Mx3000P™ Real-Time PCR System Instruction Manual

Software version 2.0

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Getting Started

Introduction

The Mx3000P™ real-time PCR system is a fully integrated real-time PCR detection system. The system includes a state-of-the-art thermal cycler, a quartz-tungsten halogen lamp to excite fluorescence, a photomultiplier tube for high-sensitivity detection, and real-time quantitative detection and analysis software. With the Mx3000P real-time PCR system, monitoring PCR in real time is fast, easy, and reliable. The Mx3000P system has an open format that allows closed-tube real-time PCR detection with many detection chemistries including SYBR® Green dye and fluorogenic probe systems. The Mx3000P system supports both real-time and plate-read experiments.

The software features a variety of specific experiment types with customized plate setup, thermal profile setup, and analysis screens that streamline the process of collecting and analyzing data for specific applications. Some of the features that differentiate the Mx3000P real-time PCR system from other real-time detection instruments include the following:

- View and analyze data in multiple formats, including: amplification plots, scatter plots, sample value screens that display all dyes for the entire plate, fluorescence intensity screens, final call results, melting curves, annealing range and text reports
- Enhanced analysis algorithms, including adaptive baseline, to improve results and reduce the need for manual adjustments
- Melting curve analysis for Molecular Beacon confirmation with automatic melting temperature (Tm) calculation
- Ability to plot individual dyes and wells from an experiment without exporting data
- Real-time amplification plots can be viewed and results analyzed during PCR run
- All results available as raw data

Real-Time Experiments

Real-time experiments monitor and report the accumulation of PCR product, as detected by increased fluorescence, during thermal cycling. Data collection during the early exponential phase of PCR allows the software to accurately calculate initial template quantities. In addition, the status of the experiment may be monitored as the run progresses.

Plate Read Experiments

Plate read experiments add flexibility to the laboratory. A one-time fluorescence reading can be taken on a set of samples and the results viewed immediately. The Mx3000P system can also be used to take and analyze pre-cycling and post-cycling readings for a set of samples that are cycled in another thermal cycler.

Revision #044002

Getting Started with the Mx3000P Instrument

Instrument Status Indicator LED's

The status indicators on the front of the Mx3000P instrument consist of two LED's.

The lower LED is a power status indicator. When this LED is lit, the Mx3000P instrument is plugged in and powered on.

The upper LED is a ready status indicator for the instrument and has three separate modes.

Steadily lit mode: the Mx3000P control software is operational and the unit is ready to run an experiment.

Blinking mode: The unit is not currently available to run an experiment. Either an experiment is already in process or a self-test is being conducted.

Off mode: The unit is not currently available to run an experiment. Either the power is off or the Mx3000P instrument control software is not operational.

Cautions and Warnings

Safety Symbols

On/Off power supply indicator

Caution

Caution, hot surfaces

Other Warnings

Be careful when loading or unloading samples from the thermal block; the thermal block can be hot.

Never touch the glass areas of the filters or the lamp.

Do not open the instrument door during a run. When an experiment is running on the instrument the upper LED on the instrument front is in blinking mode. Opening the door while the instrument is running will abort the run. A warning dialog box will appear on screen if the door has been opened and the run has been aborted.

Sample Loading and Unloading

Mx3000P Thermal Block Hardware

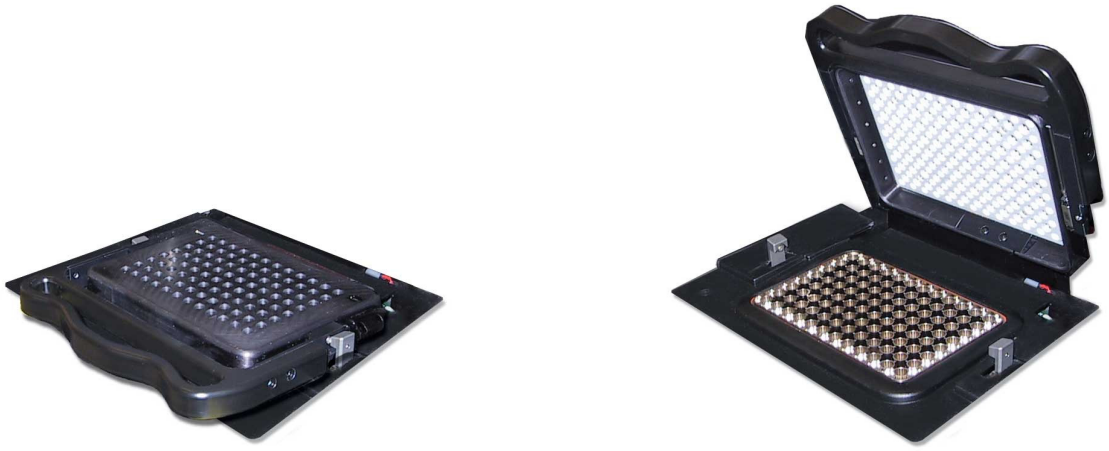
Use care when loading and unloading the samples; the thermal block can be hot.

Prior to loading samples, verify that the status LED (upper LED on the instrument front) is solidly lit (glowing green), indicating that the instrument is **Ready** for use. (If the LED is not illuminated, the instrument is not powered on. If the LED is blinking, the instrument is warming up or a run is in progress and the door should not be opened.)

Open the door located at the front of the instrument by sliding it all the way to the top.

Open the hot-top assembly to expose the thermal block by pulling forward on the hot-top handle and then lifting the hot-top up and away from the thermal block. The hot-top assembly is shown below in the closed (left) and opened (right) positions.

Note *The hot-top assembly houses a 96-hole grid (used by the instrument for light path entry) which may be mistaken for a thermal block. Ensure that the hot-top is raised, exposing the thermal block, prior to loading samples.*



Use of the Perfect Fit Frame with Single Tubes and Strip Tubes

When using individual tubes or strip tubes, insert the Perfect Fit frame (shown below in the proper orientation for installation) on the thermal block platform before loading the samples.

The Perfect Fit frame optimizes the contact between the sample tubes and the hot-top. It is not necessary to use the Perfect Fit frame with 96-well plates. One Perfect Fit frame is supplied with the Mx3000P System; additional frames are available from Stratagene (Catalog #401421).

To install the Perfect Fit frame, position the frame around the border of the thermal block. When properly installed, each of the six notches on the bottom of the frame will be positioned in the spaces between wells on the perimeter of the thermal block surface. The Perfect Fit frame should be seated on the thermal block platform with limited freedom of movement. There are no audible sounds associated with the installation. (The frame does not "click" into place.)

Preparation of Samples Prior to Loading

Application of Caps

Wear powder-free gloves while applying caps to the sample containers. Caps should be placed on the samples before the samples are loaded into the instrument. For best results, apply the caps while the samples are firmly seated in a 96-well working rack. Apply direct downward force to each cap, and then view the rack from the side to make sure all the caps are seated evenly. Alternatively, caps can be applied using a Corning Storage Mat Applicator.

Centrifugation of Samples

Prior to loading samples in the Mx3000P thermal block, briefly centrifuge the tubes or 96-well plate to collect any drops of liquid on the tube sides or lid.

Positioning of Tube Strips in the Thermal Block

When using tube strips, the 8-tube strips must be oriented vertically in the 8-position columns. Do not load strip tubes horizontally within the 12-position rows.

Consumables

Plasticware that meets the performance specifications of the Mx3000P system may be purchased from Stratagene:

96-well PCR plates, 0.2-ml, non-skirted, Stratagene Catalog #410088
8× strip tubes, 0.2-ml, Stratagene Catalog #401428
8×optical strip caps, Stratagene Catalog #401425
Single PCR reaction tubes (without caps), 0.2-ml, Stratagene Catalog #410023

Accessories

Stratagene Centrifuges

Tube-Strip PicoFuge®, for 8-tube 200-µl tube strips or individual 200-µl tubes [Catalog #400540 (115-120V) Catalog #400542 (230V)].

96-Well Working Racks

96-well benchtop working racks are available from Stratagene (Catalog #410094).

Storage Mat Applicator

A storage mat applicator may be used for seating caps on tubes [Corning Model #3081 (sold by VWR Scientific, Catalog #29445-134)].

Multiple Instrument Set-Up: Optional Use of Multiple Instrument Control

When multiple Mx3000P instruments are used in a laboratory, the instruments may be set up to be controlled either each by individual computers or all by a shared computer. To set up the instruments using single instrument control by individual computers, simply follow the setup instructions provided on the Installation Guide poster or in the Setup and User's Guide. To set up the instruments to be controlled by a shared computer, follow the guidelines below.

Hardware Connections for Multiple Instrument Control

Using the Multiple Instrument Control mode of the Mx3000P software, up to six instruments may be controlled simultaneously from a single computer.

1. Assess the availability of ports on the computer for connection to Mx3000P instruments. Each instrument will be connected using the provided serial cable (Stratagene part #22316-00). Any serial port on the computer may be used without any additional hardware. Any available USB port may also be used, after connecting the port to a USB-to-serial adapter (Stratagene part #22490). If additional ports are required to accommodate a greater number of instruments, a USB hub along with the appropriate number of USB-to-serial adapters may be used.

2. Connect each of the instruments to a power source. Verify that the power switch located at the rear of the instrument is toggled off. Attach the power cord to the plug port, adjacent to the power switch. Plug the instrument into a properly grounded outlet.

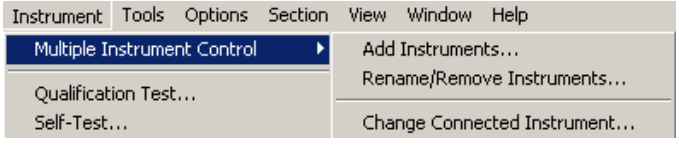
3. For each instrument, connect either end of the provided serial cable to the RS-232 serial port at the rear of the instrument.

4. Connect the free end of the serial cable to the computer, connecting either to a serial port or to a USB port outfitted with a USB-to-serial adapter.

5. Start-up the system. Power up each of the Mx3000P instruments using the switch located on the back of the instrument. Power up the PC. When the Windows® desktop appears, click on the Mx3000P icon to start the Mx3000P application.

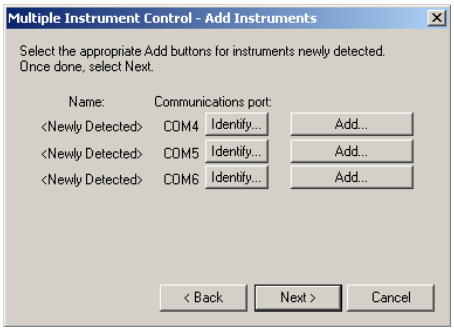
Setting Up the Mx3000P Software for Multiple Instrument Control

To establish the associations between the computer and multiple instruments in the Mx3000P software, expand the **Multiple Instrument Control** section of the **Instrument** menu and then select **Add Instruments**.



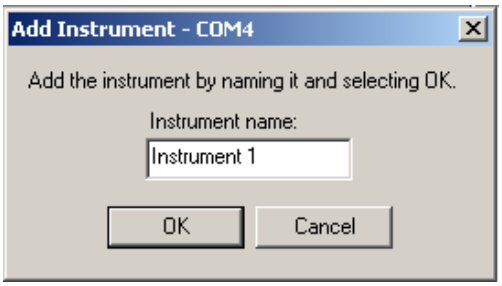
Note *It is recommended that instruments not be running experiments while setting up multiple instrument control or while adjusting the settings.*

A Wizard will guide you through the process of adding instruments. After a short series of advisory dialog boxes, the following dialog box will appear with newly detected instruments in the list.



Clicking the **Identify** button associated with a particular communications port causes the front LED of the instrument connected to that port to flash, allowing identification of the detected computers in the list.

To add an instrument to the group of instruments controlled by the computer, click the corresponding **Add** button. When the **Add Instrument – COM#** dialog box appears, enter the **Instrument name** and then click **OK**.

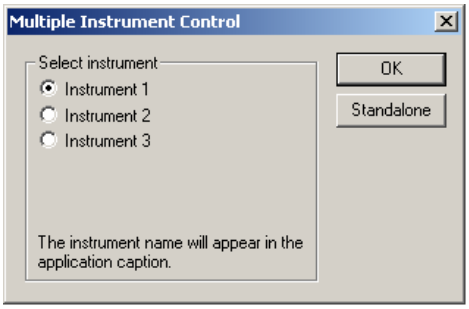


Repeat this process for any other instruments connected to other communications ports. When all instruments have been added and their names entered, click **Next**, and the Wizard will guide you through completion of the process.

Using the Mx3000P Software in Multiple Instrument Control Mode

Selection of Instrument at Start-Up

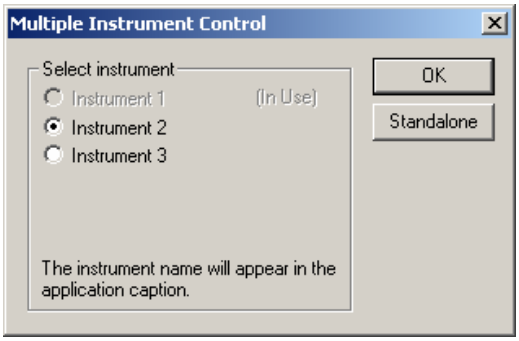
Once the computer has been set up to recognize multiple instruments, you will be prompted to specify the instrument to be used each time you launch the Mx3000P software application.



Each open copy of the Mx3000P application may only be connected to a single instrument. After launch, the specific instrument connected to the Mx3000P application is indicated in the title bar.

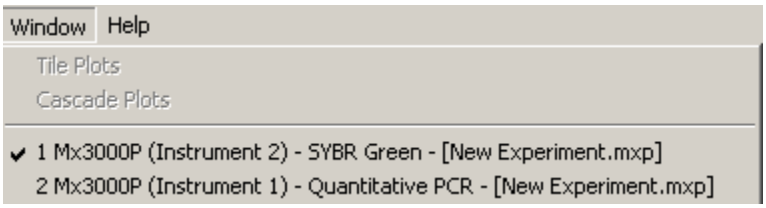
Simultaneous Use of Multiple Instruments

The multiple instruments controlled by a single computer may be used simultaneously by launching multiple copies of the Mx3000P application on the computer. Each time an additional application is launched, select the appropriate instrument from the **Multiple Instrument Control** dialog box.



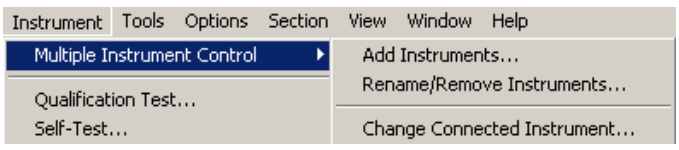
Instruments that are unavailable are indicated by an **In-Use** status. Typically an instrument is unavailable because it is being used by another Mx3000P application. Other possible reasons include that the instrument is not powered on or that communications between the computer and the instrument are disrupted (e.g. disconnection of the serial cable or adapter).

