Gene Study file is capable of comparing approximately 5,000 total wells of data. This implies a maximum comparison of approximately 52 .opd files containing 96 wells of single-fluor (non-multiplexed) data, or 10 .opd files containing 96 wells of five-fluor (multiplexed) data. Many other combinations of file number, well capacity, and fluor number are possible. The absolute well maximum will depend on the amount of RAM and virtual memory available to your computer.

Enable for Gene Study Button

All .opd files must be enabled for gene study analysis from within the Data Analysis module of the iQ5 software. Enabling an .opd file for gene study analysis allows the iQ5 software to extract only the information critical to gene expression analysis between different files. This step is

performed by activating the **Enable for Gene Study** button found in the Gene Expression window of the Data Analysis module (Figure 6.57).

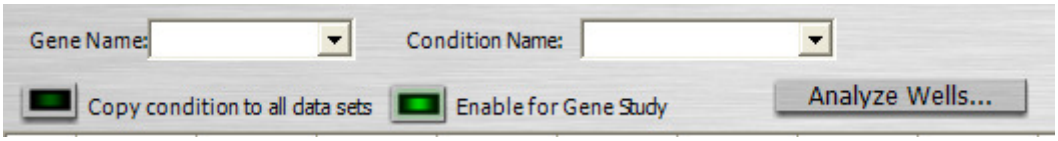


Fig. 6.57. The Enable for Gene Study button is located above the plate interface section of the Gene Expr tab window.

When a file has been saved with the Enable for Gene Study button active, it allows the saved file to be added to a Gene Study. The default setting is that this button is not enabled. You must save your data file after activating this option from within the Gene Expr tab window.

Gene Study Errors

The file must be saved with the **Enable for Gene Study** button selected. If a file with the Enable for Gene Study is not selected and there is an attempt to add the file to a Gene Study, an error will be received (Figure 6.58).

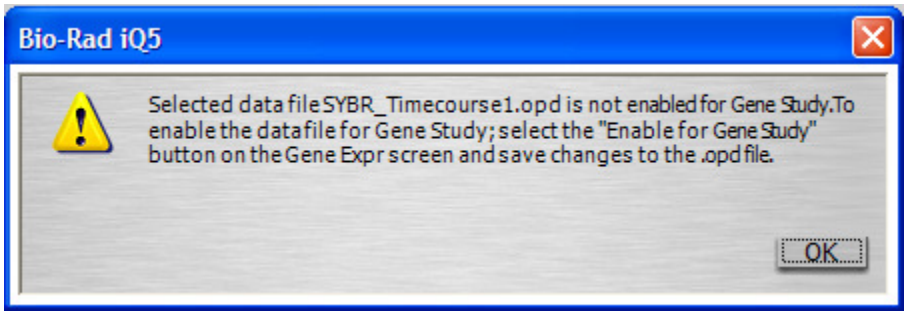


Fig. 6.58. Gene study error message indicating that the file was not enabled.

To correct this error:

1. Click OK to clear the error message.
2. Re-open the file that will be added to the Gene Study.
3. Confirm that all wells to be analyzed have valid C_T values and activate the Enable for Gene Study button.
4. Save the file.
5. Attempt to add that file to the Gene Study once again.

6.10.2 Creating a Gene Study File

A Gene Study file must contain data from at least two different .opd files. The process of creating a Gene Study file is a 2-step process.

STEP 1: Prepare .opd files for gene expression analysis.

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

   NOTE: Additional wells can be excluded or re-included later from the gene expression plate interface.

2. Click on the Gene Expr tab. Make sure that all files to be included in the Gene Study have the **Enable for Gene Study** button selected. The default setting is that this button is not enabled.
3. After enabling the file for multi-file analysis, save the .opd file.
4. Repeat steps 1–3, above, for all additional files to be included in the Gene Study.

STEP 2: Add files to a new Gene Study.

1. From the menu toolbar, select File > New > Gene Study. The Gene Expression Study Manager will be displayed.
2. Select Add .opds. The Windows file explorer dialog box will appear.
3. Locate and select the .opds needed for the Gene Study. Multiple files may be added to the Gene Study at one time.
4. Click OK when finished.

Once the files are added to the Gene Expression Study Manger, selecting OK opens an unsaved Gene Study file in the Gene Expression window of the iQ5 software. The Gene Study, which contains all the files selected from the Gene Expression Study Manager, can now be analyzed as a normal gene expression file by selecting the analysis method, assigning Gene and Condition Names from the gene expression plate interface and assigning attributes from the Gene List.

NOTE: Gene Study files are **NOT** automatically saved at the time of file creation. It is highly recommended to save the newly created .gxd file after initiating a Gene Study.

When the files are added to the Gene Expression Study Manager, the file information is separated into four columns:

- **File Selection:** This column is used in conjunction with the Remove button, and features a checkbox in the column header. The "header" checkbox will select or deselect all the displayed files chosen for the Gene Study. To select or deselect an individual file, click the checkbox within the corresponding row. If one or more files have been selected, clicking Remove will remove those file(s) from the Gene Study.
- **File Name:** This column displays the full name of the .opd files to be included in the Gene Study.
- **File Directory:** This column displays the directory location of the .opd files to be included in the Gene Study.

- **Created Date:** This column displays the creation dates of the .opd files to be included in the Gene Study. Note that these dates are NOT the dates of the most recent save event for the listed files.

6.10.3 Gene Expression Study Manager

The columns and rows of the Gene Expression Study Manager (Figure 6.59) are adjustable by clicking, holding and dragging the column/row separator lines. The overall size of the Gene Expression Study Manager box can also be resized by clicking, holding and dragging the outermost horizontal or vertical edge.

The Notes section, located at the bottom of the Gene Expression Study Manager, allows for input of text details pertaining to the study.

Clicking Show Details, located below the .opd spreadsheet, displays two additional columns of information. The two additional columns provide information about the Cycle and Step used for data collection and analysis in the source .opd file.

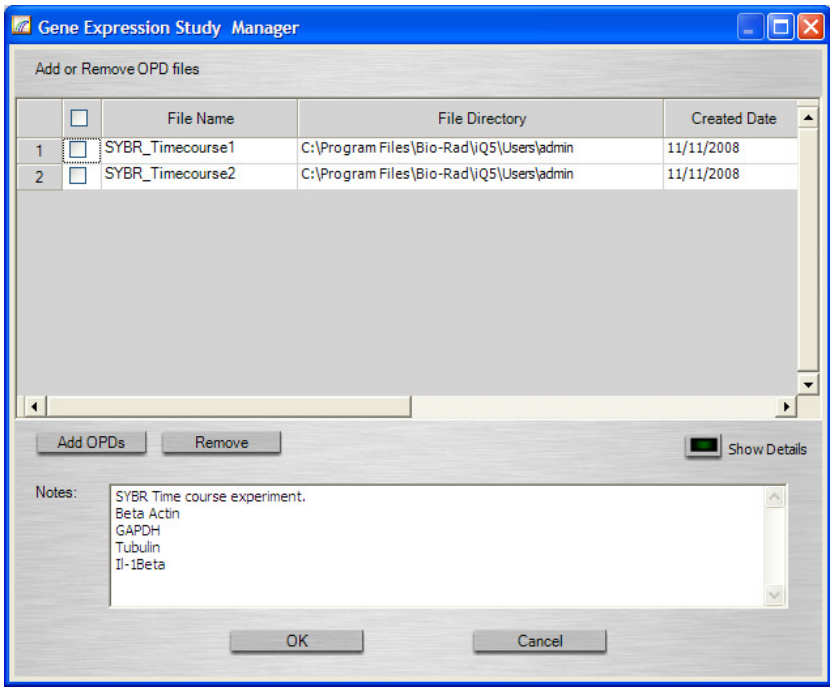


Fig. 6.59. Show Details view of the Gene Expression Study Manager Window.

6.10.4 Editing a Gene Study

A minimum of two different .opd files are required to create a new Gene Study file. Additional .opd files may be added to an existing Gene Study file by clicking the Edit Study button from within the Gene Expr window. The Edit Study button is only displayed in the Gene Expr window when a Gene Study file is being analyzed.

When you click on the Edit Study button, the iQ5 software will display the Gene Expression Study Manager. Individual files can be added to the Gene Study by clicking Add .opds. To remove files from the current Gene Study, To select or deselect an individual file, click the checkbox within the

corresponding row. If one or more files have been selected, clicking the Remove button will remove those file(s) from the Gene Study.

The Notes field is also editable from the Gene Expression Study Manager. Once the desired changes have been made, click OK to close the Gene Expression Study Manager and return to analysis of the Gene Study file in the gene expression module. Remember to immediately save your edits to the Gene Study before continuing with your analysis.

NOTE: The Edit Plate module of the iQ5 software is not available when a Gene Study file (extension, .gxd) is opened. All plate information changes to the data in a Gene Study (that is. sample ID), must be made from the plate interface of the Gene Expression window.

6.10.5 Excluding/Including Samples Using the Gene Expression Plate Interface

When a Gene Study file is displayed, the Analyze Wells button that is available when viewing a single Gene Expression file, is no longer displayed. In its place is the Include Sample/Exclude Sample drop down list located above the plate interface (Figure 6.60).

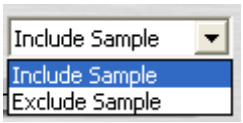


Fig. 6.60. The Include Sample/Exclude Sample Selection Pull-Down Menu.

This drop down list works similar to Analyze Wells button (see Analyze Wells for details). To exclude wells from the Gene Study analysis, select the specific wells from the plate interface. Click on the drop down arrow box to select Exclude Sample from the list. The highlighted wells will be grayed out to indicate that they are no longer included in the Gene Study analysis. To include wells for the Gene Study analysis, select the specific wells from the plate interface. Click on the drop down arrow box to select Include Sample from the list. The selected wells will be displayed with the correct Gene and Condition Name colors to indicate that they are included in the Gene Study analysis.

6.10.6 Inter-Run Calibration

Inter-run Calibration is unique to the Gene Study module. Inter-run Calibration is automatically performed by the iQ5 software in every Gene Study. The resulting Inter-run Calibration calculations are then used to normalize inter-run variations between genes assayed in separate real-time PCR runs (that is, different .opd files).

The following conditions must be met for Inter-run Calibration:

- A given sample must have the exact same Condition Name on each plate (per assay) and for each gene – this allows the sample to be designated as an "inter-run calibrator".
- At least one "inter-run calibrator" sample must be present in the Gene Study.

When the above conditions are met, an algorithm is used to calculate the pair-wise differences between the C_T values (dC_T) for all samples that qualify as inter-run calibrators (that is, having the same Condition Name per gene and per assay).

Inter-run Calibration Algorithm

All data within the Gene Study is normalized to the inter-run calibrator that yields the smallest average dC_T value. The inter-run calibrator with the smallest average dC_T value becomes the

dominant inter-run calibrator. The average dC_T value of the dominant inter-run calibrator will be used to adjust all C_T values within the Gene Study.

To find the dominant inter-run calibrator, the iQ5 Inter-run Calibration algorithm first calculates the average of the dC_T values for all inter-run calibrators of a given gene. The iQ5 software uses a multi-tiered algorithm to determine the dominant inter-run calibrator.