After launch, each copy of the application will show the connected instrument in the title bar. Navigate between Mx3000P applications connected to the different instruments by selecting the appropriate application/instrument from the Mx3000P software **Window** menu or by using the taskbar at the bottom of the screen.



Administration of Multiple Instrument Control

To add, remove or rename instruments controlled by the computer, or to change the instrument connected to a specific copy of the Mx3000P application, use the commands available from the **Multiple Instrument Control** section of the **Instrument** menu.

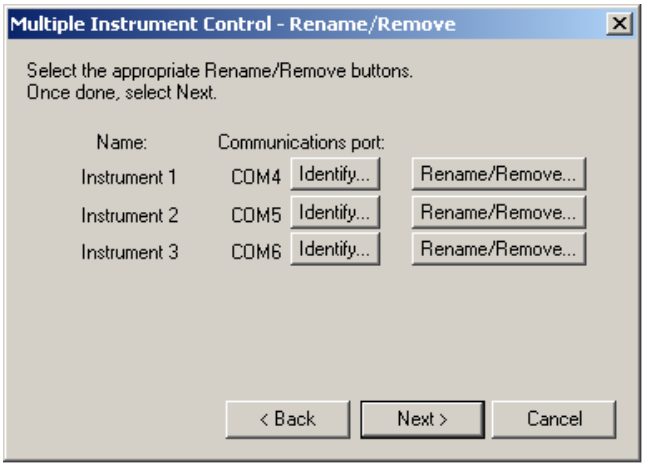


Adding an Instrument to the Group Controlled by the Computer

Adding an instrument after initial setup involves the same steps used during the initial setup of multiple instrument control. First, establish the hardware connections between the new instrument and the computer (see *Hardware Connections for Multiple Instrument Control*). Next, complete the software setup steps, beginning by selecting **Add Instruments** from the **Multiple Instrument Control** menu. Follow the steps outlined by the Wizard. (See *Setting Up the Mx3000P Software for Multiple Instrument Control*, above, for more information).

Renaming or Removing an Instrument

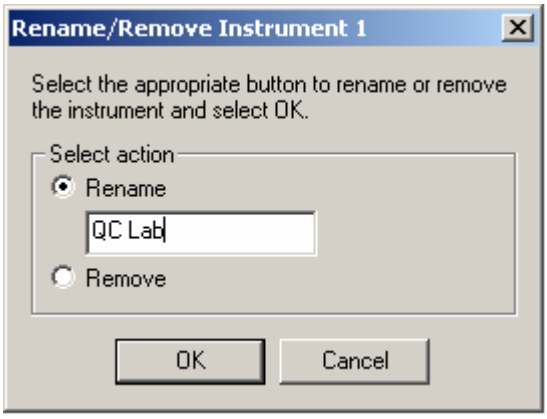
To rename an instrument or to remove an instrument from the group of instruments controlled by the computer, select **Rename/Remove Instruments** from the **Multiple Instrument Control** menu. A Wizard will guide you through the process. After one or more advisory dialog boxes, the following dialog box will appear:



To verify the identity of the instrument to be renamed or removed, click the **Identify** button associated with the instrument name. The front LED of the specified instrument will then flash.

When ready to proceed, click the appropriate **Rename/Remove** button.

To rename an instrument, select the **Rename** radio button, enter the new name in the box, and then click **OK**. The Wizard will guide you through completion of the process.



To remove the instrument, select the **Remove** radio button and then click **OK**. The Wizard will guide you through completion of the process, providing advice or instructions for the following special cases:

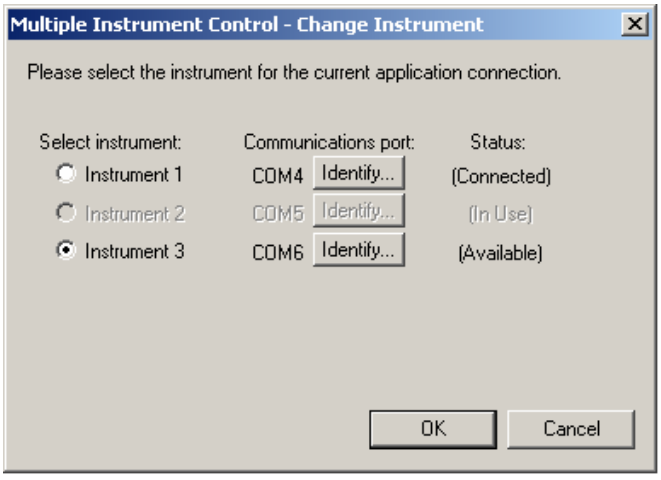
If you are removing the instrument that is currently connected to the open Mx3000P application, the Wizard will direct you to select another instrument for connection to the application.

If, after removing instruments, only one instrument remains connected, the Wizard will notify you that the computer is being switched to the single-instrument control mode.

If you attempt to remove the last instrument, the Wizard will prevent this action, since running an instrument-connected version of the Mx3000P application requires that at least one instrument be connected to the computer. If you wish to run the application without connection to an instrument, open an additional **Standalone** copy of the Mx3000P application.

Changing the Instrument Connected to the Software Application

To change the instrument connected to the open copy of the Mx3000P software application, click **Change Connected Instrument** from the **Multiple Instrument Control** menu. When the following dialog box appears, select an **Available** instrument and then click **OK**. The newly selected instrument name will appear in the title bar for the open application.



Choosing a Detection Chemistry and Experiment Type

Overview of QPCR and Detection Chemistries

Overview of Quantitative PCR

The quantitative PCR method represents an important technique for quantifying messenger RNA levels (gene expression) and DNA gene levels (copy number) in biological samples. The amount of a specific target nucleic acid is generally too low in biological samples for direct detection; the target must therefore be amplified by using PCR or other protocols to achieve detection. Endpoint analysis of PCR product accumulation does not allow accurate quantitation of the initial amounts of the target in the samples. As amplification proceeds, reagents may become limiting, PCR inhibitors may accumulate, and any small differences in PCR performance among samples are magnified. Thus PCR product accumulation measured at the end of cycling fails to accurately reflect the initial amount of target in the sample. In contrast, in real-time quantitative PCR analysis, accumulation of the amplified target is monitored during the early exponential phase of the amplification process when reagents are not limiting, and differences due to other factors are minimized, providing a significantly more accurate quantification of initial RNA or DNA levels. Additional benefits of real-time quantitative PCR include sensitivity and a wide dynamic range. As few as 10 copies of an RNA target can be detected and the linearity of detection is greater than six orders of magnitude.

Mx3000P system quantitative PCR experiments use fluorescence-labeled probes or SYBR Green I fluorescent dye for both RNA and DNA detection, allowing real-time monitoring of PCR product formation during the PCR amplification process in a closed-tube format.

Detection using Fluorescence-Labeled Probes

Fluorescence-labeled oligonucleotides, most commonly molecular beacons and TaqMan® probes, are employed for QPCR detection when the most accurate quantitation of PCR product accumulation is desired. Both of these types of probes are designed as oligonucleotides that contain a region complementary to a sequence within the nucleic acid sequence being amplified. Both also contain a fluorophore and a quencher moiety, usually at opposite ends of the oligonucleotide. For both probe types, fluorescence is quenched prior to amplification due to the action of the quencher on the fluorophore. The process of amplification separates the fluorophore from the quencher (using two different mechanisms that are described below), allowing the fluorophore to fluoresce. As a result, the amount of fluorescence at any given cycle is directly proportional to the amount of specific product present at that time.

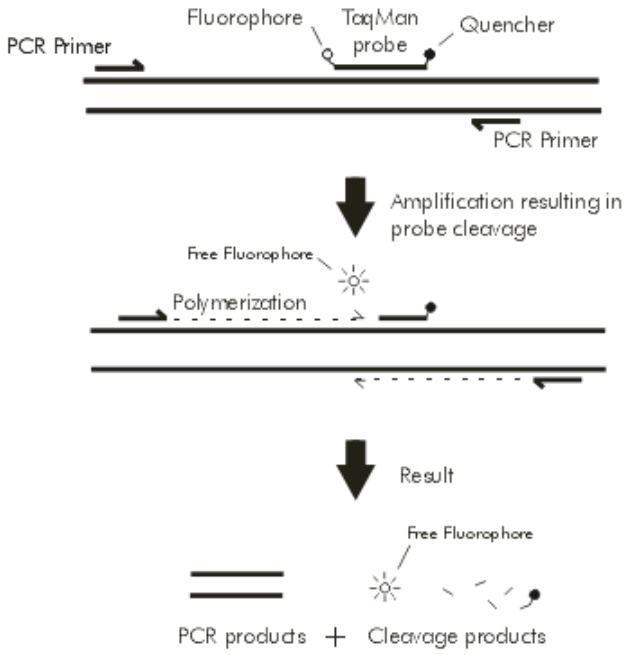
For both types of fluorescent probes, as PCR proceeds the fluorophore will produce fluorescent light of a color that is characteristic of the fluorophore used. The existence of a variety of spectrally distinct fluorophores available for labeling either molecular beacons or TaqMan probes introduces the possibility of multiplexing, or quantitating multiple targets with different probes in the same reaction well.

Molecular Beacons Probes

Molecular beacons assume a hairpin-structure when free in solution, bringing the end-bound fluorophore and quencher into close proximity, thereby allowing quenching of the fluorescence signal. The binding of the probe to its target disrupts the hairpin structure, resulting in a spatial separation of the fluorophore from the quencher, thereby allowing fluorescence. When the DNA polymerase extends the upstream primer, the bound probe is displaced and resumes its hairpin structure. The transition from free (quenched) to quantitatively bound (fluorescent) is repeated in each subsequent amplification step, allowing the real-time measurement of target levels at each cycle.

TaqMan Probes

TaqMan probes are linear oligonucleotides which are designed to allow a close proximity of the fluorophore and quencher in the intact, unstructured probe. This detection chemistry employs the cleavage of probe molecules during the amplification process to physically separate the fluorophore from the quencher moiety. TaqMan probes are complementary to a region of the target located between the upstream and downstream primer binding sites. As the DNA polymerase extends the upstream primer, it encounters the bound probe. The 5' to 3' exonuclease activity of the polymerase cleaves the probe, releasing the fluorophore into solution, where it is allowed to fluoresce. Quantitative release of additional free fluorophore is repeated in each subsequent amplification step, allowing the real-time measurement of target levels at each cycle.



Detection using SYBR Green Dye

When the most accurate quantitative data are not required, the accumulation of double-stranded DNA (dsDNA), as indicated by a fluorescent dsDNA-binding dye, can be used to quantitate the accumulation of a PCR product in a QPCR assay. The dsDNA-binding dye that is most commonly used in QPCR is SYBR Green I dye.

SYBR Green I dye has a higher affinity for dsDNA than for single-stranded DNA (ssDNA) or RNA, making it ideal for reporting the accumulation of dsDNA. Upon binding DNA, the magnitude of SYBR Green fluorescence emission increases by approximately 1000-fold, making this dye a sensitive indicator for the quantity of dsDNA present in the reaction mixture at any given time.

Specific and non-specific double-stranded PCR products generate the same fluorescence signal upon binding SYBR green dye. To distinguish between fluorescence derived from specific and non-specific products, SYBR green dye-based QPCR assays include a dissociation curve. During the dissociation curve, dsDNA product is melted into ssDNA by a stepwise increase in temperature, with fluorescence data being collected at each step. The magnitude of the reduction in fluorescence intensity at the melting temperature of the specific PCR product of interest provides a qualitative indicator of the proportion of dsDNA attributable to the specific PCR product.

A major advantage of this approach is that no fluorescence-labeled probes are required. SYBR Green I stain is compatible with fluorescence-detection instruments equipped with filters in the 520 nm range (e.g. filters for detecting 6-FAM).

Overview of Mx3000P System Experiment Types

Real-Time vs. Plate Read Experiments

The Mx3000P real-time PCR system software allows you to select from a variety of experiment types, providing setup and analysis screens that are specialized for the specific application. Both real-time experiments that monitor and report the accumulation of PCR product during thermal cycling, and plate-read (sometimes referred to as endpoint) experiments types are available.

Real-Time Experiments

Real-time experiments should be used when accurate quantitation of the levels of a specific RNA or DNA target is required. In real-time experiments, accumulation of the amplified target is monitored during the early exponential phase of the amplification process when reagents are not limiting, and differences due to other factors are minimized, providing a significantly more accurate quantification of initial RNA or DNA levels. *See Overview of Quantitative PCR* for a more detailed discussion of the advantages of using real-time analytical methods.

Note *The term* quantitative PCR *is often used to describe a variety of experimental approaches using different detection chemistries and analysis algorithms to achieve a quantitative analysis of target levels by monitoring PCR product accumulation. The Mx3000P software includes the experiment type* **Quantitative PCR** *describing a specific assay type in which fluorogenic oligonucleotide probes are used to quantitate target levels by way of a standard curve. Other specific experiments which fall under the general usage of* quantitative PCR *are featured in other experiment types.*

The Mx3000P system includes the following specific real-time experiment types:

- **Quantitative PCR**: This experiment type uses a standard curve to quantitate the amount of target present in an **Unknown** sample with high accuracy using a fluorescence-labeled probe for detection. A series of **Standard** samples, containing dilutions of a known amount of target, are amplified to generate a curve that relates the initial quantity of the specific target to the **Ct**. The standard curve is then used to derive the initial template quantity in **Unknown** wells based on their **Ct** values. This method is sometimes referred to as absolute quantitation or as standard-curve quantitation in the literature. This experiment type is also useful for primer/probe optimization experiments in the absence of a standard curve.

- **Comparative Quantitation**: This experiment type is a form of relative quantitation, comparing the levels of a target gene in test samples (referred to as **Unknowns**) relative to a sample of reference (referred to as the **Calibrator**). For example, the **Calibrator** sample might contain RNA from untreated cells, while the **Unknowns** might contain RNA from cells treated with different agents of interest. This experiment type provides an efficient method for comparing levels of RNA or DNA across samples when information about the absolute amounts of target in any sample is not required.

- **SYBR Green (with Dissociation Curve)**: This experiment type uses a standard curve to quantitate the amount of target present in an **Unknown** sample using SYBR Green I dye for detection. This experiment type is also useful for SYBR Green assay optimization experiments in the absence of a standard curve. The thermal profile includes a dissociation curve, used to discriminate between specific and non-specific PCR products.

- **Allele Discrimination/SNP's Real-Time**: This experiment type uses fluorescence-labeled probes to determine the allelic composition of DNA samples. Two fluorogenic probes, labeled with two spectrally distinct dyes, are used to discriminate between two different alleles that may differ by as little as a single nucleotide. The presence or absence of a given allele is based on the Ct value determined for the allele-specific probe.

- **Molecular Beacon Melting Curve**: This experiment type analyzes the melting characteristics of a molecular beacon for the determination of the experimental melting temperature (Tm) and optimal annealing temperature for subsequent PCR experiments. Melting curves are typically performed with the molecular beacon alone and in the presence of both perfectly-matched and mismatched oligonucleotide targets in order to determine the temperature that results in maximum specificity.