The algorithm utilizes the following hierarchy for determining the dominant inter-run calibrator:

1. Set the dominant calibrator to the gene with the highest number of common replicate groups in a given pair-wise comparison.

If multiple genes have the same number of common replicate groups, then:

2. Set the dominant calibrator to the gene with the smallest range of dC_T values in pair-wise comparisons. The range is examined by comparing the absolute value of the difference between the maximum and minimum dC_T for the inter-run calibrators of a given gene.

If multiple genes have an identical range of dC_T values, then:

3. Set the dominant calibrator to the gene with the smallest absolute value of "Average dC_T" of eligible inter-run calibrator samples.

If multiple genes have identical "Average dC_T" absolute values, then:

4. Set the dominant calibrator to the replicate group with the smallest dC_T.

NOTE: The first data file imported into the Gene Study will always serve as the "hub" file for pair-wise data comparisons during Inter-run Calibration.

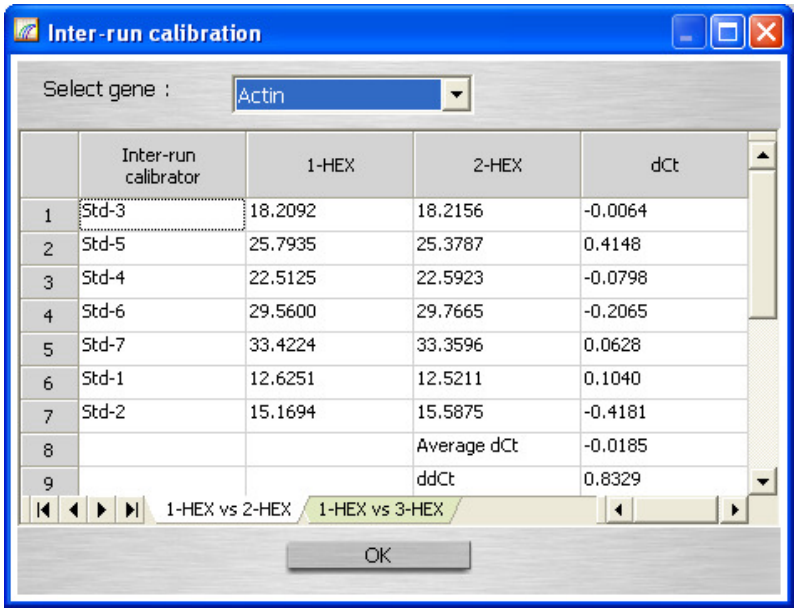


Fig. 6.61. The Inter-Run Calibrator Calculation Display.

Details of the Inter-run Calibration calculations can be accessed by clicking the Inter-run Calibration button. The resulting Inter-run Calibration window (Figure 6.61) will display the comparative fluorophore calculations per gene (pair-wise comparisons).

- You can choose to view the inter-run calibrator calculations for different genes in your assay by selecting a Gene Name from the Select Gene drop-down menu.
- You can choose to view the calculations resulting from different pair-wise comparisons by clicking on the desired tab displayed below the spreadsheet.

Inter-run Calibration Errors

When opd files are merged into a Gene Study that does not meet the above criteria, a warning message will be displayed which states that the genes do not contain common samples across all data sets (Figure 6.62). The warning message will specifically list the genes that are in violation. By clicking the warning message OK button, the analysis will proceed without any inter-run calibrators.

Clicking on the Inter-run Calibration button when there are no calibrators assigned will display a message box which states that inter-run calibrators are not available. Proceeding without inter-run calibrators may cause inaccurate representation of the data. To correct this problem change the Gene and/or Condition Names in the plate interface so that identical Gene and Condition Names are used across all data sets of the Gene Study. Use the Gene Name and Condition Name drop down menus to select the appropriate Gene and Condition Names or directly input the correct Gene and Condition Names. To change a large number of Gene and Condition well names across several plates use the Copy and Paste command from the right mouse button menu.

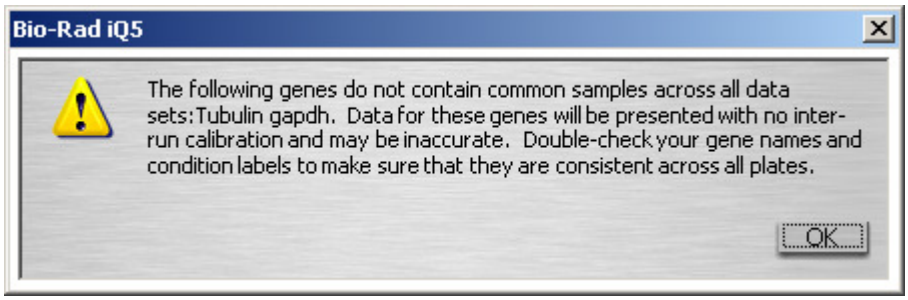


Fig. 6.62. No Inter-Run Calibrator Error.

6.11 Post-Run Plate Editing

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:

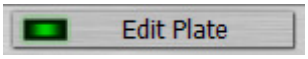
1. From the Workshop module:

    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 section 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 will open (Figure 6.63). In this modified window, you cannot add fluorophores to or remove fluorophores from the fluorophore list. Nor can you add a previously defined fluorophore or remove a previously defined fluorophore from a well.

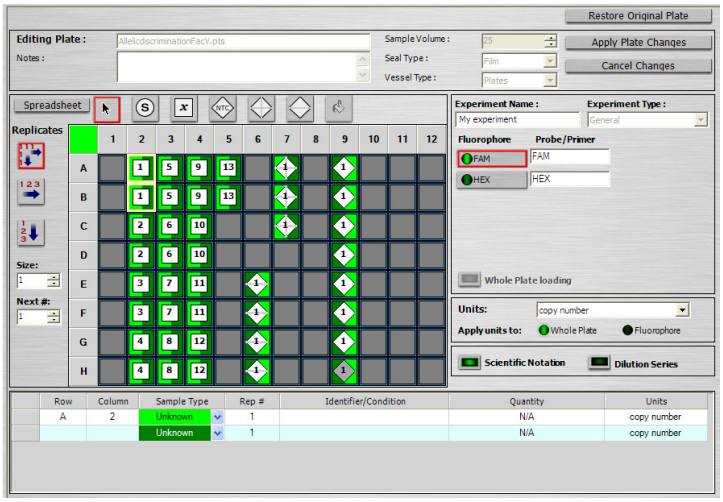


Fig. 6.63. The Edit Plate Window.

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. For most experiments the Whole Plate Loading box will be checked. With Whole Plate Loading, changes made to any fluorophore within a well are extended to the other fluorophores within the well and within the replicate group. If you are editing a plate, the Whole Plate Loading checkbox may be unavailable because it is not appropriate based on the current definition of the plate.
7. Click a fluorophore.
8. Click a sample type icon.

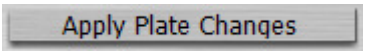
9. Select the type of replicate loading desired.
10. Click or drag across the plate to define wells with the selected fluorophore and sample type.
11. Continue defining the remaining wells that will contain the first fluorophore by changing to any other sample type icons required.

    To calculate standard concentrations automatically, click Dilution Series and enter the upper or lower concentrations and units of the standards, set the dilution factor, and click Apply Dilution Series.
12. Repeat steps 7-11 for any additional dye layers/fluorophores as required. Remember that if the Whole Plate Loading box is checked, changes made in standard concentrations will be applied to all dye layers for that well and extended to all replicates in the group.

    NOTE: To delete a previously defined well, click the Delete All icon, and then click the well. To delete the selected fluorophore from a previously defined well, click the Delete Fluorophore icon, and then click the well.

    NOTE: Use the Next # checkbox to enter a particular number to assign to the next standard or sample you define.

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



For more information about Edit Plate functions, refer to section 4.2.

6.12 The Reports Tool

The Reports tool of the iQ5 software is accessible from the menu bar of the iQ5 software. Clicking on the heading for Reports will open the Report Viewer (Figure 6.64).

The Reports Viewer pulls data, charts, and graphs directly from the displayed settings of the Data Analysis module currently in use. For this reason, it is best to complete all desired analysis and formatting of the displayed data prior to using the Reports tool.

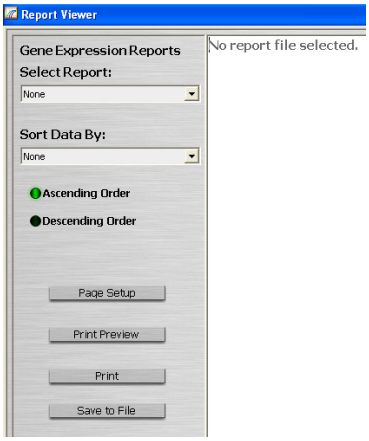


Fig. 6.64. The Report Viewer Window for Creating New Reports.

6.12.1 Report Viewer Options

The following options are available from within the Report Viewer window:

- **Select Report:** Use this drop-down menu to select the level of detail in the report for your dataset. The number and type of reports available will vary according to the Data Analysis Module currently being used
- **Sort Data By:** Within a given report, all data displayed in tables can be sorted by the parameters listed in the drop-down menu. The Sort Data By drop-down menu works in conjunction with **Ascending Order** and **Descending Order** radio buttons. Each of the parameters in the report can be sorted in ascending or descending order by the appropriate radio button. The results of the sort are immediately displayed in the report display area
- **Page Setup:** Clicking the Page Setup button opens the Windows Page Setup dialog where different page display parameters can be adjusted
- **Print Preview:** Clicking the Print Preview button opens the Windows Print Preview screen where the printed layout of the pending report can be previewed
- **Print:** Clicking the Print button opens the Print screen where different print parameters can be adjusted and the report printed
- **Save to File:** Clicking the Save to File button opens the Save report screen where a specific location can be chosen to save the report in rich text format

6.12.2 Parts of the Data Report

Each report template contains a preset report header that outlines specific information about the dataset being analyzed, such as time and date of file creation, and file path information. At the bottom of the "General Data" section, there is information that can be used to determine whether the report contains saved or unsaved changes to data analysis settings, including:

- **Report differs from last save:** The "Report differs from last save" report entry can be used to determine whether the analysis conditions displayed in the report template are different from the analysis conditions present at the time of the most recent file save event for a given dataset. If this entry reads "Yes", then the displayed data was generated from an unsaved version of the dataset being analyzed
- **Notes:** Text entered in the Notes field at the time of a data file creation, or data file save event is included in the header section of every report template
- **Protocol:** The header of each report template contains a summary of the protocol used for thermal cycling and data collection within a given dataset. Any modifications to the protocol used for data collection will be displayed in the Report Header

6.12.3 Data Analysis Report Types

There are over 15 different report templates available in the iQ5 software. The number and type of reports available at any given time will vary according to the Data Analysis module currently being used. The various report options available for each of the Data Analysis modules are summarized below.

PCR Quant Reports

- **PCR Quant Data:** This report template includes only the data included in the Standard Curve/C_T Results spreadsheet – the PCR amplification chart is NOT displayed.