Plate Read Experiments

Plate read experiments are appropriate for applications that allow endpoint analysis of fluorescence values. The plate-read function may be used to take fluorescence readings for a set of samples that are cycled in another thermal cycler, collecting either a one-time fluorescence reading or pre-cycling and post-cycling readings.

The Mx3000P software offers two specific plate-read experiment types:

- **Quantitative Plate Read**: This experiment type uses a standard curve to estimate the amount of target present in an **Unknown** sample using a fluorescence-labeled probe for detection. A series of **Standard** samples, containing dilutions of a known amount of target, are included in the experiment to generate a curve that relates the quantity of the specific target to the final fluorescence reading. The standard curve is then used to estimate the template quantity in **Unknown** wells based on their fluorescence readings.

- **Plate Read/Allele Discrimination**: This experiment type is used both for general plate-reading tasks and for allele discrimination assays with endpoint detection. In both cases, samples may be called as positive or negative for the presence of a specific sequence based on whether the endpoint fluorescence readings meet user-defined statistical criteria.

Navigating in the Mx3000P Software

Menu Commands

Menu commands available in the Mx3000P real-time PCR system software are used to manipulate files, to customize and use the experimental setup and analysis features of the software, to initiate several instrument actions and to access **Help** functions. Click on the menu name below for more information on the software commands accessed through each individual menu.

File Menu

Edit Menu

Instrument Menu

Tools Menu

Options Menu

Section Menu

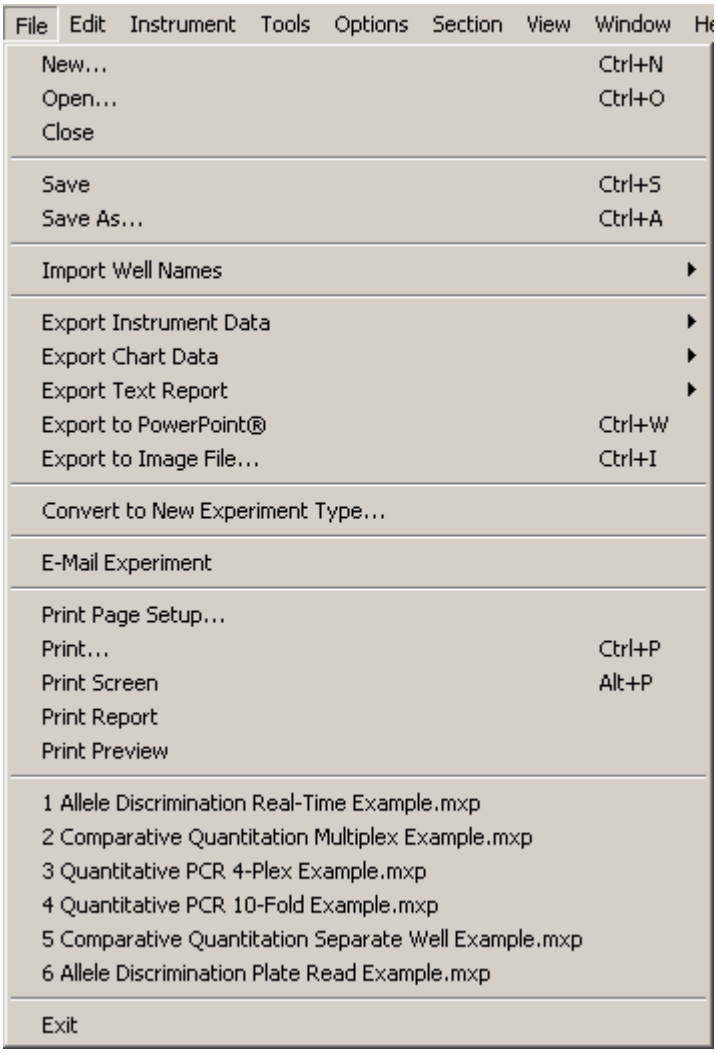
View Menu

Window Menu

Help Menu

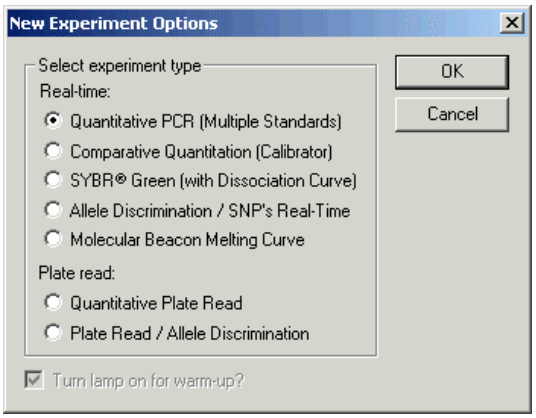
File Menu

The **File** menu contains commands used to open, close, save and print files. The menu also contains commands for exporting and converting files as well as a list of recently-opened experiments.



New - Create a New Experiment 

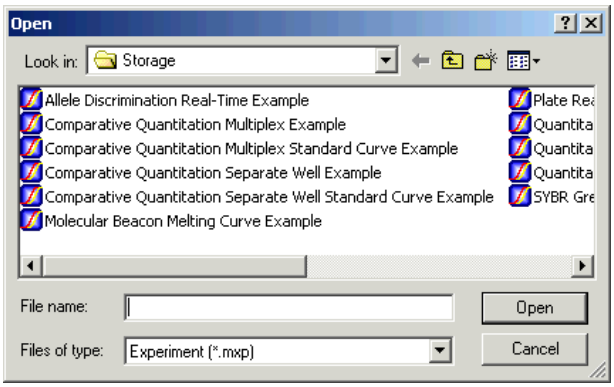
To create a new experiment, click **New** in the **File** menu, press **Ctrl+N**, or click the new experiment button  on the toolbar. The **New Experiment Options** dialog box will open, allowing you to select the type of experiment to be created. Click the radio button next to the desired experiment type and then click **OK**. Checking **Turn lamp on for warm-up?** will apply power to the lamp once **OK** is clicked. The lamp should be warmed-up for 20 minutes before an experiment is run.



After **OK** is clicked, the software will proceed to the **Plate Setup** screen for the specific type of experiment selected.

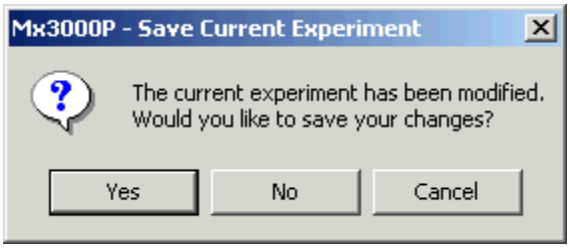
Open - Open an Existing Experiment

To open an existing experiment, click **Open** in the **File** menu, press **Ctrl+O**, or click the open button on the toolbar. The **Open** dialog box will appear. Select the experiment to be opened. (Use the **Look in** menu to browse to other storage locations if necessary.) Click the **Open** button at the bottom of the dialog box.



Close - Close the Current Experiment

To close an experiment without closing the Mx3000P software, click **Close** in the **File** menu. If the experiment has been modified, the **Save Current Experiment** dialog box will appear. Click **Yes** to save the changes and close the experiment, click **No** to close the experiment without saving the changes, or click **Cancel** to return to the experiment without saving any changes. If the **Yes** button is clicked to save the experiment, the **Save As** dialog box will appear, allowing you to specify the name that the file will be saved under.



Save

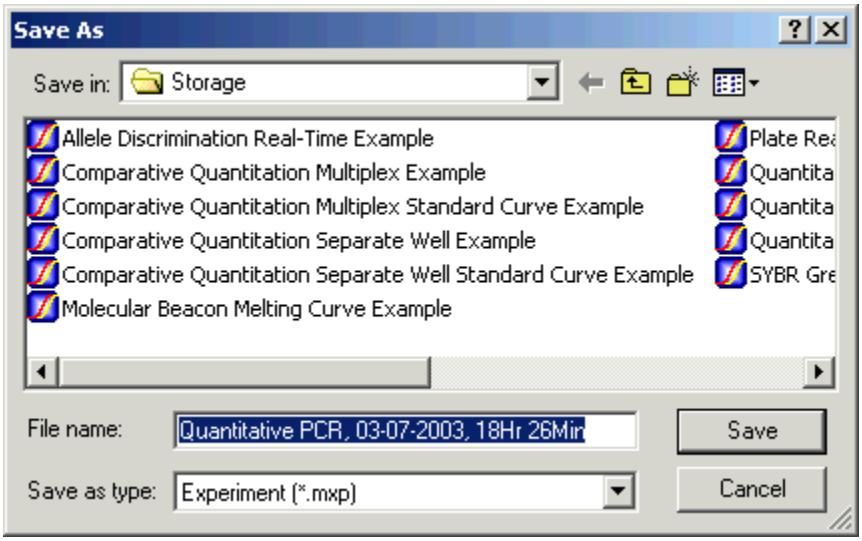
To save an experiment, click **Save** in the **File** menu, press **Ctrl+S**, or click the save button on the toolbar. If the experiment has already been saved, no dialog box will be displayed and the experiment will be saved under its current name. If the experiment has not already been saved, the **Save As** dialog box will open (see below), allowing you to specify a file name and directory for storing the experiment.

If the experiment has not been modified since it was last saved, the **Save** command and button will be disabled. The Mx3000P software automatically saves the experiment at the end of a run, therefore **Save** is disabled at the conclusion of a run. If any changes are made to the experiment after completion of the run, **Save** will be enabled.

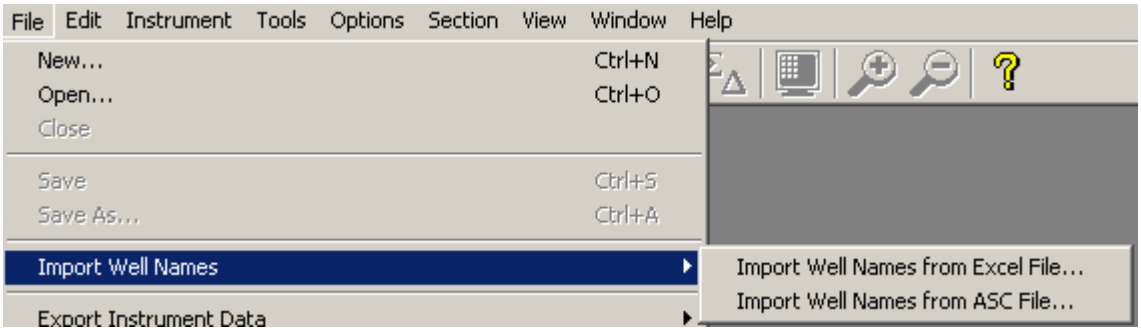
To save an experiment under a different name, use the **Save As** command in the **File** menu.

Save As

To save a new experiment or to save an existing experiment under a new name, click **Save As** in the **File** menu or press **Ctrl+A**, and the **Save As** dialog box will open. If the experiment was previously saved, the name of the experiment will appear in the **File name** field where it may be modified. If the experiment has not yet been saved, a default file name will appear in the **File name** field. You can elect to either use the default file name or specify another file name. Experiment files are stored in the default data directory (C:\Program Files\Stratagene\Mx3000P\Storage directory). The default storage location and the default file name structure may be changed on the **File** tabbed page of the **Preferences** dialog box (accessed from the **Options** menu).



Import Well Names



Well names may be imported from a Microsoft® Excel file or from certain ASC files into the open experiment. The Mx3000P software associates well names with well ID's (A1–H12) using a mechanism appropriate to the input file type.

Import Well Names from Excel File

Well names may be imported from a Microsoft Excel file formatted as shown below with column A containing Well ID's and column B containing the well names to be imported. The Well ID's may appear in any order but must contain the syntax A1–H12 or A01–H12.

	A	B	C
1	Well ID	Well Name	
2	A1	Reference RNA (1)	
3	B1	Reference RNA (10)	
4	C1	Reference RNA (100)	

To import the well names into the **Plate Setup** for the open experiment, first ensure that all wells to be associated with an imported well name are defined (have at least a **Well type** assignment). Select **Import Well Names from Excel File** from the expanded **File** menu. When the **Well Name Import** dialog box appears, browse to the Microsoft Excel file location and then click **Open**.

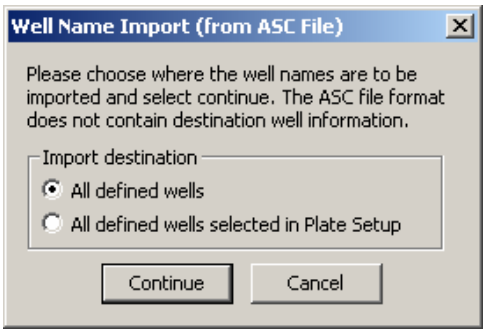
Well names may be visualized in the **Full Screen Plate** view.

Import Well Names from ASC File

Import Well Names from ASC File is a specialized feature that allows import of well names from files generated using some other automated instruments. The ASC files do not contain destination (well ID) information. For this reason, the destination wells are specified within the Mx3000P experiment and the well names must be listed in the ASC file in a specific structure. The supported structure of well names in the ASC file is vertical, left-to-right. In other words, well names imported from the ASC file are directed to user-specified wells in the Mx3000P experiment in the order of A1–H1, A2–H2…A12–H12.

To use this well name import feature, first specify the destination wells using the **Plate Setup** screen. The specified wells must be defined with at least a **Well type** assignment. Do not assign replicate symbols prior to import. Select the wells to be associated with well names from the ASC file with the mouse on the **Plate Setup** screen (all defined wells or a subset of the defined wells may be selected).

Next, select **Import Well Names from ASC File** from the expanded **File** menu. The following dialog box will appear. Select the appropriate description of the wells to be associated with a well name under **Import destination**, and then click **Continue**.