- **PCR Quant Detailed:** This report template includes all of the data available in the PCR Quant module of the iQ5 software – including the PCR amplification chart, C_T value spreadsheet, and analysis parameters. If the dataset being analyzed also contains a standard curve, the standard curve chart and spreadsheet data will also be included in this report.
- **PCR Quant Graph Only:** This report template displays only the PCR amplification chart, exactly as analyzed and formatted within the PCR Quant module.
- **Std Curve Data Only – Landscape:** This report template includes only the data included in the Standard Curve/C_T Results spreadsheet. Although the report on the screen in displayed in the Portrait orientation, the Page Layout print settings are pre-set to the Landscape orientation.

Melt Curve Reports

- **Melt Curve Detailed:** This report template includes all of the data available in the Melt Curve/Peak module of the iQ5 software – including the melt curve chart, melt peak chart, Melt data spreadsheet, and analysis parameters. In addition, if all fluorophores have been selected for display in the PCR Quant module, the PCR amplification chart and analysis parameters will also be displayed.
- **Melt Curve Graph Only:** This report template displays only the graphs available in the Melt Curve/Peak module of the iQ5 software, such as the melt curve chart and melt peak chart, exactly as analyzed and formatted within the Melt Curve/Peak module.
- **Melt Curve Only:** When creating a report from a data set performed as an Melt Curve Only run, the Melt Curve Only report option becomes available. This report template includes all of the data available in the Melt Curve/Peak module of the iQ5 software – including the melt curve chart, melt peak chart, Melt spreadsheet, and analysis parameters.

End Point Reports

- **End Point detailed:** When creating a report from a data set that includes PCR Quantification data, End Point detailed is the only report option available. This report includes a section titled "Data Analysis Parameters", which details the PCR analysis settings for the current dataset.
- **End Point only:** When creating a report from a data set performed as an End Point Only run, the End Point Only report is the only report option available. This report template contains only the End Point Analysis spreadsheet and data analysis parameters.

Allelic Disc Reports

- **Allelic Data Only:** The Allelic Data Only report template is limited to the data displayed in the Allelic Disc module only, but will not display the allelic discrimination chart.
- **Allelic Detailed:** This report template includes all of the data available in the Allelic Disc module of the iQ5 software – including the allelic discrimination chart, spreadsheet, and analysis parameters. In addition, if all fluorophores have been selected for display in the PCR Quant module, the PCR amplification chart and analysis parameters will also be displayed.
- **Allelic Only:** This report template is limited to the data displayed in the Allelic Disc module only and includes the allelic discrimination chart, spreadsheet, and analysis parameters.

Gene Expression Reports

- **Gene Expression Detailed:** This report template includes all the Gene Expression data available in the Gene Expr module, including charts, data tables, Gene List and Condition List information, Threshold Crossing Spreadsheet, and Gene Expression analysis

parameters. In addition, if all fluorophores have been selected for display in the PCR Quant module, the PCR Amplification Chart and PCR Baseline Analysis Parameters will also be displayed.

- **Gene Expression Graph only:** This report template displays only the Gene Expression chart, exactly as analyzed and formatted within the gene expression module.
- **Gene Expression only:** This report template includes all of the data tables, Gene List and Condition List information, Threshold Crossing Spreadsheet, and Gene Expression analysis parameters available in the gene expression module. The gene expression chart is NOT displayed.

Gene Study Report

- **MultiFiles Gene Expression Detailed**: When creating a report from within a Gene Study, "MultiFiles Gene Expression Detailed" is the only selection available. This report format is similar to the Gene Expression Detailed report; however, no PCR Quant data is available for this report

Section 7. Calibrating the Instrument

This section contains information on the following topics:

7.1 Calibration Overview

To run experiments on the MyiQ2, MyiQ and iQ5 systems requires that the instruments are calibrated. There are four different calibrations required for the MyiQ2 and iQ5 systems: mask alignment, background calibration, persistent well factor generation, and pure dye calibration.

NOTE: Pure dye calibration is **NOT** performed on the MyiQ Real-Time PCR Detection System.

Calibration should be performed when;

- The instrument is installed
- Any of the components that affect the light path are replaced such as the lamp, filters, optical lid or optics module
- Whenever prompted by the software as calibrations expire

Calibration factors are specific for the optical path you are using, which includes the reaction vessel and sealing mechanism used in the experiment you wish to run. Consequently calibrate using the reaction vessel type (plates or tubes) and sealing method (film or caps) you will use for your future experiments.

The iQ5 software requires persistent well factor calibration for all combinations of reaction vessel, sealing product, and reaction volume that you anticipate using, even if dynamic well factor collection is selected. This feature prevents the run aborting if dynamic well factor collection from the experimental plate fails.

7.2 Components Required for Calibration

The following reagents and accessories are required to calibrate the MyiQ2 instrument:

- MyiQ2 Calibrator Dye Solution kit (catalog #170-8791)
- External Well Factor Solution (catalog #170-8794)
- 3 x 96-well PCR plates or preferred reaction vessel
- Optical-quality sealing tape or preferred sealing method

The following reagents and accessories are required to calibrate the iQ5 instrument:

- iCycler iQ calibrator dye solution kit (catalog #170-8792), which contains iQ5-specific pure dye solutions and external well factor solution
- 3 x 96-well PCR plates or preferred reaction vessel
- Optical-quality sealing tape or preferred sealing method

NOTE: Calibrator dye solutions are NOT required for calibrating the MyiQ system.

7.3 Preparing Calibration Plates

Preparation of an External Well Factor Plate

Preparation of this plate is required for mask alignment and persistent well factor generation for the MyiQ2, MyiQ and iQ5 systems.

To prepare an external well factor plate:

1. Dilute the 10x External Well Factor Solution to 1x with water.
2. Pipet 25 µl of the 1x external well factor solution into each of 96 wells of your preferred reaction vessel.
3. Seal using your preferred sealing method you will use in your future experiments.

Preparation of a Background Calibration Plate

Preparation of this plate is required for background calibrations for the MyiQ2, MyiQ and iQ5 systems.

Leaving the wells completely empty, seal 96 wells of your preferred reaction vessel with your preferred sealing method.

Preparation of the Pure Dye Calibration Plate

Preparation of this plate is required for pure dye calibration for the MyiQ2 and iQ5 systems.

To prepare a pure dye calibration plate:

1. In clean wells, pipet 25 µl of the appropriate 1x calibrator pure dye solution into 8 wells of your preferred reaction vessel.
2. Repeat for all fluorophores you will use in your future experiments. See example of Pure Dye Plate Setup.
3. Seal using your preferred sealing method you will use in your future experiments.

NOTE: Pure Dye Calibration is **NOT** performed on the MyiQ Real-Time PCR Detection System.

7.4 Performing Calibrations

The MyiQ2, MyiQ and iQ5 systems are calibrated using the calibration plates described in section 7.3 above and using the Calibration Module of the iQ5 software. Allow the instrument optics to warm up for 20 min prior to performing the mask alignment, background calibration, persistent well factor collection, or pure dye calibration procedures.

Calibrations are performed in the order as they appear in the tabs of the Calibration module:

- Mask Alignment
- Background Calibration
- Persistent Well Factor Generation
- Pure Dye Calibration

7.4.1 Performing Mask Alignment

Before you align the mask, complete the following steps:

1. Prepare an external well factor plate (see section 7.3)
2. Place the external well factor plate in the iCycler thermal cycler.
3. Select the Mask tab in the Calibration module to open the image window (Figure 7.1).

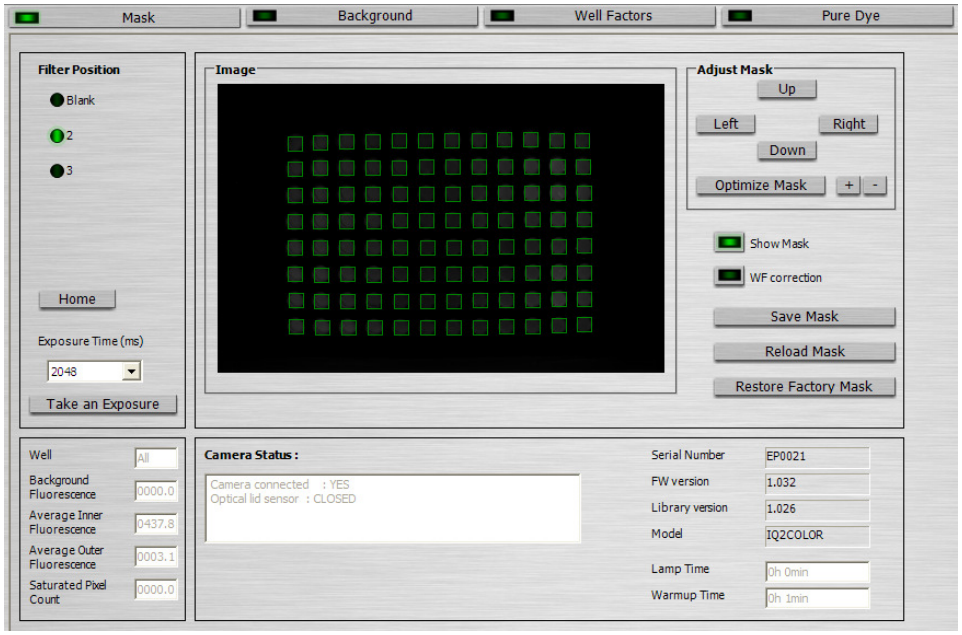


Fig. 7.1. The Mask Image Window for the MyiQ2 Real-Time PCR Detection System.

The main use of the Mask Image window 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.

To align the mask:

1. Click Home.
2. Click the Filter Position 2 option button.

3. Click Take an Exposure. 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
4. Click Show Mask. The Mask is displayed as an array of green boxes (Figure 7.2).

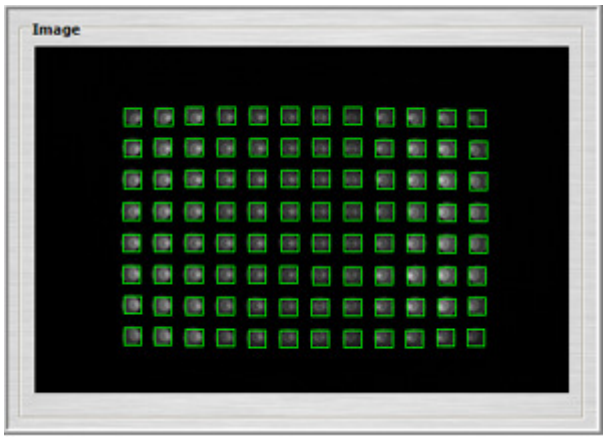


Fig. 7.2. Camera Image with Overlying Mask Array.

5. If any pink (saturated) 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. Each individual box should be centered over the well so that all the fluorescent signal from the well falls within the box.
7. Click Save Mask to save mask-data.xml file in the Mask folder of the iQ5 Folder.

The Mask can be displayed or hidden using the Show Mask button. The Mask array can be moved as a group by clicking on the Up, Down, Left or Right buttons. An individual box within the array can also be moved by first clicking on that box to select it, then using the Up, Down, Left or Right buttons to move it.

The Mask can be restored to the last saved Mask by clicking Reload Mask. The default, factory Mask can be restored by clicking Restore Factory Mask.

This completes Mask Alignment calibration. Next, perform background calibration.

7.4.2 Performing Background Calibration

Background calibration is performed to account for fluorescence in the experimental system that is due to the reaction vessel and sealing mechanism.

What You Will Need

You need the following to calibrate the camera:

- 96-well PCR plate or preferred reaction vessel (referred to as "plate")
- Optical-quality sealing tape or preferred sealing method

Before you perform background calibration, complete the following steps:

1. Prepare a background calibration plate (see section 7.3)
2. Ensure that the mask has been aligned.

3. Place the background calibration plate in the iCycler thermal cycler.
4. Select the Background tab in the Calibration module.

The Background window is shown in Figure 7.3.

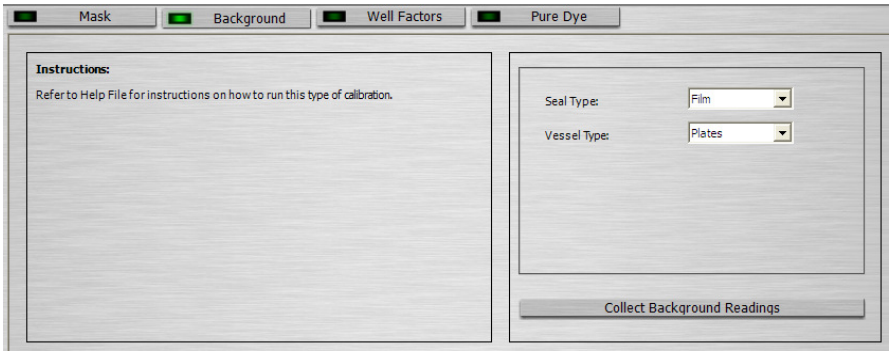


Fig. 7.3 The Background Window.

To perform background calibration:

1. Select a well sealing type (film or caps) from the drop-down list.
2. Select a vessel type (plates or tubes) from the drop-down list.
3. Click Collect Background Readings.
4. When the iQ5 software completes the background calibration run, a dialog box appears with the message, "Background Calibration Run Complete."
5. Click OK to exit.

NOTE: If you wish to calibrate for more than one vessel and sealing type combination, repeat the process above to collect additional background calibration files. During a run the software will automatically use the correct file for the vessel and sealing parameters specified in plate setup

This completes background calibration. Next, perform persistent well factor calibration.

7.4.3 Generating Persistent Well Factors

Before you perform a persistent well factor calibration run, complete the following steps:

1. Prepare an external well factor plate (see section 7.3)
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 cycler.
4. Select the Well Factors tab in the Calibration module.

The Well Factor window is shown in Figure 7.4.

To generate persistent well factors:

1. Select a well sealing type (film or caps) from the drop-down list.
2. Select a vessel type (plates or tubes) from the drop-down list.
3. Click Collect Persistent Well Factors.
4. When the iQ5 system completes the persistent well factor calibration run, a dialog box appears with the message, "Persistent Well Factor Calibration Run Complete".
5. Click OK to exit.

NOTE: If you wish to calibrate for more than one vessel and sealing type combination, repeat the process above to collect additional external well factor files. During a run, the software will automatically use the correct file for the vessel and sealing parameters specified in plate setup.

This completes the generation of persistent well factors. This completes calibration of cameras on the MyiQ system. To calibrate the cameras on the MyiQ2 and iQ5 systems, perform pure dye calibration as described in section 7.4.4.

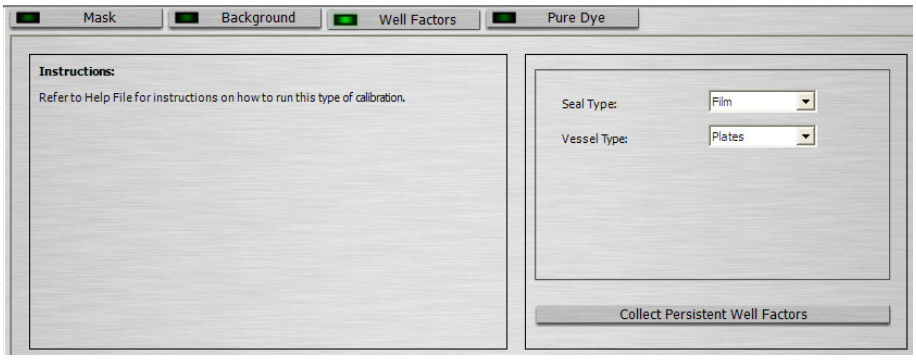


Fig. 7.4. The Well Factors Window.

7.4.4 Performing Pure Dye Calibration

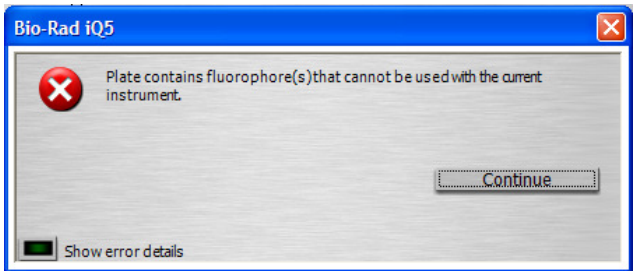
Before you perform a pure dye calibration run, complete the following steps:

1. Prepare a pure dye calibration plate (see section 7.3).
2. Ensure that the mask has been aligned.
3. Ensure that background calibration has been performed.
4. Ensure that persistent well factors have been generated.
5. Place the pure dye calibration plate in the iCycler thermal cycler.
6. Select the Pure Dye tab in the Calibration module.

The Pure Dye Calibration window is shown in Figure 7.5.

To perform a pure dye calibration run:

1. Select from the Pure Dye Plate Setup file selector window or click Create New. Ensure appropriate dyes are selected that are compatible with the filter setup of your connected instrument. If incompatible dyes are selected the following error will appear.



2. Click Run Pure Dye Calibration.
3. When the iQ5 software completes the pure dye calibration run, a dialog box appears with the message, "Pure Dye Calibration Run Complete".
4. Click OK to exit.

NOTE: if you wish to calibrate for more than one vessel and sealing type combination, repeat the process above to collect additional dye calibration files. During a run the software will automatically use the correct file for the vessel and sealing parameters specified in plate setup.

NOTE: Pure dye calibration is not required for SYBR® Green (dye designators "SYBR" and "SYBR1" – " SYBR5").

This completes calibration of the camera on the MyiQ2 and iQ5 systems.

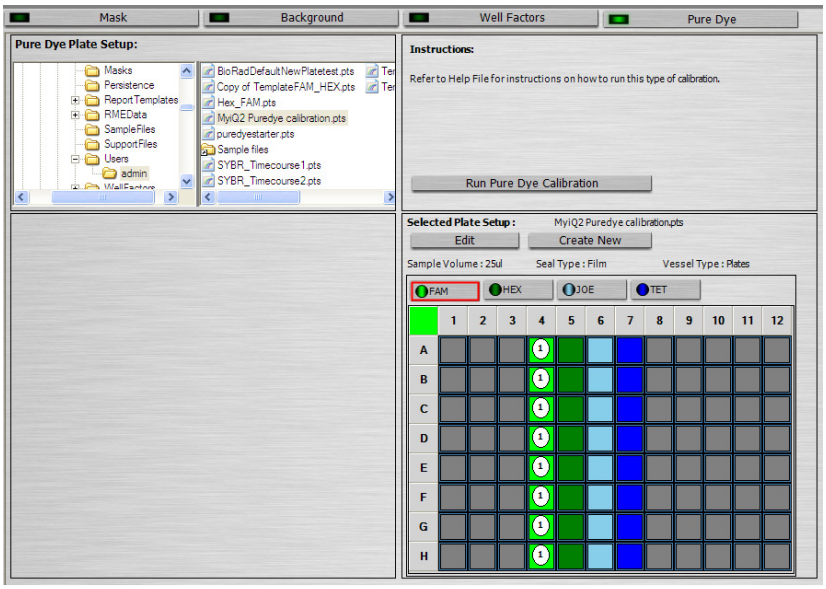


Fig. 7.5. The Pure Dye Calibration Window.

7.4.5 Editing and Creating Pure Dye Plate Setups

Selecting a Pure Dye Plate Setup

Select a Pure Dye Plate Setup from the file selector window within the Pure Dye Calibration tab. When loaded, the fluorophores on the plate will display within the plate display in the lower right-hand corner of the Pure Dye Calibration tab.

Check the Seal Type and Vessel Type fields. If these fields match the intended procedure and the correct fluorophores are displayed, proceed with pure dye calibration. If any of these particulars vary from the intended experiment parameters, the Pure Dye Plate Setup must be edited or a new Pure Dye Plate Setup created. An example of a Pure Dye Calibration Plate Setup window is shown in Figure 7.6.

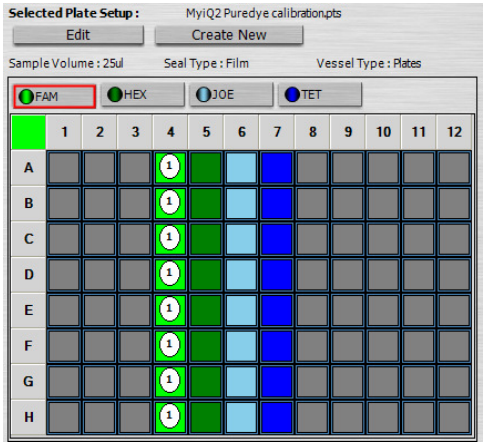


Fig. 7.6. Pure Dye Calibration Plate Setup Window.

Editing or Creating a Pure Dye Plate Setup

To edit a Pure Dye Plate Setup, select the Pure Dye Plate Setup that most closely resembles the experimental parameters desired, and click Edit. The Pure Dye Plate Setup editor will load as shown in Figure 7.7.

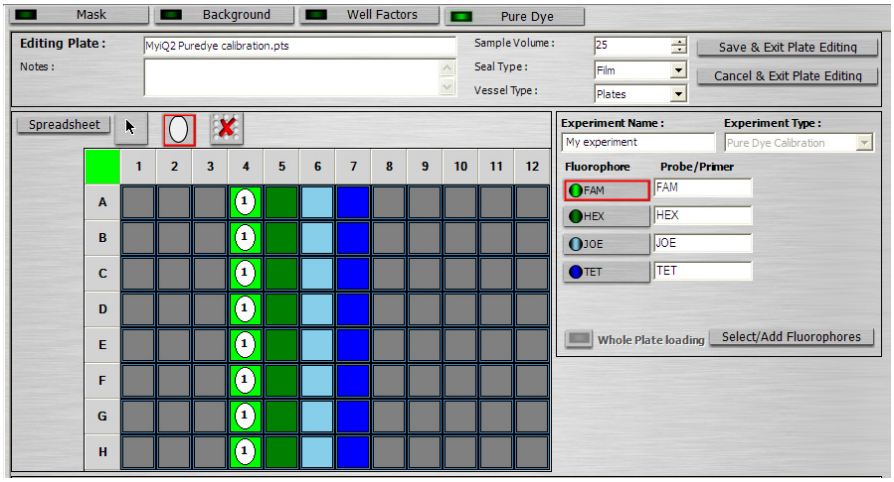


Fig. 7.7. Creating a Pure Dye Calibration Plate Setup.