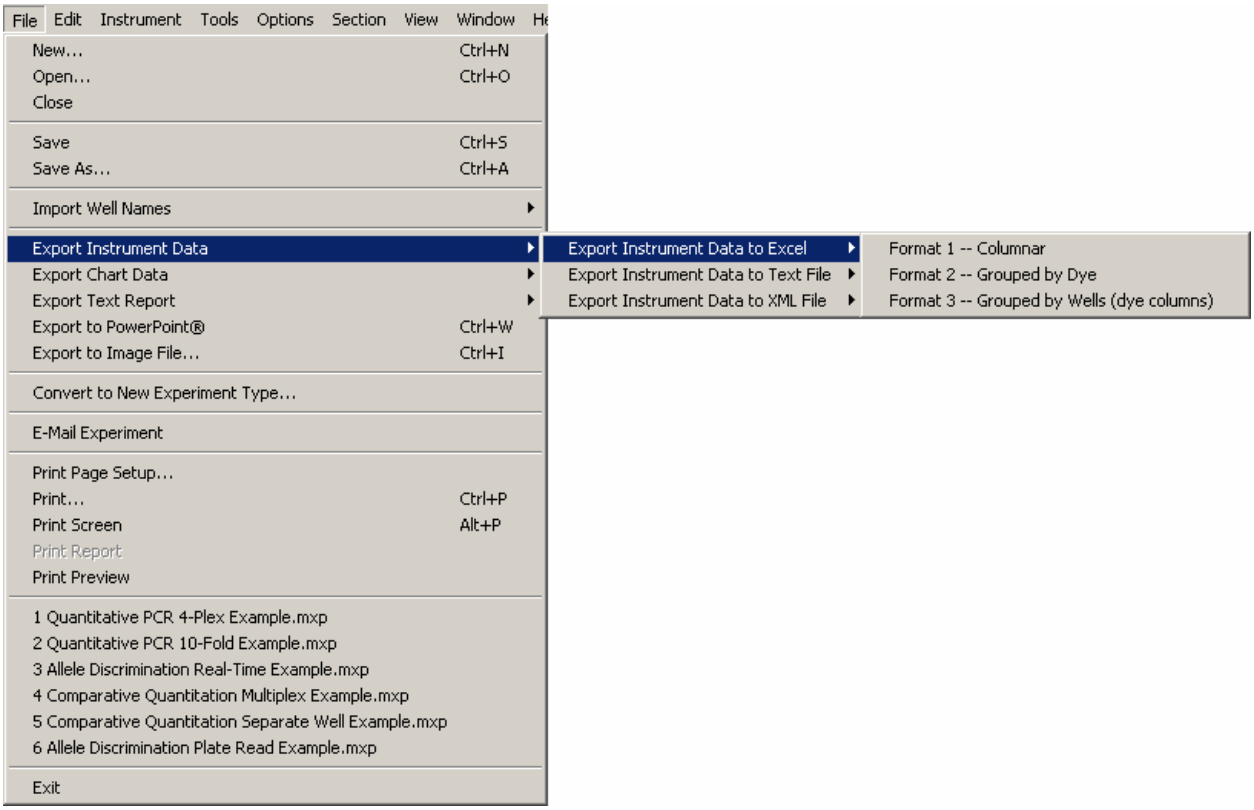


In the next dialog box, browse to the ASC file location, select the file name, and then click **Open**.

Well names may be visualized in the **Full Screen Plate** view.

Exporting Data to Other Applications

The **File** menu export commands allow raw instrument data, chart data, or text report data to be exported to a Microsoft Excel file, a text file or an XML file in a variety of formats. In addition, Mx3000P software screens may be exported to Microsoft PowerPoint® or image files. Each of the menu items marked with arrows may be expanded to reveal output file options and formatting options.



Exporting Data to Excel

Raw instrument data (cycle-by-cycle fluorescence readings), chart data points, or text report data may be exported to the Microsoft Excel application.

Selecting **Export Instrument Data to Excel, Export Chart Data to Excel**, or **Export Text Report to Excel** from the **File** menu exports the corresponding data to Microsoft Excel. To maintain the new file as an Excel workbook, execute the **Save As** command from the Excel application and select the Excel workbook file type from the **Save as type** menu. If you exit the Excel software without saving the file as a workbook, then the file will be saved as a tab-delimited text file.

Formatting options for instrument or chart data output to the Excel file are described below.

Exporting Data to a Text File

Raw instrument data (cycle-by-cycle fluorescence readings), chart data points, or text report data may be exported to a tab-delimited text (.txt) file. The text file can be opened in any word-processing program.

Formatting options for instrument or chart data output to the text file are described below.

Exporting Data to an XML File

Raw instrument data (cycle-by-cycle fluorescence readings), chart data points, or text report data may be exported to an Extensible Markup Language (XML) file to allow viewing in a browser window and to allow importing of the data into databases.

Selecting **Export Instrument Data to XML File, Export Chart Data to XML File**, or **Export Text Report to XML File** from the **File** menu exports the corresponding data to an XML file (filename.xml). At the same time, two support files (filename.xsl and filename.xsd) are also created. All three files are stored in the same folder [by default, the Mx3000P data storage folder specified in the **Preferences-File** dialog box (e.g., C:\Program Files\Stratagene\Mx3000P\Storage)].

Note *The .xml, .xsl and .xsd files should be maintained in the same file directory to allow browser viewing.*

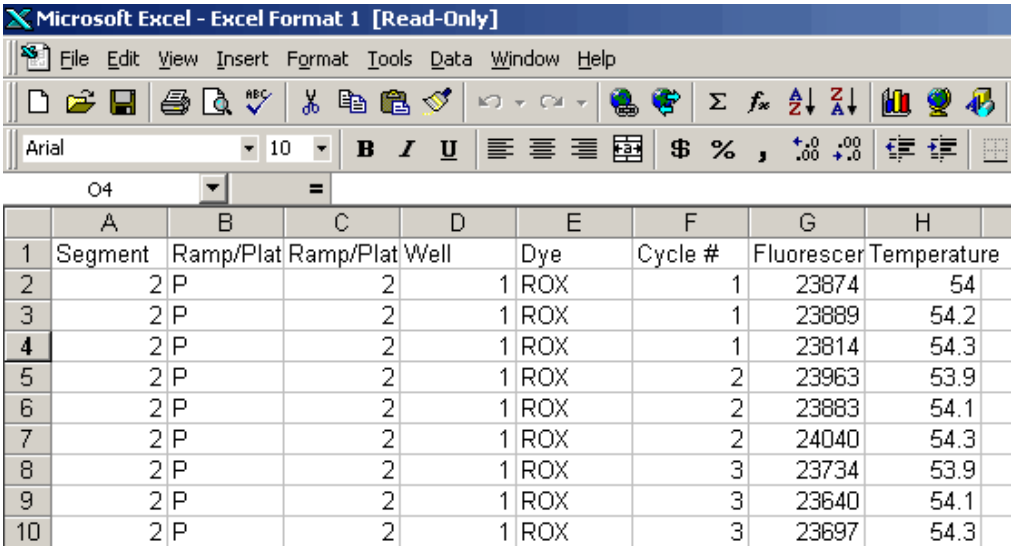
The XML file may be opened using your computer's browser (typically, double-clicking on the XML file icon is sufficient).

Formatting options for instrument or chart data output to the XML file are described on the following pages.

Formatting Options for Exported Instrument Data

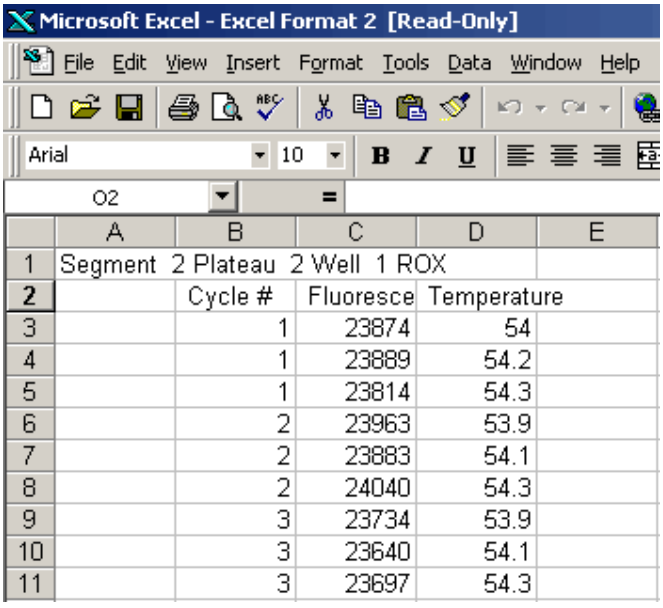
Three different formats are available from the **Export Instrument Data to Excel** and the **Export Instrument Data to Text File** and **Export Instrument Data to XML File** submenus. Examples of the three formats are shown below.

Format 1--Columnar -- Cycle-by-cycle data are listed for each well.



	A	B	C	D	E	F	G	H
1	Segment	Ramp/Plat	Ramp/Plat	Well	Dye	Cycle #	Fluorescer	Temperature
2	2	P	2	1	ROX	1	23874	54
3	2	P	2	1	ROX	1	23889	54.2
4	2	P	2	1	ROX	1	23814	54.3
5	2	P	2	1	ROX	2	23963	53.9
6	2	P	2	1	ROX	2	23883	54.1
7	2	P	2	1	ROX	2	24040	54.3
8	2	P	2	1	ROX	3	23734	53.9
9	2	P	2	1	ROX	3	23640	54.1
10	2	P	2	1	ROX	3	23697	54.3

Format 2--Grouped by Dye -- Cycle-by-cycle data for each well are grouped by dye paths.



	A	B	C	D	E
1	Segment	2 Plateau	2 Well	1 ROX	
2		Cycle #	Fluoresce	Temperature	
3		1	23874	54	
4		1	23889	54.2	
5		1	23814	54.3	
6		2	23963	53.9	
7		2	23883	54.1	
8		2	24040	54.3	
9		3	23734	53.9	
10		3	23640	54.1	
11		3	23697	54.3	

Format 3--Grouped by Wells (dye columns) -- Cycle-by-cycle data are grouped by wells with the data for each dye path shown in separate sets of columns.

Microsoft Excel - Excel Format 3 [Read-Only]

File Edit View Insert Format Tools Data Window Help

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	A	B	C	D	E	F	G	H	I	J
1	Segment 2 Plateau 2 Well 1									
2		ROX			FAM			CY5		
3		1	23874	54	1	19826	54	1	1353	54
4		1	23889	54.2	1	19923	54.2	1	1359	54.2
5		1	23814	54.3	1	19996	54.3	1	1354	54.3
6		2	23963	53.9	2	19611	53.9	2	1362	53.9
7		2	23883	54.1	2	19877	54.1	2	1368	54.1
8		2	24040	54.3	2	20279	54.3	2	1367	54.3
9		3	23734	53.9	3	19548	53.9	3	1373	53.9
10		3	23640	54.1	3	19926	54.1	3	1377	54.1
11		3	23697	54.3	3	20211	54.3	3	1377	54.3

Formatting Options for Exported Chart Data

Two different formats are available from the **Export Chart Data to Excel** and the **Export Chart Data to Text File** and **Export Chart Data to XML File** submenus. Examples of the two formats are shown below.

Format 1—Vertically Grouped by Plot – The coordinates of each plotted data point are listed for each well/dye vertically. (The contents of the x- and y-coordinate columns are dependent on the specific chart type.)

Microsoft Excel - Chart Data Excel.txt [Read-Only]

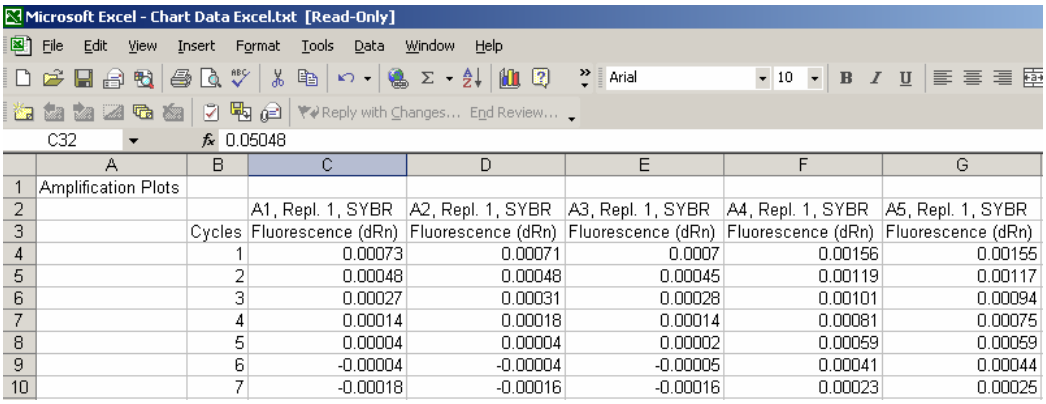
File Edit View Insert Format Tools Data Window Help

Reply with Changes... End Review...

A1 Amplification Plots

	A	B	C	D	E
1	Amplification Plots				
2					
3	A1, Repl. 1, SYBR	Cycles	Fluorescence (dRn)		
4		1	0.00073		
5		2	0.00048		
6		3	0.00027		
7		4	0.00014		

Format 2—Horizontally Grouped by Plot – The coordinates of each plotted data point are listed with each well/dye shown in a separate column. (The contents of the x- and y-coordinate columns are dependent on the specific chart type.)



	A	B	C	D	E	F	G
1	Amplification Plots						
2			A1, Repl. 1, SYBR	A2, Repl. 1, SYBR	A3, Repl. 1, SYBR	A4, Repl. 1, SYBR	A5, Repl. 1, SYBR
3		Cycles	Fluorescence (dRn)	Fluorescence (dRn)	Fluorescence (dRn)	Fluorescence (dRn)	Fluorescence (dRn)
4		1	0.00073	0.00071	0.0007	0.00156	0.00155
5		2	0.00048	0.00048	0.00045	0.00119	0.00117
6		3	0.00027	0.00031	0.00028	0.00101	0.00094
7		4	0.00014	0.00018	0.00014	0.00081	0.00075
8		5	0.00004	0.00004	0.00002	0.00059	0.00059
9		6	-0.00004	-0.00004	-0.00005	0.00041	0.00044
10		7	-0.00018	-0.00016	-0.00016	0.00023	0.00025

Exporting Content to a PowerPoint File

Selecting **Export to PowerPoint** exports the contents of the active section of the experiment to a Microsoft PowerPoint file. This command may be used to export the content of the screens from any section (**Setup**, **Run**, or **Analysis**) of the Mx3000P software. The PowerPoint application is automatically launched when this command is executed, and the content of the current screen is copied into a new slide.

Exporting Content to an Image File

Selecting **Export to Image File** exports the contents of the active section of the experiment to a bitmap (.bmp) image file. This command may be used to export the content of the screens from any section (**Setup**, **Run**, or **Analysis**) of the Mx3000P software. By default, the new image file is stored in the Mx3000P data folder (e.g., C:\Program Files\Stratagene\Mx3000P\Storage) and can be opened using a variety of graphics applications.

Convert to New Experiment Type

After an experiment has been set up and run as one experiment type, the data may be reanalyzed as different experiment type. When the **Convert to New Experiment Type** command is executed, the Mx3000P software creates a new experiment file and applies the analysis algorithms and display schemes of the newly selected experiment type to the existing data, using the well assignments and fluorescence readings from the original run. If well assignments made in the original run are unavailable in the new experiment type, a dialog box containing information about well type assignment incompatibilities will appear. The Mx3000P software will convert any of the available experiment types to any other available experiment type.

E-mail Experiment

The **E-mail Experiment** command is a shortcut for creating an email that contains the open experiment file in the Mx3000P System file format (.mxp). Clicking **E-mail Experiment** launches Microsoft Outlook® and creates a new message with the open Mx3000P experiment file attached to the new message.

Print Page Setup

Print Page Setup is used to adjust the size of the image printed when either **Print** or **Print Screen** is executed. To adjust the image size, click **Print Page Setup.** When the dialog box appears, enter the desired image size (25–100% of the original size) in the **Scale** box and then click **OK**.

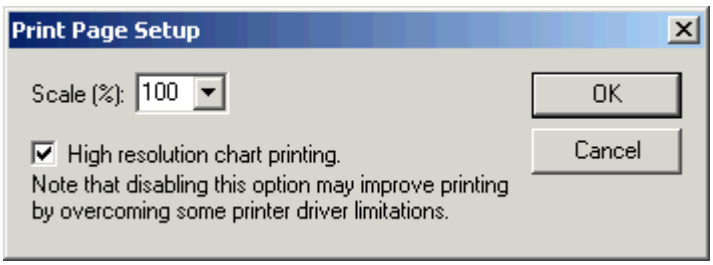


Chart printing using most printers will be enhanced by using the **High resolution chart printing** option. If you are experiencing chart print quality problems, try deselecting the **High resolution chart printing** checkbox. Limitations of certain printer drivers cause incompatibilities with this option.

Print 

To print any view within the Mx3000P software, click **Print** in the **File** menu, press **Ctrl+P**, or click the print button  on the toolbar.

To resize the image prior to printing, use the **Print Page Setup** dialog box.

If you want to print the contents of the full screen, including toolbars and taskbars, use **Print Screen** instead of **Print**.

Print opens the printer driver dialog box which allows printing configuration and execution.

Print Screen 

To print the full contents of the screen including toolbars and taskbars, click **Print Screen** in the **File** menu, press **Alt+P** or click the print screen button  on the toolbar.

To resize the image prior to printing, use the **Print Page Setup** dialog box.

If you want to print only the active Mx3000P software module view, use **Print** instead of **Print Screen**.

Print Report

To print a text report of the well-based information from **Plate Setup**, click **Print Report** on the **File** menu. The printer driver dialog box opens and allows printing configuration and execution.

Print Preview

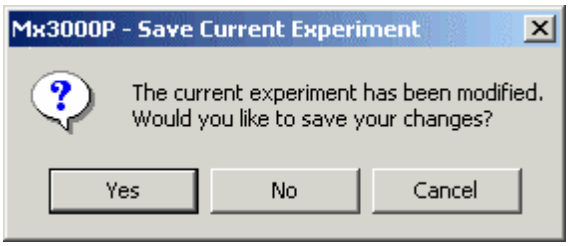
Clicking the **Print Preview** command generates a new window that displays the image that would be printed after the execution of a **Print** command. You may print directly from the **Print Preview** screen by clicking the **Print** button.

Recent Experiments

The **File** menu displays a list of the six most recently used experiments. Any experiment in the list may be opened by clicking on its file name.

Exit

To exit the program, click the **Exit** command in the **File** menu or click the **Close** button in the upper right-hand corner of the screen. If the open experiment has not been saved, the **Save Current Experiment** dialog box appears.



Note *A running experiment must be either completed or aborted before exiting the program.*

If you attempt to exit the software while the lamp is on, a dialog box will appear that prompts you to turn off the lamp.

Edit Menu

The **Edit** menu features two pairs of commands: the **Undo** and **Redo** command pair and the **Copy** and **Paste** command pair.

Undo

Undo allows you to undo multiple actions in **Plate Setup** or **Thermal Profile Setup**. Select **Undo** to effect each undo action.

Redo

Select **Redo** to reinstate the last action undone by the **Undo** command. (These commands are available for use during **Plate Setup** or **Thermal Profile Setup**.)

Copy

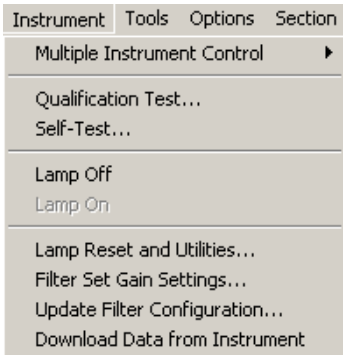
The **Copy** command is available in **Plate Setup**, allowing you to copy the information from one or more selected wells to the clipboard for later pasting into other well positions within the same experiment. To copy well information, first select the wells that contain the information to be copied by clicking on one well and then dragging the cursor across the set of wells to be selected. Release the mouse button, then click **Copy** on the **Edit** menu or press **Ctrl+C**.

Paste

The **Paste** command is available in **Plate Setup**, allowing you to paste well information from the clipboard into a selected set of wells. Using the mouse, first select the wells that you want to paste into. To paste the well information, click **Paste** on the **Edit** menu or press **Ctrl+V**.

Instrument Menu

The **Instrument** menu contains commands that control several functions of the instrument.



Multiple Instrument Control

Multiple Mx3000P instruments may be controlled using a single PC. The multiple instrument control mode is set up and administered by using the **Multiple Instrument Control** portion of the **Instrument** menu. See *Multiple Instrument Set-Up: Optional Use of Multiple Instrument Control* in the *Getting Started* section of the manual for more information on the use of this feature.

Qualification Test

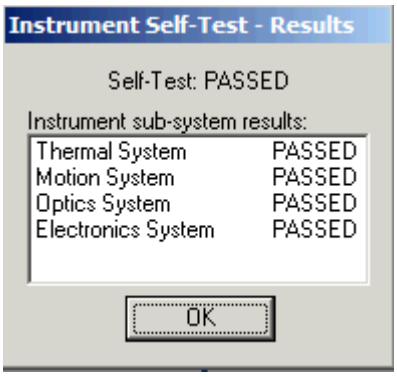
Clicking **Qualification Test** opens the **Instrumentation Qualification Test - Start** dialog box.

A **Qualification Test** is completed during the initial instrument setup, using the predispensed qualification plate that was supplied with the instrument. This test verifies that the biological performance of the instrument is within factory specifications for a Quantitative PCR experiment. The **Qualification Test** run uses a preconfigured plate setup and thermal profile setup. The software provides test results upon completion of the run.

The first time that a new experiment is opened using instrument-connected software, the **Instrumentation Qualification Test - Start** dialog box will automatically open. To initiate the run, click **Next**. Follow the instructions that are presented in subsequent dialog boxes for completing the run and analyzing the results. If **Cancel** is selected, the dialog box will open every time a new experiment is opened, until the test has been completed.

Self-Test

The **Self-Test** command initiates a set of tests run by the Mx3000P instrument to validate a variety of performance parameters. When the self-test is complete, a dialog box will appear indicating that instrument passed or failed. In case of a failure result, subsystem failures will be specified; contact Technical Services for assistance.



Lamp Off

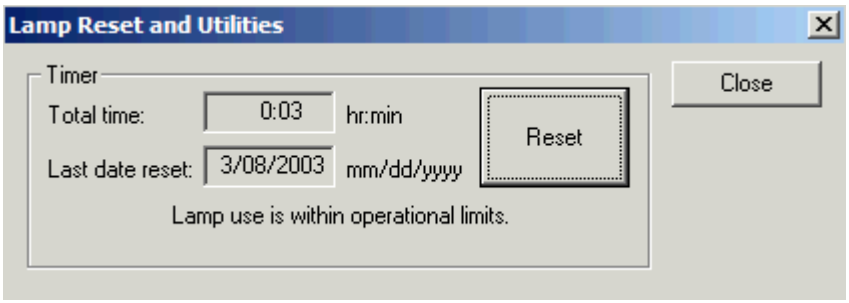
This command turns off the quartz-tungsten halogen lamp. Turning the lamp off when it is not needed extends the life of the bulb.

Lamp On

This command turns on the quartz-tungsten halogen lamp. Turn the lamp on at least 20 minutes prior to a run in order to allow the lamp to warm up. Stratagene recommends that no data be taken during this 20-minute warm up period.

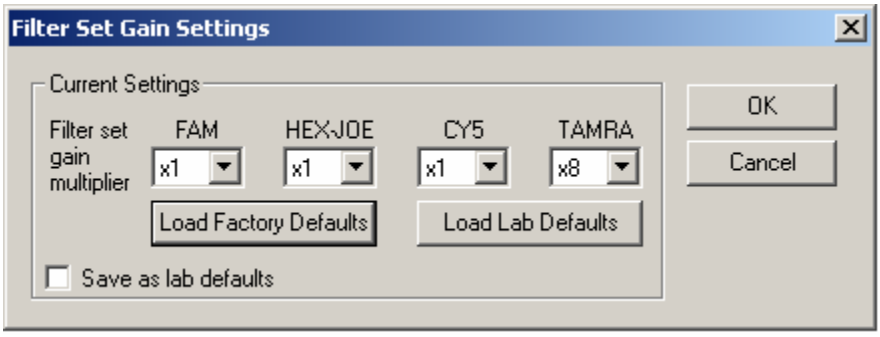
Lamp Reset and Utilities

Lamp Reset and Utilities is used to reset the lamp timer to 0 after the quartz-tungsten halogen lamp has been changed. The **Lamp Reset and Utilities** dialog box displays the **Total time** of lamp usage accrued, the **Last date** the lamp was reset, and whether or not the lamp is within operational limits (the lamp has an average life expectancy of 2000 hours). To reset the timer, click the **Reset** button. The **Total time** will be set to 0 and the current date will appear in the **Last date reset** field.



Filter Set Gain Settings

Filter Set Gain Settings determine the amplification of the fluorescence signal readings for each of the four optical paths. Select **Filter Set Gain Settings** from the **Instrument** menu to open the **Filter Set Gain Settings** dialog box, in which the gain settings may be viewed and modified.



Factory Defaults are gain settings calibrated to the four filters (optical paths) fitted in the Mx3000P instrument during manufacturing.

If you want to adjust the gain settings (for example, if the relative signal levels for your samples are not optimal using the current settings), use the **Filter set gain multiplier** dropdown menus to change the appropriate gain value(s). Click **OK** for the settings to take effect. To save new settings as **Lab Defaults**, select the **Save as lab defaults** check box before clicking **OK**. Only one set of **Lab Defaults** can be stored at a time.

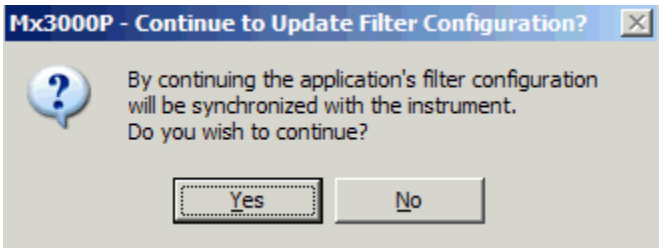
Click **Load Factory Defaults** to retrieve the original factory settings. Click **Load Lab Defaults** to retrieve the last user-defined defaults saved by using the **Save as lab defaults** checkbox.

Note *The fluorescence signal is saturated at 65536 and the accuracy of the fluorescence signal is reduced above 35000. As a general guideline, the gain should be set to a value that causes the highest signals seen from samples to be less than 35000.*

Update Filter Configuration

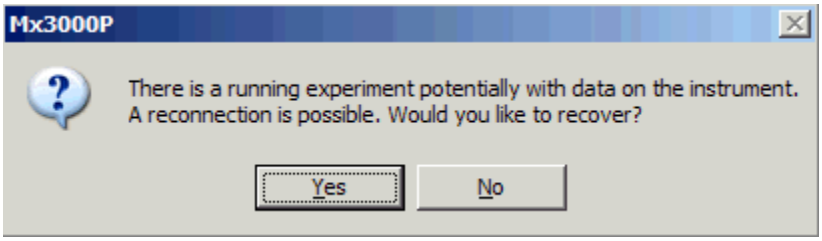
The Mx3000P software stores filter configuration settings that specify the four filters fitted in the optical paths of the instrument during manufacturing. During routine use of the software connected to an individual instrument, these settings do not need to be changed.

If it is necessary to change or reinstate the filter configuration settings (e.g. if the software is used in conjunction with multiple Mx3000P instruments with different filters installed), use the **Update Filter Configuration** command to revise the filter configuration settings. When **Update Filter Configuration** is selected from the **Instrument** menu, a dialog box similar to the one shown below will appear. Click **Yes** to update the filter configuration used by the software to the configuration of the connected instrument.



Download Data from Instrument

In rare situations, when an instrument-connected Mx3000P application is re-started, such as after a PC hardware or instrument communication problem, a dialog box similar to the one below may be displayed.



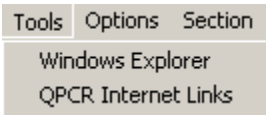
This dialog box indicates that there may be data on the instrument that can be recovered, and in this case, that the run may be resumed. Selecting **Yes** will perform the necessary data download and resume the run, if appropriate.

Alternatively, the **Download Data from Instrument** command can be executed through the **Instrument** menu.

The downloaded data will automatically be stored in the last open experiment, retaining its original filename. The instrument stores data only for the last run started on the instrument.

Tools Menu

The **Tools** menu provides access to tools located outside of the Mx3000P software.



Windows Explorer

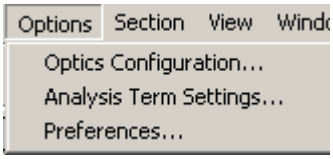
Clicking on **Windows Explorer** opens the Windows Explorer application and displays the contents of the current Mx3000P software data storage folder.

QPCR Internet Links

If the computer is connected to the Internet, clicking **QPCR Internet Links** will open Microsoft Internet Explorer to a Stratagene web page that contains a number of useful links for primer/probe design and other QPCR-related topics.

Options Menu

The **Options** menu is used to access several dialog boxes which contain important instrument and software settings. The menu commands are summarized below; see the manual subsections (on following pages) dedicated to each of the three menu commands for more details.



Optics Configuration

The **Optics Configuration** dialog box is used for making **Dye Assignments** to filters, for defining properties of **Dyes & Filters** and for specifying the **Scan Order**.

Analysis Term Settings

Clicking **Analysis Term Settings** opens the appropriate dialog box: **Analysis Term Settings-Real-Time** or **Analysis Term Settings-Plate Read**. These are used for managing several settings that impact data analysis including specifications for background, threshold, curve smoothing and confidence interval calculations.

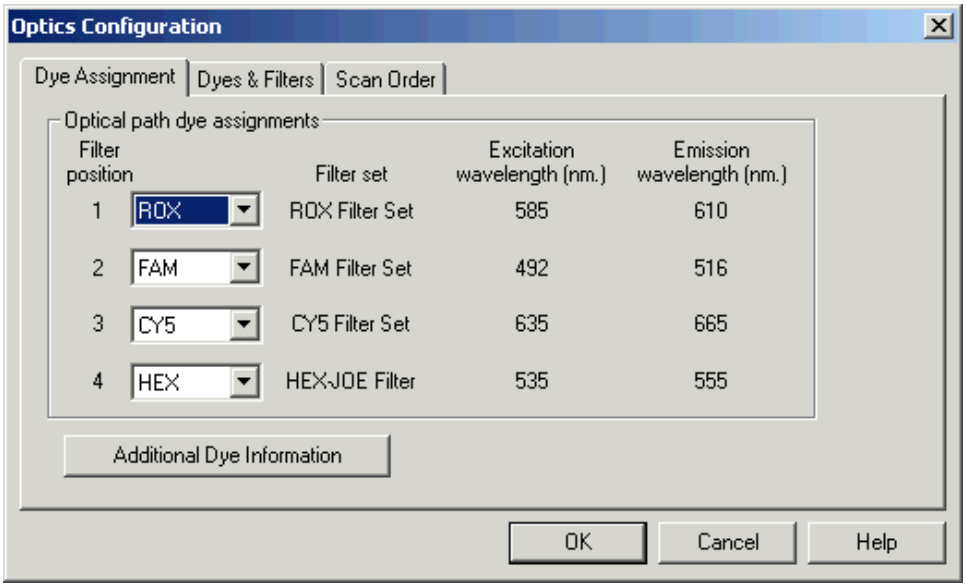
Preferences

The **Preferences** dialog box allows you to personalize several software settings. These include **File** storage and naming conventions, **Colors** used in the software displays, screen **Display** options and the use of **Defaults** in experimental setup and analysis.