Select/Add Fluorophores

To choose the fluorophores needed to calibrate the instrument, select from the predefined fluorophores or add custom fluorophores by clicking Select/Add Fluorophores. The Fluor Selector will be displayed (Figure 7.8).

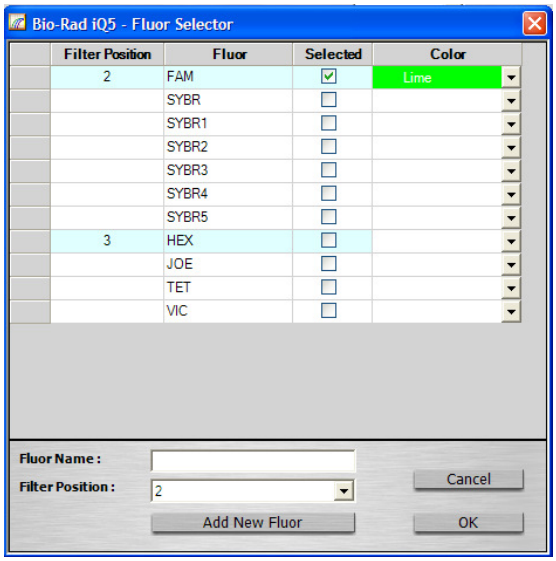


Fig. 7.8 The Select/Add Fluorophores Dialog Box

Add a fluorophore from the list displayed by clicking the checkbox next to the fluorophore name. To add a custom fluorophore that is not on the list, select the appropriate filter pair from the drop-down menu. Refer to section 9.2.1 for information about system filter specifications and recommended fluorophores.

Specify the name and color of the custom fluorophore by typing the name in the Fluor Name field and clicking Add New Fluor. The new fluorophore name appears in the list for the selected filter pair. Click the checkbox next to the new fluorophore name, then select the color to specify the fluorophore in the graphic by clicking on the color field that appears. Choose contrasting colors for maximum clarity, then click OK.

NOTE: Two or more different dyes may be used with the same filter pair; however, two dyes using the same filter pair may not be used in the same well.

Select new Sample Volume, Seal Type and Vessel Type from the drop-down menus as appropriate for this experiment, then click Save & Exit Plate Editing. Choose a meaningful file name, and save the Pure Dye Plate Setup.

IMPORTANT: Pure dye calibrations are made for each instrument, dye type, sample volume, seal type, and vessel type, and are instrument- and experiment-specific. Do not attempt to use pure dye calibration results on a different instrument, seal or vessel type, or sample volume.

7.5 Viewing Calibration Files

Background and well factors calibration files are saved to their respective folders within the iQ5 program folder. Pure dye calibration files are saved in the RMEData folder in the iQ5 program folder. Previous calibrations are saved in the subdirectory called "Backup". It is advisable to periodically backup your calibration files by copying the calibration folders and their contents.

To determine what fluorophore dyes an instrument has been calibrated for within the iQ5 software, select Calibration Data from the View menu (Figure 7.9) to open the Calibration Data file view. Fluorophore dyes for which the instrument has been calibrated will be listed by name in the summary table.

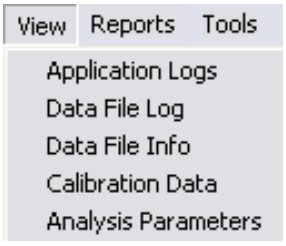


Fig. 7.9. The View Menu.

Similarly, the contents of the background and external well factor calibration files can also be accessed in this manner.

7.6 Troubleshooting Optics with the Mask Image Window

7.6.1 Filter Position

The Filter Position radio buttons (Figure 7.10) are used to position the filter wheels. The exposure time can be changed from this screen and an exposure taken. Home locates the filter wheel to the blank position. Refer to section 9.2.1 for information about system filter specifications and recommended fluorophores.

Fig. 7.10. The Filter Selection Frame for a MyiQ2 System.

7.6.2 Camera Status

Fig. 7.11. The Camera Status Screen.

The Camera Status screen (Figure 7.11) provides feedback about the camera. In this screen you can determine if:

- The camera is connected
- The optical lid is closed
- The serial number, firmware version, library version, and model of the camera
- The length of time the camera lamp has been in use since it was installed
- The length of time the lamp has been on since the camera was last turned on (referred to as Warmup Time)

Section 8. User Profiles

This section contains information on the following topics:

To assist with the management of files and data created by the iQ5 software, one or more user profiles may be created for users of the MyiQ2, MyiQ or iQ5 systems.

User profiles in the iQ5 software consist of:

- Login name (required)
- Password (optional)
- Permission settings for collecting, saving, and analyzing data
- Preferred defaults (user preferences) for collecting, saving, and analyzing data

NOTE: All user profile information resides on the hard drive of the computer where the software has been installed, this means that user profile information cannot be shared over a network.

When the software is installed, the default user profile has the ability to add and configure additional users through the User Profile module. The User Profile module is where users can be added, their permissions controlled and their preferences set. This module of the iQ5 software consists of 2 tabs:

- User Preferences
- Administration

8.1 User Preferences

User Preferences, found in the User Profile module, can be used to customize the preferences of the currently logged in User.

For each User created in the iQ5 software, user-specific preferences can be set for:

- File Paths
- Plate Setup options
- Protocol options
- File Selection at application startup
- PCR Quant module analysis settings

- Allelic Discrimination module analysis settings
- End Point module analysis settings
- Gene expression module analysis settings

8.1.1 File Paths Preferences

Each file created by the iQ5 software is destined to a specific folder location on your computer, this is known as a "file path". The File Paths preferences box (Figure 8.1) can be used to select the folders that you wish to save your files into when logged into the iQ5 software. The default File Paths for all users is a folder created with the current user's User Name.

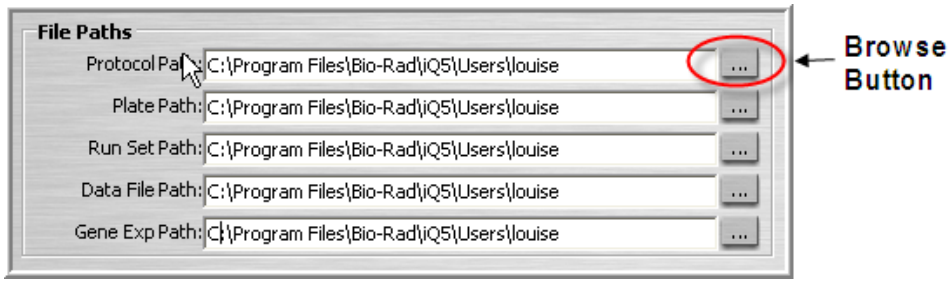


Fig. 8.1. The File Paths dialog box.

File Paths can be specified for: protocol files, plate setup files, run sets, data files and Multi file Gene Expression Analysis Files (Gene Exp Path).

To set the file path to an existing directory, click the browse button and navigate to the appropriate folder. To set the file path to a new directory, click the browse button, then click Make New Folder in the Browse For Folder dialog box. A new folder will appear within the currently selected folder (Figure 8.2). To rename this folder, right-click, select Rename, and then enter the new name of the folder in the text field next to the newly created folder. Click OK when finished.

NOTE: Changes to default File Paths DO NOT become active until the software has been restarted.

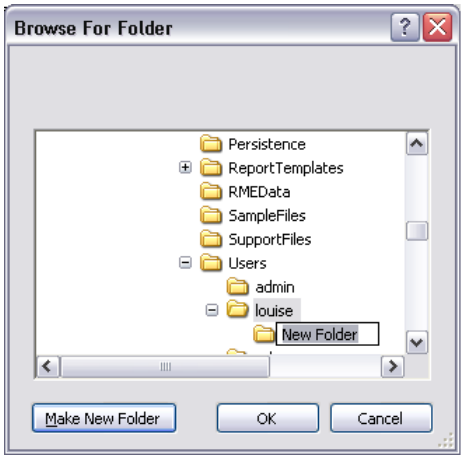


Fig. 8.2. Creating New Folders in the Browse For Folder Dialog Box.

8.1.2 Plate Setup Preferences

The Plate Setup preferences box (Figure 8.3) can be used to define the default conditions when a user creates a new plate setup.

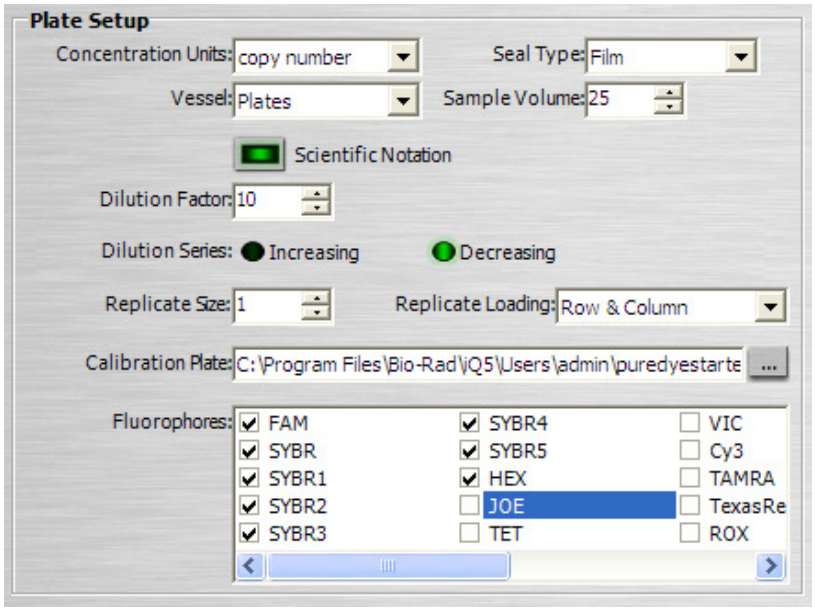


Fig. 8.3. Plate Setup Preferences.

The following default conditions can be set for the Plate Setup:

- **Concentration Units:** Choose from 14 different units of measure to match the units used in your experiment
- **Seal Type:** Chose from Film, Domed Cap and Flat Cap
- **Vessel:** Chose from Plates, Strips (tube strips) and Tubes
- **Sample Volume:** Set the sample volume (in µl) that will be used as the default value
- **Dilution Series:** Set the defaults for the dilution factor, and specify whether the series is increasing or decreasing in quantity. You may specify if scientific notation is to be used when the dilution series values are displayed
- **Replicate Size:** Used to specify the default replicate size
- **Replicate Loading:** Used to specify the default direction replicates will be loaded. You can choose from Row (replicates added horizontally), Column (replicates added vertically), and Row and Column (replicates added in a block). See Specifying Replicates for more information
- **Calibration Plate:** This is used to set the default file to be selected for the pure dye calibration plate. This file will be used after Application Startup
- **Fluorophores:** Place a checkmark next to the fluorophores that you wish to have automatically selected when creating a new plate setup. No more than 5 fluorophores can be selected. At least one fluorophore must always be selected

IMPORTANT NOTE: The Fluorophores preference box will display all fluorophores available to the MyiQ2, MyiQ and iQ5 systems. If both dyes that can and cannot be detected by the connected instrument are selected in user preferences, only fluorophores that are compatible with the connected instrument will be displayed. If only fluorophores that are not available for detection by the connected instrument are selected, by default the Plate Setup window will display FAM only.

8.1.3 Protocol Preferences

The Protocol preferences box can be used to set the defaults for the current user for Melt Curve, Gradient, and Well Factor Collection Type (Figure 8.4).

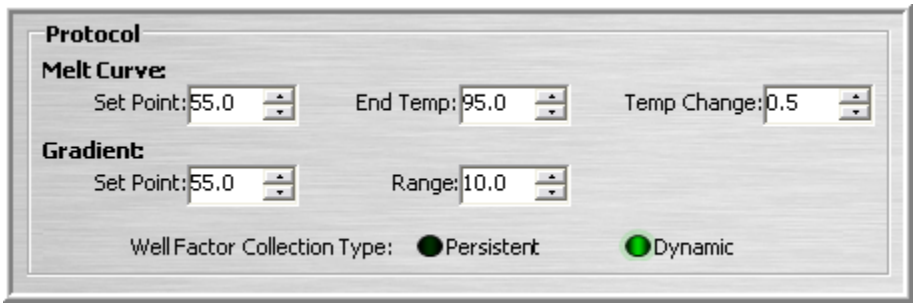


Fig. 8.4. Defining User-Specific Protocol Preferences.

The following default conditions can be set for the Protocol:

- **Melt Curve:** Enter the desired beginning temperature (Set Point), ending temperature (End Temp), and the change in temperature that will occur at each repeat (Temp Change). Click Save
- **Gradient:** Enter the desired lowest temperature (Set Point) and the Range for the gradient in the appropriate text boxes (or use the scroll buttons to enter these values). Click Save
- **Well Faction Collection Type:** Select the radio button associated with the well factor that you wish to use as the default type. Click Save. If Dynamic is chosen as the default type, dynamic well factors will be used unless restricted by the Plate Setup

8.1.4 File Selection at Application Startup Preferences

The file that you wish to have automatically selected when the iQ5 software is opened is determined using the File Selection at Application Startup radio buttons (Figure 8.5).

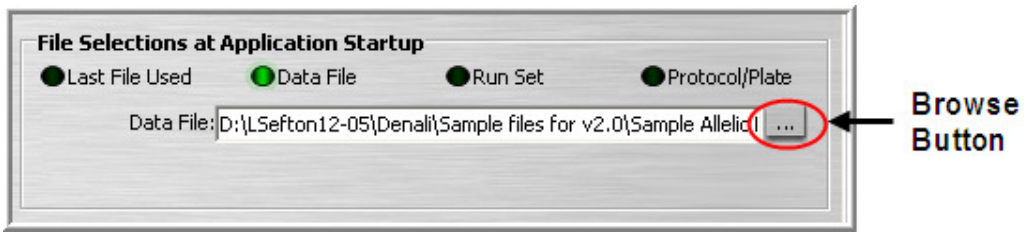


Fig. 8.5. Selecting Custom File Defaults for Application Startup.

- **Last File Used:** This option will select the last Protocol, Plate Setup, Run Set or Data File used as the file to display in the Workshop screen when the iQ5 software starts up
- **Data File:** If you wish to have a specific data file selected when the iQ5 software opens, select Data File and then use the browse button to navigate the data file that you wish to have selected on application startup
- **Run Set:** If you wish to have a specific Run Set file selected when the iQ5 software opens, select Run Set and then use the browse button to navigate the run set file that you wish to have selected on application startup
- **Protocol/Plate:** If you wish to have a specific protocol or plate setup file selected when the iQ5 software opens, select Protocol/Plate and then use the browse button to navigate either the protocol or plate file that you wish to have selected on application startup

8.1.5 PCR Quant Screen Preferences

The PCR Quant preference box (Figure 8.6) can be used to set the defaults for the PCR Quant screen. You can set the defaults for four major settings that will impact the analysis or display of data collected from your real-time PCR detection system.

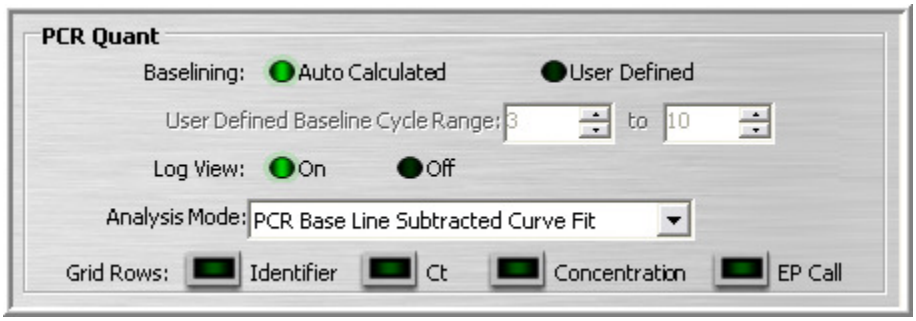


Fig. 8.6. Defining User Specific PCR Quant Analysis and Display Options.

- **Baselining:** By default, PCR baselines are auto-calculated. With the Baselining user preference setting, you can override auto-calculation by clicking User Defined. After you click User Defined, the iQ5 software activates the User Defined Baseline Cycle Range boxes. Enter the desired values for the start cycle and ending cycle for all traces. Auto Calculated is the factory default
- **Log View:** Use this option to select either a semi-logarithmic or linear view of the PCR amplification chart data. When the Log View option is On, a semi-logarithmic view of the amplification data will be displayed. The factory default for Log View is Off
- **Analysis Mode:** You can select from three analysis modes in the Analysis Mode drop-down list boxes. The three analysis modes include: Background Subtracted, PCR Base Line Subtracted, PCR Base Line Subtracted Curve Fit. PCR Base Line Subtracted Curve Fit is the factory default
- **Grid Rows:** The Grid Rows options are a set of checkboxes that you can use to display additional details about the data displayed in the PCR Quant screen. These optional details will be displayed in the results spreadsheet of the PCR Quant screen, and include the following information about each sample: Identifier, Concentration, Threshold Cycle, and End Point Call

8.1.6 Allelic Discrimination Module Preferences

The Allelic Discrimination preference box (Figure 8.7) can be used to set the default display conditions used in the Allelic Discrimination screen. The Display Mode can be set to Threshold Cycle or RFU using the radio buttons. The radio buttons for the Normalize Data option become active only when the Display Mode is set to RFU.

Fig. 8.7. Defining Allelic Discrimination Display Options.

8.1.7 End Point Module Preferences

The End Point module preference box (Figure 8.8) allows you to predetermine several analysis and display options associated with analyzing final RFU in an end point analysis assay.

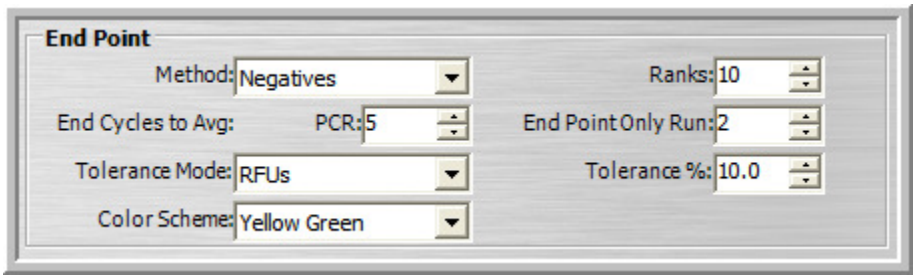


Fig. 8.8. Defining Analysis and Display Options for the End Point Module.

- **Method:** 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, Positives, and Positives & Negatives. Analysis by comparison to negative controls (Negatives) is the default method.
- **Ranks:** The number of ranks allows assignment of samples into distinct groups based on their RFU values. The default Rank value is 10 and the minimum number of Ranks is 3.
- **End Cycles to Avg:** 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 Avg field defaults to 5 for non-end point only (PCR) runs.
- **End Point Only Run:** The End Cycles to Avg field defaults to 2 for end point only runs.
- **Tolerance Mode:** End Point Tolerance defines the margins for sorting unknowns as positives or negatives. The End Point Tolerance drop-down list box consists of two choices: RFUs and Percent of Range.
- **Tolerance %:** Select a value between 1 and 99 percent for this setting. The default percent of range tolerance is 10 percent.
- **Color Scheme:** Colored rank boxes symbolize the number and order of ranks in the end point analysis. There are five options to choose from that allow a change in the color scheme of the rank boxes, once the data are analyzed.

8.1.8 Gene Expression Module Preferences

The Gene Expression module preference box (Figure 8.9) allows you to predetermine several analysis and display options associated with analyzing C_T values for gene expression analysis.

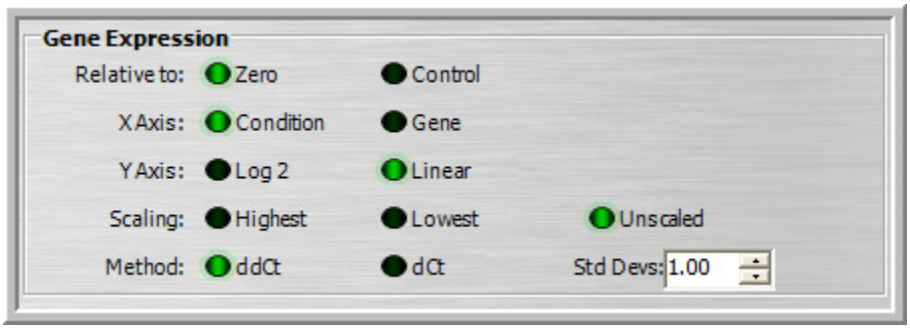


Fig. 8.9 Defining Analysis and Display Options for the Gene Expression Module.

- **Relative to:** This option allows you to present data with axes originating at 1 (relative to control) or at zero (relative to zero).
- **X Axis:** This option allows you to graph either genes or conditions on the x-axis.
- **Y Axis:** This option allows you to display the graph with the y-axis in a log2 scale or on a linear scale.
- **Scaling:** "Scale to control" is another option, which is accomplished by assigning a control in the Condition List.
- **Method:** These options allow you to set the default analysis mode for your gene expression analysis. You can select **ddCt** (for normalized expression) or **dCt** (for relative expression) as a default setting.
- **Std Devs:** 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.

8.2 User Administration

The Administration tab can be used by a user with an "iQ5 Administrator" role to:

- Add or delete users
- Edit user details (that is, full name and Email information)
- Set or change passwords
- Control the permissions of each user

Only an iQ5 Administrator can access and modify information contained in the Administration window.

Users specified as iQ5 Principal, iQ5 Operator, and iQ5 Guest have read-only access to the Administration window, where they can review permission settings for their role.

8.2.1 Adding New Users

Users are added using the Defining Users spreadsheet (Figure 8.10).