Options-Optics Configuration

Optics Configuration - Dye Assignment

To assign a specific dye to an optical path, select **Optics Configuration** from the **Options** menu. When the **Optics Configuration** dialog box opens, click the **Dye Assignment** tab.



When the software is running on an instrument-connected PC, the **Filter sets** shown correspond to the four filter sets installed in the instrument. To assign a specific dye, select from the dyes associated with the **Filter set** from the corresponding menu and then click **OK**. Note that if the dye to be assigned is a custom dye, its association with a particular filter set must first be defined in the **Dyes & Filters** tabbed page of the dialog box.

When running the software on a PC that is not connected to an instrument, a dye may be assigned to any of the filter positions by selecting the appropriate dye from the dropdown menu corresponding to each path. The **Filter set** associated with each filter position will change accordingly. When assignments are complete, click **OK**.

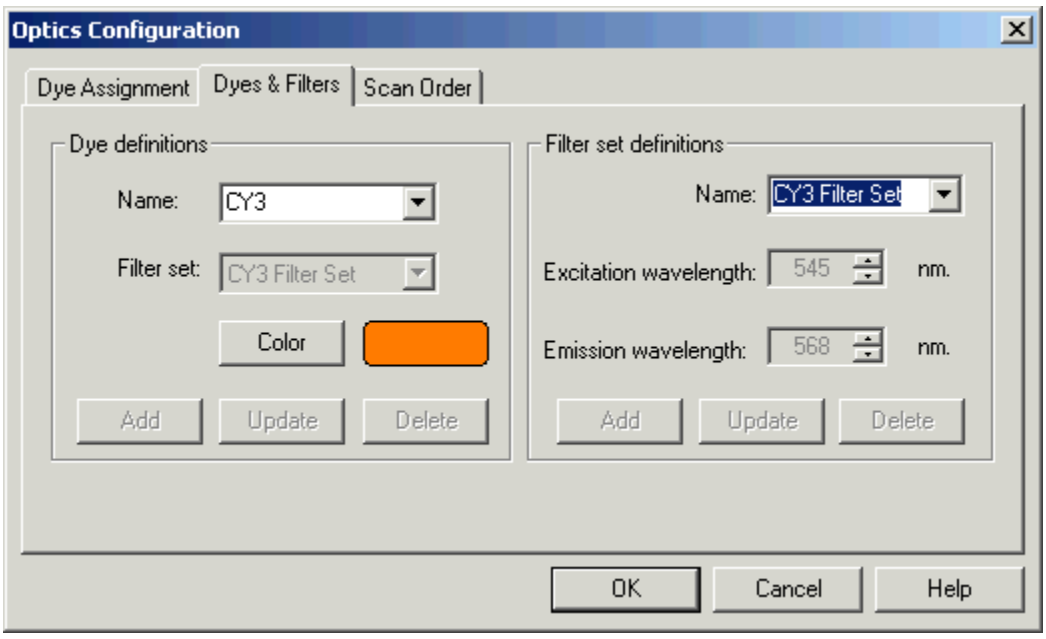
Specific information about dye and filter set compatibility is available by clicking **Additional Dye Information**.

Changing a Dye Assignment After Data Collection

To change a dye/filter position assignment in a post-run experiment, two steps are required. First, open the **Dye Assignment** page of the **Optics Configuration** dialog box and select the new dye from the dropdown menu corresponding to the appropriate filter set. (Menus will be available only for the filter sets that are compatible with multiple dyes.) Second, go to the **Plate Setup** screen for the open experiment. Using the **Collect fluorescence data** checkboxes, deselect the dye that was originally assigned to the path. The newly-assigned dye will now appear under **Collect fluorescence data**; re-select the dye checkbox to display the newly-assigned dye on all screens within the experiment.

Optics Configuration - Dyes & Filters

To define a custom dye or filter set, or to view the properties of defined dyes and filter sets, select **Optics Configuration** from the **Options** menu. When the **Optics Configuration** dialog box opens, click the **Dyes & Filters** tab.



Dye Definitions

The **Dye definitions** section allows you to view the **Filter set** associated with a dye, to view or modify the display **Color** assigned to a dye, and to add new custom dyes. To view information about a particular dye, select the dye from the **Name** menu. The filter set associated with that dye is displayed; for the pre-defined dyes, the assigned **Filter set** may not be changed. The display color for the selected dye is shown. To change the color, click the **Color** button and the **Color** dialog box will open, allowing you to select a new color.

To add and define a custom dye: enter the new dye **Name**, select the **Filter set** with which the dye should be associated, and then click **Add**. The **Define New Dye Color** dialog box opens. Select a display color for the dye and click **OK**. The custom dye will then be available for assignment to a filter position on the **Dye Assignment** tabbed page of the **Optics Configuration** dialog box.

Filter Set Definitions

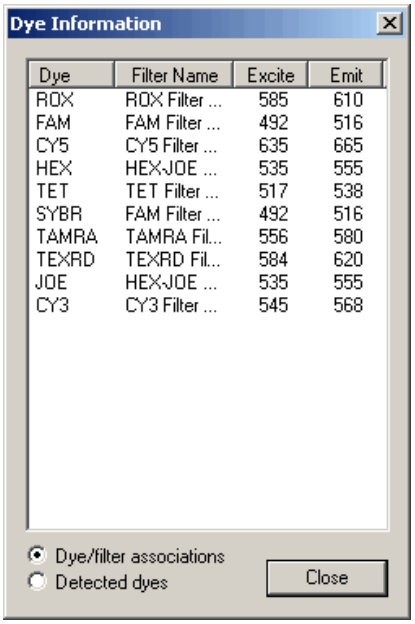
When the software is running on an instrument-connected PC, the **Filter set definitions** section of the dialog box will report the excitation and emission wavelength for each of the four filter sets installed in the instrument. Select the filter of interest from the **Name** menu to view the wavelength information.

When running the software on a PC that is not connected to an instrument, you may view information for any defined filter set by selecting the filter set of interest from the **Name** menu. In addition to defining custom dyes, you may also define a custom filter set. To do this, enter the custom filter set **Name**, enter the **Excitation wavelength** and the **Emission wavelength**, and then

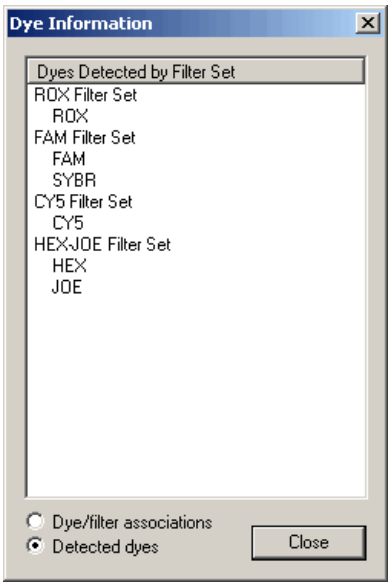
click **Add**. The custom filter set will then be available for dye assignment under **Dye definitions**. To change the wavelengths defined for a custom filter set, enter new wavelength value(s) and then click **Update**.

Dye Information Dialog Box

The **Dye Information** dialog box provides information about each dye: its associated filter set, excitation wavelength, and emission wavelength. This dialog box may be accessed by clicking the **Additional Dye Information** button at the bottom of the **Dye Assignment** tabbed page of the **Optics Configuration** dialog box.



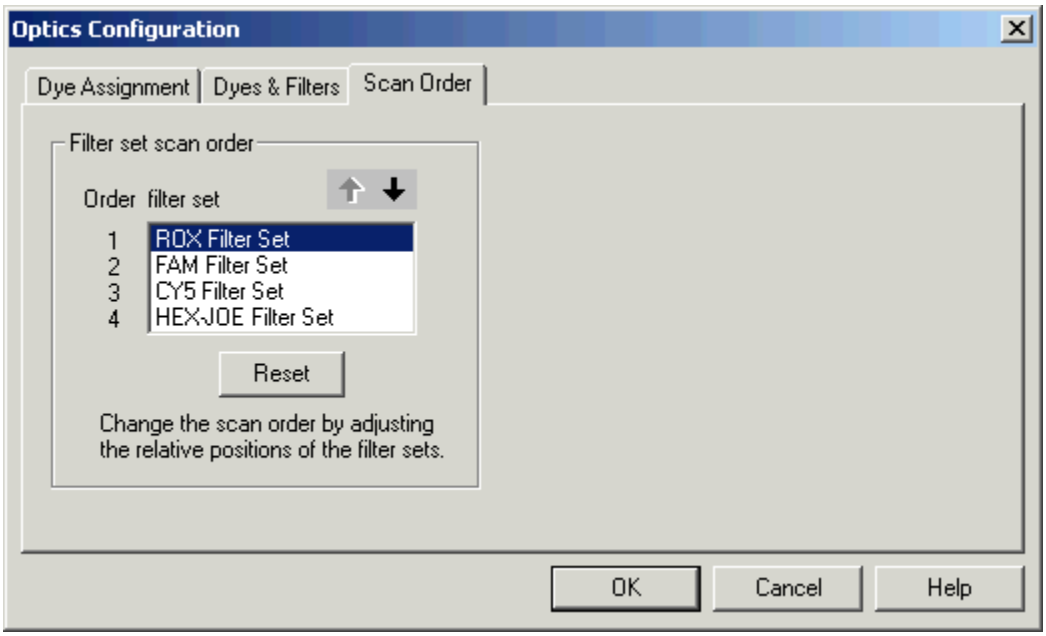
Selecting the **Detected dyes** radio button displays the filter sets installed in the instrument and lists the defined dyes that are compatible with each of the installed filter sets.



Optics Configuration - Scan Order

Filter sets are scanned in the order shown on the **Scan Order** page of the **Optics Configuration** dialog box (accessed from the **Options** menu).

To change the order in which the four filter sets are scanned, select the filter set label to be placed in a different position, and then use the arrow buttons to change the position of the filter set in the list. Alternatively, by using the mouse, drag the filter set label to the new position. Click **OK** to institute the new scan order.

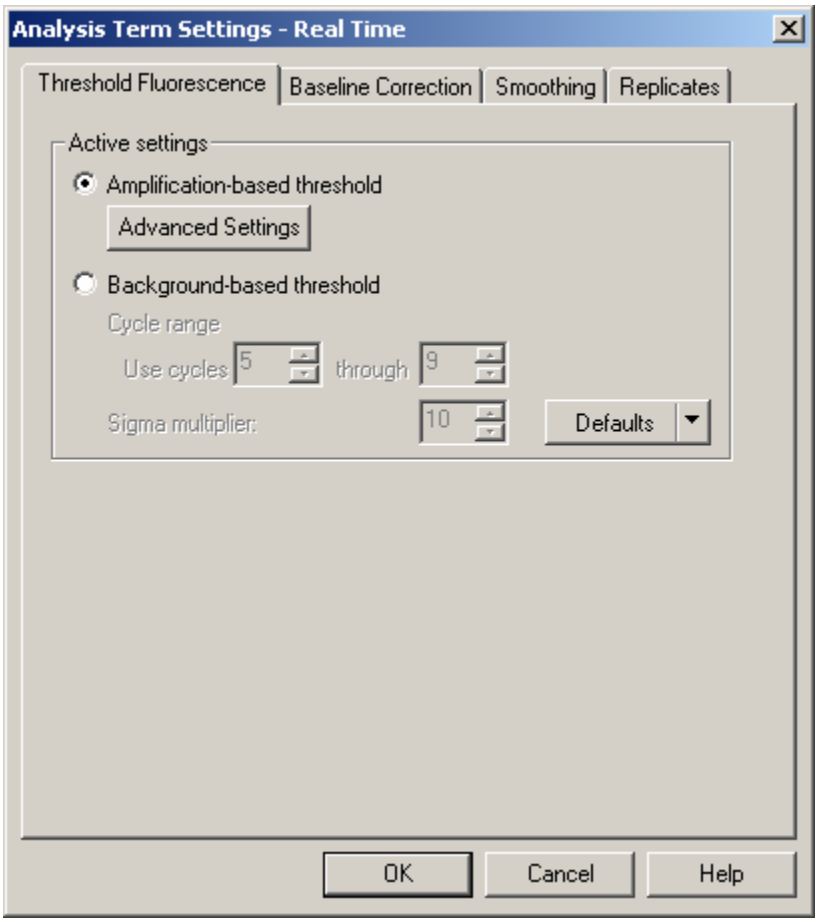


Options-Analysis Term Settings for Real-Time Experiments

Analysis Term Settings - Real-Time - Threshold Fluorescence

The **Threshold Fluorescence** tabbed page of the **Analysis Term Settings-Real Time** dialog box allows you to select an automatic threshold-determination algorithm. Options include **Amplification-based** and **Background-based** threshold determination algorithms. For each algorithm, the result is one threshold value for each dye, excluding the reference dye. The threshold fluorescence may also be manually adjusted for each individual dye in any given experiment.

The threshold value is used in determining the dye's individual well product threshold cycle (Ct) results. Real-time final results, such as initial template quantities for **Unknown** wells, are based on Ct values.



Amplification-based Threshold

When the **Amplification-based threshold** radio button is selected, the Mx3000P software automatically determines threshold values by one of two methods. The first method performs a search of fluorescence values within product exponential amplification to minimize Ct spread among replicates. The fluorescence associated with minimum Ct spread is assigned as the dye-specific threshold value. The second method assigns fluorescence associated with a fixed amplification position as the threshold value. The search range for the first method, and the fixed position used in the second method, can be set through the **Advanced Settings** dialog box.

Method 1

A minimum Ct spread algorithm is used for experiments that include at least two wells per replicate set for all replicates measuring a given dye. The algorithm calculates the Ct standard deviation for each replicate at each potential fluorescence value within the search range. The result is the fluorescence where the overall spread of the Ct standard deviation is minimized for all replicates, indicating the best overall position during amplification. The algorithm takes into account experiments with large dynamic ranges. Typically, the exponential amplification phase of all plots of interest (**Standards** and **Unknowns**) is included in the search. Whether or not all products' amplification are included is primarily determined by the search range specified under **Advanced Settings**.