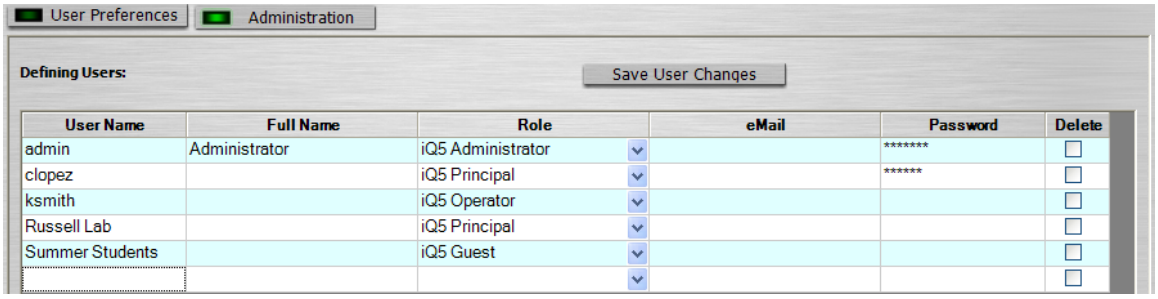


Fig. 8.10. The Defining Users interface of the User Profile Module.

- **User Name** (REQUIRED): The User Name is a set of alphanumeric characters that uniquely defines each user. It can be up to 15 characters long and composed of upper and/or lower case characters.
- **Full Name** (optional): Use this field to specify the full name corresponding to the User Name
- **Role** (required): There are 4 roles in the iQ5 Software: Administrator, Operator, User, and Guest. Each of these Roles gives users within that role permission to access specific features and functions of the software. Permissions granted to all of the Roles, with the exception of Administrator, can be customized by the Administrator
- **eMail** (optional): The eMail cell is an informational area for contact information.
- **Password** (optional): The password can be any combination of letters, numbers or symbols. It can be of any length. An Administrator can also change the password of users. This is useful if a user forgets the password.

To Add a new user:

1. Type the desired login name into an empty User Name box of the Defining Users spreadsheet.
2. (Optional) Type the full name of the user.
3. Define the Role of the user by using the pull-down menu in the Role cell. Select Administrator, Operator, User, or Guest. The features/functions that each of these user roles is permitted/restricted to use is defined by an Administrator using the Defining Roles spreadsheet.
4. (Optional) Type the email or phone contact information of the user.
5. (Optional) Type the password for the user. This password, defined here by the Administrator, can later be changed by the user using the Change Password option found in the Tools menu.
6. Click Save User Changes.

Adding a user will create a folder with the new user's name. This folder will be created in the Bio-Rad/iQ5/Users folder and will be the default folder for all files saved when the user is logged into the software.

8.2.2 Deleting Existing Users

Users are removed using the Defining Users spreadsheet.

1. In the Defining Users spreadsheet, check the box in the Delete column for the user that is to be removed.
2. Click Save User Changes.

NOTE: No messages will appear to confirm this action. The User Profile will immediately be removed from the Defining Users spreadsheet.

8.3 Logging on to the iQ5 Software

There are two ways for a user to sign in to the iQ5 software:

- At the start up of the iQ5 program
- Through the Switching Users option in the Tools menu

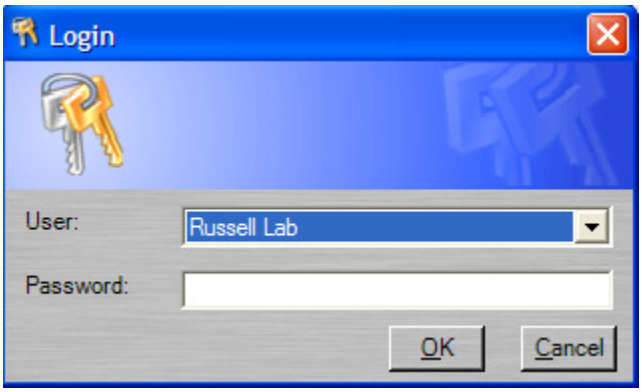


Fig. 8.11. The User Login Dialog Box.

1. The user name can be selected from the pull-down menu in the Login dialog box (Figure 8.11). Enter the password, and click OK to log on to the iQ5 software.
2. If the user name or password is incorrect, an error message will be displayed (Figure 8.12).

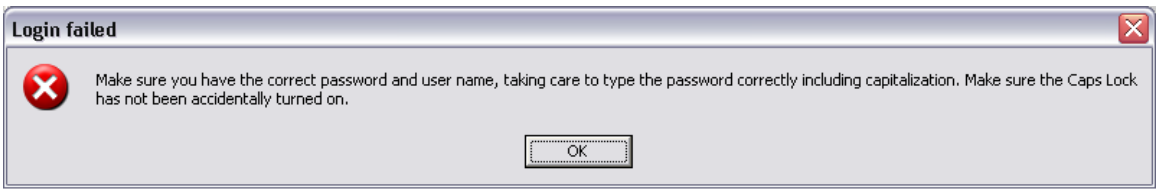


Fig. 8.12. Incorrect Login Error Message.

3. Click OK and enter the correct information. If you have forgotten your password, it may be reset by the iQ5 Administrator.
4. The current user can be determined by looking at title bar (in the top-right corner) of the software. The current user name will be displayed in parentheses next to the Bio-Rad iQ5 header (Figure 8.13).

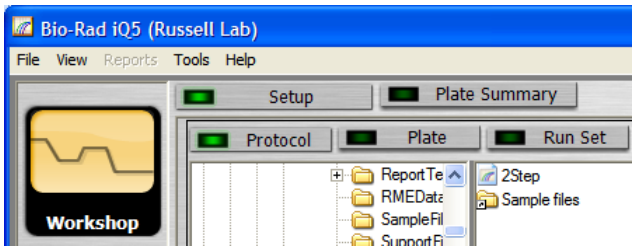


Fig. 8.13. User Name Displayed in the iQ5 Software Title Bar.

8.3.1 Switching Users

To switch a user:

1. Click on the Workshop module icon, then select Switch User from the Tools menu.
2. The Login dialog box will appear.
3. Select the desired user from the User pull-down list. Enter the password (if any) in the Password text box and click OK.
4. If the user name you want is not available, you will need to add it in the User Profile module in the Administration tab.

8.3.2 Changing Password

To change the password of the current:

1. Click on the Tools menu and select Change Password. The Change Password window opens (Figure 8.14).
2. Enter the old password in the top text box. Enter the new password in the New Password and Confirm New Password text boxes.
3. Click OK.

Fig. 8.14. The Change Password Dialog Box.

8.3.3 Defining Roles

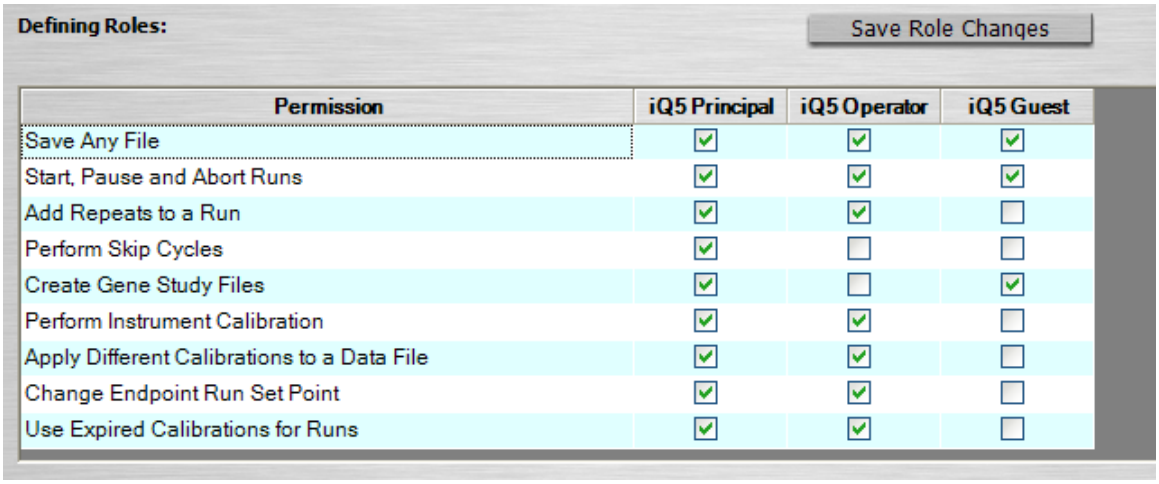


Fig. 8.15. The Defining Roles interface of the User Profile module.

Figure 8.15 lists the set of features and functions (permission) that can be granted by the iQ5 Administrator to users assigned to one of three non-administrator roles. The Administrator can customize the roles of a Principal, Operator, and Guest by checking or unchecking the box associated with each function and role. To apply the selected changes, click Save Role Changes. To restore factory default roles, click Restore Factory Roles.

NOTE: In the factory default roles, users assigned to the iQ5 Guest role do not have any permission granted to them. By default, these users are granted "read-only" access to the software, unless the ability to start runs and save files is granted by the iQ5 Administrator.

Viewing User Permissions

To view the current permission settings for existing iQ5 Users, access the User Administration window by clicking on the Administration button.

All non-administrator users will have read-only access to the information in the User Administration window.

Section 9. Instrument Maintenance

This section contains information on the following topics:

9.1 Cleaning the Real-Time PCR Detection System

Take care not to spill liquids onto or into the iCycler thermal cycler or the 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.

9.2 Filter Description and Installation Instructions

The filters designed for use in the MyiQ2, MyiQ and iQ5 optics modules are made of glass and mounted in plastic holders. The filter holders are held in either the excitation or emission filter wheel of the optics module. Each filter wheel holds six filters. Every position in a filter wheel must have a filter or an opaque filter blank to avoid damage to the CCD detector. 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 optics module. Section 9.2.1 summarizes the positions of the filter pairs for the systems, optical characteristics, and the recommended fluorophores with which the filters are compatible.

9.2.1 Recommended Fluorophores and Filter Specifications

Recommended fluorophores for the MyiQ2 Real-Time PCR Detection System

- Filter position 2 485/30X 530/30M Fluorescein (FAM), SYBR® Green I
- Filter position 3 530/30X 575/20M HEX, TET, VIC, JOE

Recommended fluorophores for the MyiQ Real-Time PCR Detection System

- Fluorescein (FAM), SYBR® Green I

Recommended fluorophores for the iQ5 Real-Time PCR Detection System

- Filter position 2 485/30X 530/30M Fluorescein (FAM), SYBR® Green I
- Filter position 3 530/30X 575/20M HEX, TET, VIC, JOE

- Filter position 4 545/30X 585/20M TAMRA, Cy3
- Filter position 5 575/30X 625/30M Texas Red, ROX
- Filter position 6 630/30X 685/30M Cy5, LC640

NOTE: The filter designation 485/30X indicates that this filter will allow light between 475 and 595 nm to pass through. The first number, 485, indicates the center of the wavelength of light. The second number, 30, 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.

9.2.2 Accessing the Filters

To access the existing filters, proceed as follows:

1. Turn off the power to the optics module.
2. Reach behind the 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 instrument 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. Be sure that every position in the filter wheel has either an excitation or emission filter or a filter blank before powering on the system.
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 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.

9.2.3 Cleaning the Filters

Each optics module 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.