Method 2

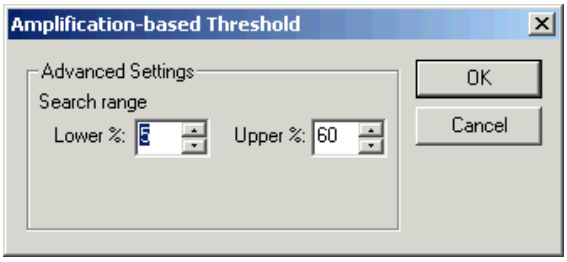
If method 1 cannot be performed due to an insufficient number of wells per replicate (i.e. fewer than two wells per replicate), a fixed amplification position is used. When it is necessary to employ this backup method, the software assigns the fluorescence associated with the midpoint of the search range (specified under **Advanced Settings**) as the threshold value.

Advanced Settings for Amplification-based Threshold

To view or modify the algorithm **Search range**, click the **Advanced Settings** button to open the **Amplification-based Threshold** dialog box. Valid **Search range** entries are between 0% and 100%, and the **Original Settings** are 5% to 60%.

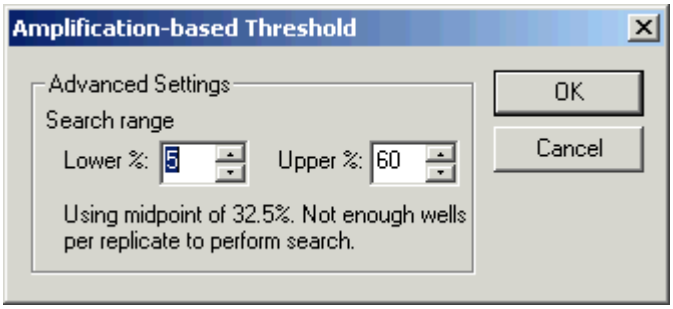
The 0% position on an amplification plot is the point at which amplification begins to be detected. Typically for baseline corrected data, this point has a fluorescence value very close to 0 (e.g. 0.001). The 100% fluorescence value represents the maximum of the first derivative. It is near the ending of the exponential amplification phase and is sufficiently past the optimal search range of interest.

The algorithm will use the Ct spread minimization method (method 1) if two or more wells per replicate set are included in the experiment. The appearance of the **Amplification-based Threshold** dialog box under these conditions is shown below.

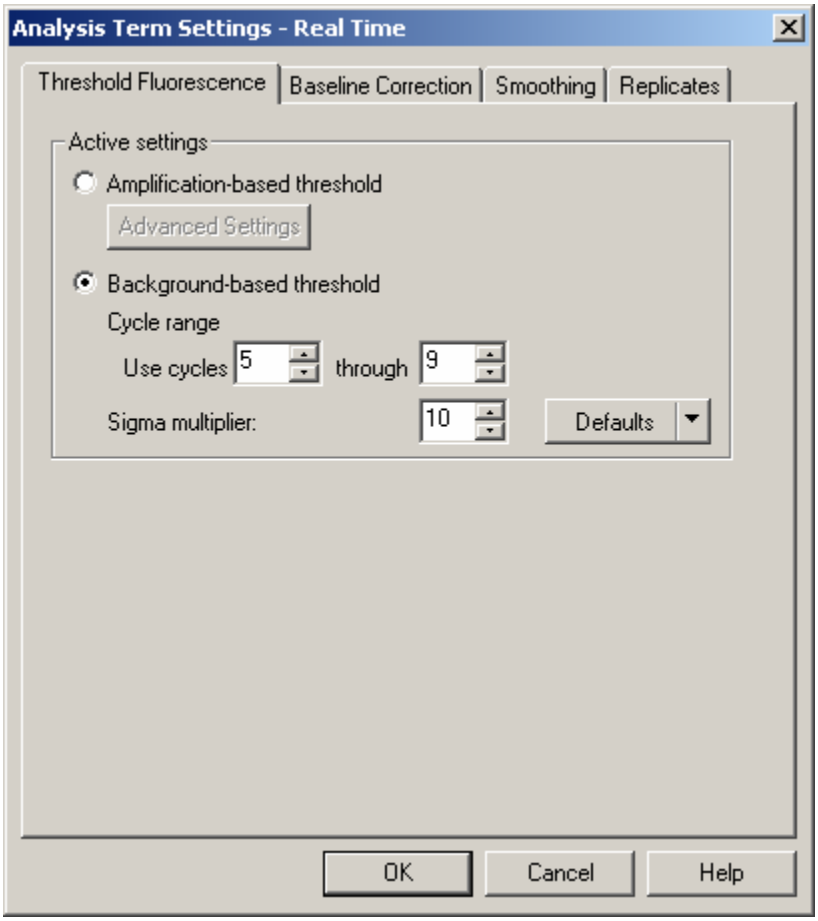


Changing the **Lower %** and **Upper %** range limits biases the threshold result accordingly. For example, if the threshold result is consistently too high (based on personal preference), the **Upper** limit may be lowered, resulting in a lower threshold result. If the threshold result is consistently too low, the **Lower** limit may be increased. This modification will further separate the beginning of the search range from the noise level and may improve results derived from certain data, increasing the confidence of the individual plot results. Alternatively, raising the **Lower** limit can be avoided by increasing the number of wells per replicate.

If fewer than two wells per replicate are included, the midpoint of the specified range is used for the fixed position method of threshold determination (method 2). When this occurs, the **Amplification-based Threshold** dialog box appears as shown below, specifying the range midpoint used. The **Upper** and **Lower** limits of the range may be modified, biasing the threshold result accordingly.



Background-based Threshold



To analyze data from the experiment using a threshold fluorescence that is determined based on the noise in the background, select the **Background-based threshold** radio button. Enter the desired settings in the **Background cycle range** and **Background sigma multiplier** spin boxes.

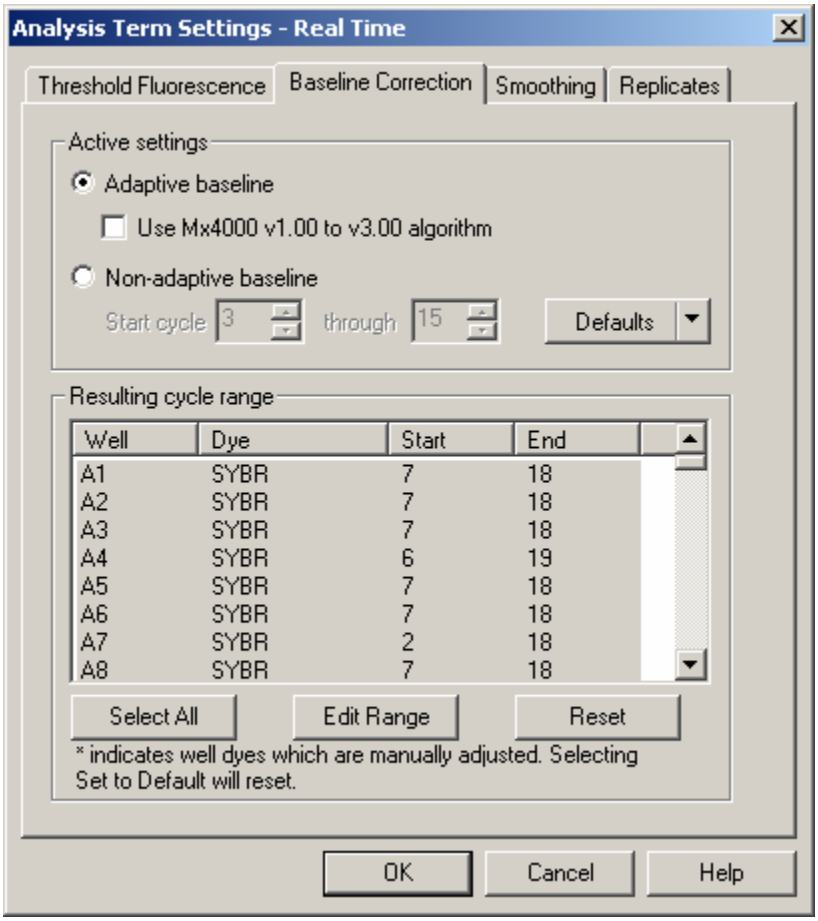
Background cycle range specifies the cycles that will be used to calculate the threshold fluorescence levels. Threshold fluorescence is determined by calculating the standard deviation (sigma) of the raw fluorescence for all selected wells for the specified cycles and multiplying this value by the **Background sigma multiplier** constant. Typically, the **Background cycle range** should encompass only early cycles, before the PCR process starts to affect the fluorescence values. The **Original Settings** values for the background cycle range are cycles 5 through 9, and for the **Background sigma multiplier** is 10.

See *Preferences-Defaults* for information about the use of the **Defaults** menu and the application of defaults sets to the settings specified in this dialog box.

Analysis Term Settings - Real-Time - Baseline Correction

The **Baseline Correction** tabbed page of the **Analysis Term Settings-Real Time** dialog box allows you to specify how baseline fluorescence should be determined in order to generate baseline-corrected fluorescence (dR) data.

For each plot, the raw fluorescence data over a specified range of cycles are fit to a line using a linear least mean squares algorithm to produce a baseline. The value of the baseline function is calculated for every cycle and subtracted from the raw fluorescence to produce baseline corrected fluorescence (dR). The cycles used to determine the baseline may be the same for all plots in the experiment or may be defined differently for each individual plot.



The **Baseline Correction** page is divided into two sections. Use the **Active settings** section to specify the baseline corrections settings prior to analysis. During experimental analysis, use the **Resulting cycle range** section to view and adjust the baseline corrections cycle ranges determined for each plot.

Options for baseline correction include **Adaptive baseline**, where the Mx3000P automatically determines the optimal baseline cycle range for each dye in each experiment, or **Non-adaptive baseline**, where user-defined settings are used to determine the baseline.

Active Settings-Adaptive Baseline

When **Adaptive baseline** is selected, the Mx3000P software will automatically determine the optimal background cycle range for each plot individually, resulting in the most accurate Ct value for each well/dye combination. After the experiment is run and analyzed using an adaptive baseline calculation, the resulting cycle range may be modified for any or all plots by the user as described in **Baseline Correction-Resulting Cycle Range**.

Note *If you want use the Mx3000P software to analyze data that was collected using Stratagene's Mx4000® software (versions 1.00 to 3.00), select the* **Use Mx4000 v1.00 to 3.00 algorithm** *checkbox.*

Active Settings-Non-adaptive Baseline

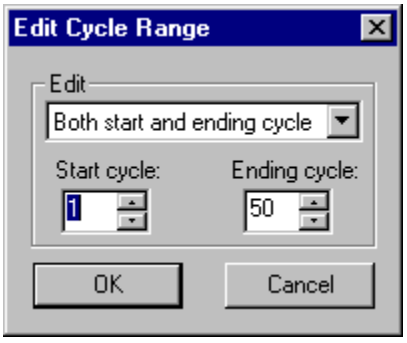
When **Non-adaptive baseline** is selected, the Mx3000P software will determine the baseline function using the range of cycles specified in the **Start cycle** and **through** menus. The starting and ending cycles in the software's **Original Settings** are 3 and 15, respectively.

See *Preferences-Defaults* for information about the use of the **Defaults** menu and the application of defaults sets to the settings specified in this dialog box.

Resulting Cycle Range

After running the experiment, use the **Resulting cycle range** section to view and adjust the baseline correction cycle ranges for each plot. Prior to completing the run, this section will be empty. After the run is complete, the starting and ending cycles being used to calculate the baseline are listed in this section. If **Adaptive baseline** is selected, the starting and ending cycles listed will be variable, since the Mx3000P software calculates the optimal baseline cycle range for each plot individually. If **Non-adaptive baseline** is selected, the starting and ending cycles listed will be constant and equal to the values entered in the **Active settings** section.

The baseline correction cycle range for one or more plots may be modified manually. To specify the range of cycles used to calculate the baseline for a particular plot, select a well/dye combination by highlighting its entry in the **Resulting cycle range** list. Multiple plots can be selected by holding down the **Ctrl** key when selecting, or all plots can be selected by clicking the **Select All** button. Once the desired well/dye combinations are selected, click the **Edit Range** button. The **Edit Cycle Range** dialog box appears.

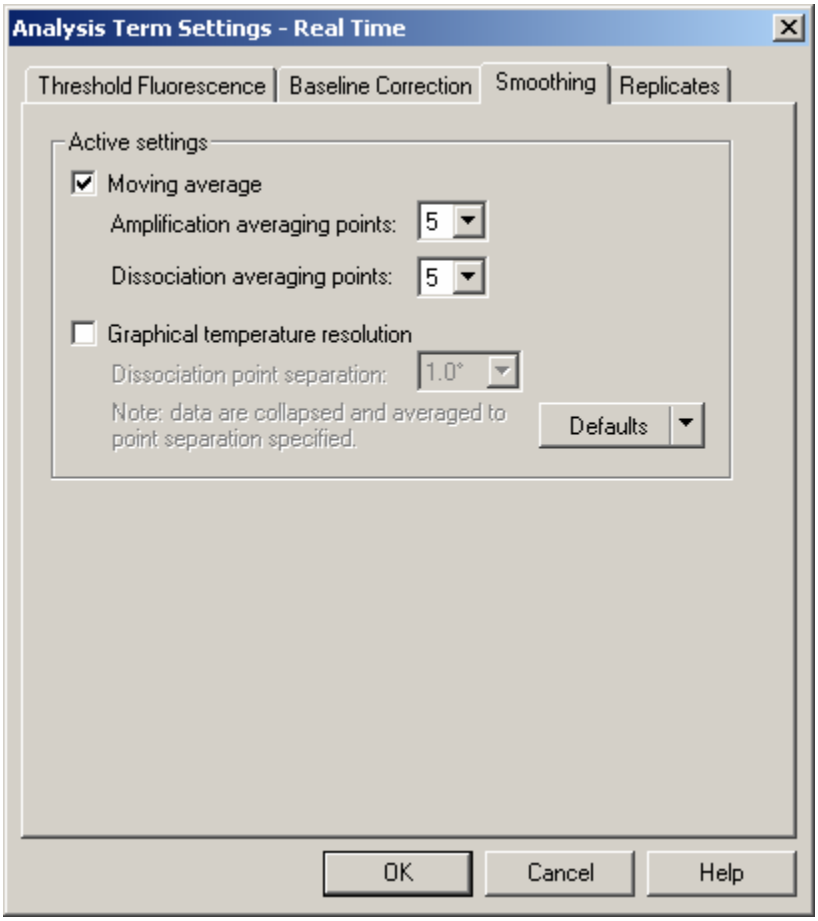


In the spin control boxes, enter the desired start and ending cycle for the baseline algorithm to use to calculate the baseline correction.

The top menu provides 3 options for choosing the endpoints for the baseline calculation. **Both start and ending cycle** allows you to set both endpoints for all selected wells. **Start cycle only** allows the start cycle to be set for all selected wells without changing the ending cycle values. **Ending cycle only** allows the ending cycle to be set for all selected wells without changing the start cycle values.

To reset the start and end cycles to the **Active settings** values (for **Non-adaptive baseline**) or to the Mx3000P system-calculated values (for **Adaptive baseline**), select the desired well/dye combinations and then click **Reset**.

Analysis Term Settings - Real-Time - Smoothing



The **Smoothing** tabbed page of the **Analysis Term Settings-Real Time** dialog box allows you to apply curve-smoothing to amplification plots and dissociation curve plots.