You may clean the optical filters by wiping them gently with lens paper and 70% ethanol. You may obtain lens paper and lens cleaning solution by ordering a Lens Cleaning Kit from Bio-Rad (catalog #170-7731). After cleaning both sides of an optical filter, hold it up to the light and make sure that no smudges, fingerprints, debris, or water marks remain. Once cleaned, reinsert the optical filters as described above.

9.3 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, if 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 MyiQ2, MyiQ or iQ5 camera module before removing the lamp.

Lamp Replacement Procedure

1. Turn off the power to the optical module.
2. Reach behind the 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 instrument casing. Lift upward to remove the cover of the optical module.
4. The lamp is located on the right side of the optical module. Push up on the lamp spring clip to release the lamp from the bracket.
5. Lift the lamp out of the socket.
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, then close the case and secure the lid.

Appendix A: Warranty

The MyiQ2, MyiQ and iQ5 Real-Time PCR Detection Systems are warranted against defects in materials and workmanship. For specific warranty information, contact your local Bio-Rad office.

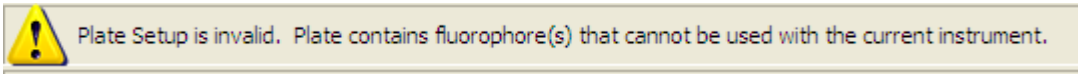
If any defects should occur during the warranty period, Bio-Rad will replace the defective parts without charge. However, damage or defects resulting from any of the following causes are specifically excluded:

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 MyiQ2, MyiQ or iQ5 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.

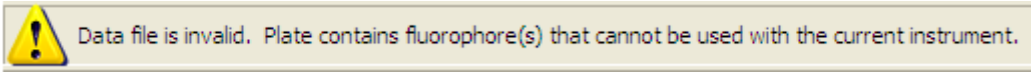
For inquiry or request for repair service, contact Bio-Rad Laboratories after confirming the model and serial number of your instrument. For technical support, call your local Bio-Rad office, or in the US, call 1-800-4BIORAD (1-800-424-6723), or visit us on the Web at discover.bio-rad.com.

Appendix B: Troubleshooting Error Messages



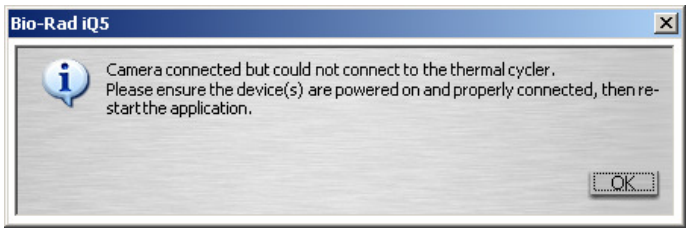
Cause:
Selecting or creating plate setup files that contain fluorophores not compatible with the connected instrument. The warning message above will be displayed at the bottom of the screen. This message indicates that while the plate setup can be viewed and edited, the plate setup contains fluorophores that cannot be used to collect new data.

Solution:
If you wish to view or edit the plate setup, no corrective action is required. If you want to run this plate setup using the currently connected instrument, you will need to edit the plate setup to remove fluorophores that are incompatible with the connected instrument. Refer to section 4.2 for further details



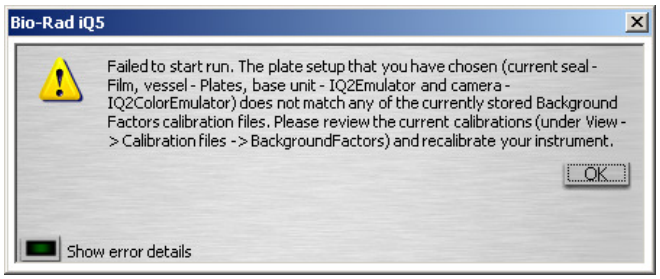
Cause:
Data files collected using an iQ5 system can be opened when the computer running iQ5 software (version 2.1) is connected to a MyiQ2 or MyiQ system. The warning message above indicates the plate setup contains fluorophores not compatible with the connected instrument. This message means that while the data can be viewed and analyzed, the plate setup contains fluorophores that cannot be used to collect new data. Similarly, opening a data file collected on the MyiQ2 system when a MyiQ system is connected to the software will result in the same message if MyiQ-incompatible fluorophores are present in the plate setup.

Solution:
If you wish to view or edit the data, no corrective action is required. If you want to repeat the run using the plate setup associated with the data file, you will need to edit the plate setup to remove fluorophores that are incompatible with the connected instrument. Refer to section 4.2 and 4.5 for further details



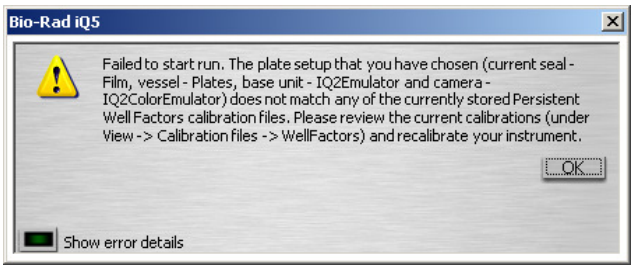
Cause:
This error appears when the software/computer is unable to communicate with the camera. The iQ5 software and camera are on but the iCycler base unit is powered off.

Solution:
Close the software, turn the camera on and restart the iQ5 software.
Check that the USB cable is securely connected to both the camera and computer.
Ensure that the 2.0 high-speed enabled USB port on the computer is being used and all hibernate and power save settings are disabled. Refer to section 1.2.5 and 1.4 for further details.



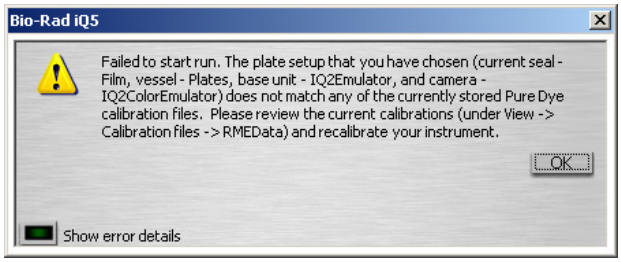
Cause:
Background factor data not found. This error occurs when a run start is attempted using a vessel and seal combination the instrument has not been calibrated for.

Solution:
The background vessel and seal type selected for your plate setup must match collected background calibration files. The instrument should ideally be re-calibrated for the desired vessel and seal type combination. If a run must be started immediately, select a vessel and seal type for which the instrument has already been calibrated. After the run has completed, recalibrate the instrument for the desired vessel and seal combination and back-apply this new background calibration to your data file. Refer to section 4.5.5 for further details.



Cause:
Well factor data not found. This error occurs when a run start is attempted using a vessel and seal combination the instrument has not been calibrated for.

Solution:
Well Factors vessel and seal type for your plate must match collected Persistent Well Factor calibration files. The instrument should ideally be re-calibrated for the desired vessel and seal type combination. If a run must be started immediately, select a vessel and seal type for which the instrument has already been calibrated. After the run has completed recalibrate the instrument for the desired vessel and seal combination and back-apply this new Well Factor calibration to your data file. Refer to section 4.5.5 for further details.



Cause:
Pure dye calibration data not found. This error occurs when a run start is attempted using a fluorophore with a vessel and seal combination the instrument has not been calibrated for.

Solution:
The instrument should ideally be re-calibrated for the fluorophore with desired vessel and seal type combination. This is important for correct dye de-convolution in multiplex experiments.

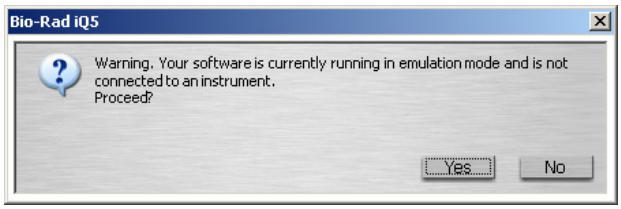
NOTE: If a run must be started immediately, select a vessel and seal type for that fluorophore for which the instrument has already been calibrated. After the run has completed recalibrate the instrument for the desired vessel and seal combination and back-apply this alternate pure dye calibration (.RME file) to your data file. Refer to section 4.5.4 for further details.

Cause:
Bad protocol file. There is no step defined for data collection or analysis in the thermal protocol.

Solution:
At least one data acquisition step must be present in the thermal protocol. Open the protocol editor and specify a data collection at one step of the protocol. A data collection step is indicated by a yellow (real-time data collection) or green (melt curve data collection) camera icon at the appropriate step in the graphical display of the protocol. Refer to section 4.3 for further details.

Cause:
Fluorophores have been selected in the plate setup; however, no wells on the plate have been edited 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.



Cause:
This is not an error message but a warning reminder that the iQ5 software is running in emulation mode. In emulation mode the camera is not detecting and collecting data from the plate and is generating emulated run data for demonstration purposes only.

Solution:
If you wish the camera to detect and collect data from your experimental plate, exit the software. From the Start Menu, select programs > Bio-Rad > iQ5 >iQ5 to restart the software in standard data collection mode.

Appendix C: Product Ordering Information

Catalog Number	Product Description
170-9790	**MyiQ2 Real-Time PCR System**, includes iCycler thermal cycler, 96-well optical reaction block, MyiQ2 optics module, iQ5 system software on CD-ROM, optical-quality 96-well PCR plates, sealing tape, communication cables, system accessories, power cords, instructions
223-9441	96-well Thin Wall PCR Plates, 25 per box
HSS-9601	Hard-Shell Full-Height 96-Well Semi-Skirted PCR Plates
MSB-1001	Optical Quality Sealing Tape, 100 sheets
TBS-0201	0.2 ml 8-Tube Strips Without Caps
TCS-0803	Optical Flat 8-Cap Strips
170-8756	Replacement Halogen Lamp
170-9753	iQ5 Software Installation Disk
170-8791	MyiQ2 Calibrator Dye Solution Kit. 1x calibration dye solutions, 3 tubes each of FAM, HEX, TET, Joe
170-8794	External Well Factor Solution, 5 tubes
170-8780	Fluorescein Calibration Dye
Reagents	
170-8860	iQ Supermix, 100 x 50 µl reactions, 2x mix
170-8862	iQ Supermix, 500 x 50 µl reactions, 2x mix
170-8880	iQ SYBR® Green Supermix, 100 x 50 µl reactions, 2x mix
170-8882	iQ SYBR® Green Supermix, 500 x 50 µl reactions, 2x mix
170-8890	iScript cDNA Synthesis Kit, 25 x 20 µl reactions, includes 5x iScript reaction mix, iScript enzyme, nuclease free water
170-8891	iScript cDNA Synthesis Kit, 100 x 20 µl reactions,
170-8896	iScript Select cDNA Synthesis Kit, 25 x 20 µl 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 µl reactions,
170-8892	iScript OneStep RTPCR Kit With SYBR® Green, 50 x 50 µl reactions
170-8893	iScript OneStep RTPCR Kit With SYBR® Green, 200 x 50 µl reactions
170-8894	iScript OneStep RTPCR Kit for Probes, 50 x 50 µl reactions
170-8895	iScript OneStep RTPCR Kit for Probes, 200 x 50 µl reactions

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Canada *905 364 3435* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***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 4029300* ***Israel*** *03 963 6050*
Italy *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500*
Norway *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723*
Spain *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*

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