Moving Average

Moving average is an optional algorithm enhancement that uses curve-smoothing to help improve the precision and accuracy of the Ct determination from amplification plots and the Tm determination from dissociation curves by decreasing the effect of signal noise. When this enhancement is selected, the value for each data point in the curve represents the average of the values for a specified range of cycles surrounding a given point. Selecting a greater number of points increases the magnitude of curve smoothing that is achieved but decreases the resolution of the curve data.

To perform amplification data analysis employing a moving average calculation, select the **Moving average** checkbox and select the number of data points to be averaged in the **Amplification averaging points** box.

To perform dissociation data analysis employing a moving average calculation, select the **Moving average** checkbox and select the number of data points to be averaged in the **Dissociation averaging points** box.

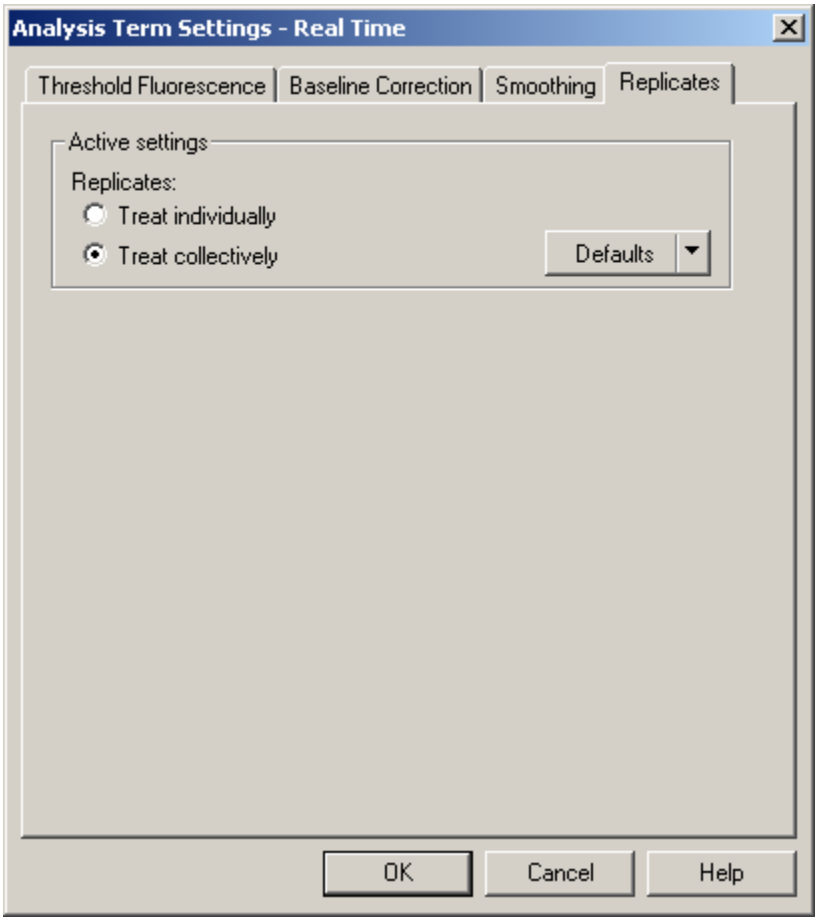
Graphical Temperature Resolution

Graphical temperature resolution is a second optional algorithm enhancement available for dissociation curve smoothing. This algorithm is based on collapsing data by averaging adjacent data points to arrive at the desired point separation. Application of this algorithm increases the ratio of dissociation peak height to noise level.

To perform dissociation data analysis employing this algorithm, select the **Graphical temperature resolution** checkbox. Next, select the desired **Dissociation Point Separation**. Note that as the point separation is increased, the ratio of peak height to noise will increase. Note also that in some cases, the ability to differentiate between products with unusually closely spaced melting temperatures may decrease as the point separation is increased. The final results are displayed in the dissociation chart display.

See *Preferences-Defaults* for information about the use of the **Defaults** menu and the application of defaults sets to the settings specified in this dialog box.

Analysis Term Settings - Real-Time - Replicates

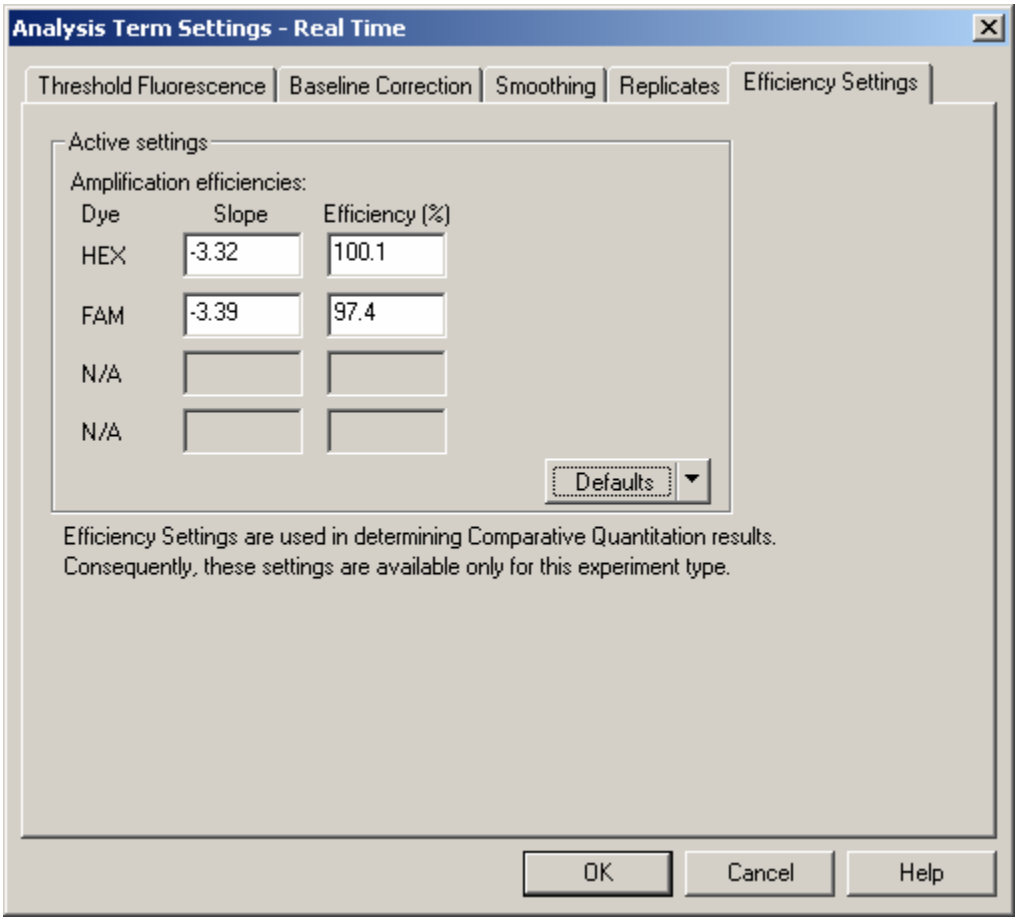


Wells identified as replicates in **Plate Setup** may be treated either individually or collectively during **Analysis**. Selecting **Treat individually** under **Replicates** will cause the software to analyze each well independently and display or report results for each well separately. Selecting **Treat collectively** will cause the software to average the data from the replicate wells and report data from all replicate wells as a group, effectively treating the measurements as if they came from the same well. See *Treatment of Replicates* in the *How-To (Detailed Protocols)* section for more information on replicate treatment during analysis.

See *Preferences-Defaults* for information about the use of the **Defaults** menu and the application of defaults sets to the settings specified in this dialog box.

Analysis Term Settings - Real-Time - Efficiency Settings

When the Analysis Term Settings - Real Time dialog box is opened from a Comparative Quantitation experiment, the dialog box includes a tabbed page for Efficiency Settings. This dialog box is accessed through the **Options** menu.

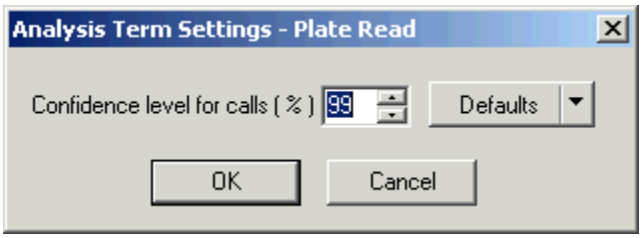


This feature allows you to specify the amplification efficiency for the gene of interest and Normalizer targets used in a Comparative Quantitation experiment, in order to compensate for any difference in the efficiencies while calculating results. The amplification efficiency for each target is determined by performing a Comparative Quantitation Standard Curve.

After the standard curve slopes have been determined, enter the Slope from the standard curve for each dye. Alternatively, enter the amplification Efficiency for each dye. (The efficiency may be calculated from the slope using the Amplification Efficiency Calculator on the Standard Curve results screen.) The efficiency settings recorded in Analysis Term Settings are used by the Mx3000P software in calculating the Relative quantity results.

See *Preferences-Defaults* for information about the use of the Defaults menu and the application of defaults sets to the settings specified in this dialog box.

Options-Analysis Term Settings for Plate Read Experiments



Confidence Level For Calls (%)

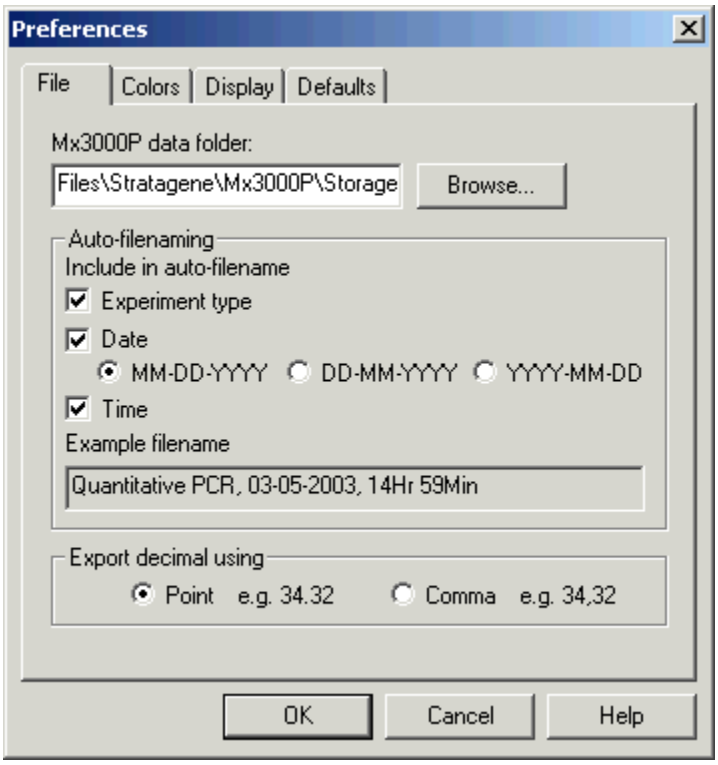
The user-defined confidence level for calls is the statistical probability required before the algorithm will call an amplification occurrence in a well as positive. See *Positive and Negative Calling* in the *How-To (Detailed Protocols)* section for more information. In the **Confidence level for calls** box, type or select the desired value. The factory-set default confidence level is 99%.

See *Preferences-Defaults* for information about the use of the **Defaults** menu and the application of defaults sets to the settings specified in this dialog box.

Options-Preferences

Preferences - File

To view or change the default file storage and file naming settings, select **Preferences** from the **Options** menu. When the **Preferences** dialog box opens, click the **File** tab.



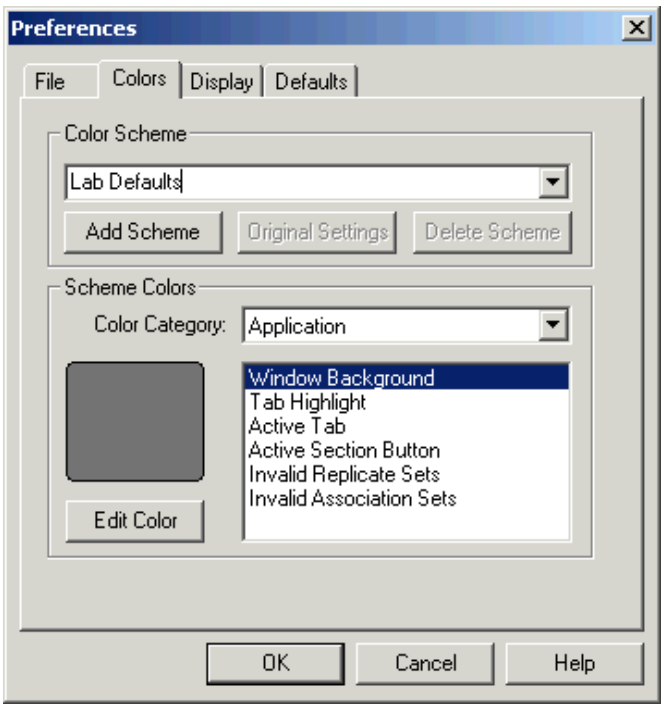
The default storage location for saved Mx3000P experiments is specified using the **Mx3000P data folder** box. The factory default location is C:\Program Files\Stratagene\Mx3000P\Storage. If you wish to specify a different default file storage location, type the directory address into the text box or click **Browse** to navigate to another directory.

The **Auto-filenaming** feature allows you to select the contents of the default file name for all Mx3000P instrument experiments. Three components (**Experiment type**, **Date**, and **Time**) can be excluded or included using the selection checkboxes, and the format of the date may be selected using the radio button controls. View the resulting filename structure under **Example filename**.

In the **Export decimal using** box, specify the form of the decimal used in the numerical values reported in **Text Reports** as either **Point** (period character) or **Comma** by selecting the appropriate radio button.

Preferences - Colors

To view or change the color settings for the various elements of the Mx3000P software screens, select **Preferences** from the **Options** menu. When the **Preferences** dialog box opens, click the **Colors** tab.



Color Schemes

By using color schemes, you can define new sets of colors for various software elements and save the settings as a group. The **Color Scheme** menu allows you to select one of several built-in color schemes or to add and delete user-defined color schemes. To add a scheme, type in a new scheme name (e.g. Lab Defaults, in the example above) and click the **Add Scheme** button. The scheme will be added and the colors can then be defined under **Scheme Colors**.

To change the color scheme used by the software, select the scheme of interest from the color scheme menu and then click **OK**.

To restore the colors of all software elements to the colors that were in place when the instrument was originally installed, click **Original Settings** and then click **OK**.

Scheme Colors

The colors of individual Mx3000P software display components can be defined in the **Scheme Colors** section of the dialog box. The components whose colors may be defined are grouped into five categories under **Color Category** pull-down menu: **Application**, **Charts**, **Plate Wells**, **Stock Well Types** and **Thermal Profile**. Selecting one of these categories causes a corresponding list of graphical elements to appear in the list below the menu. To display the color used for a specific element, click the element name in the list and the color assigned to that element will appear in the color box to the left.

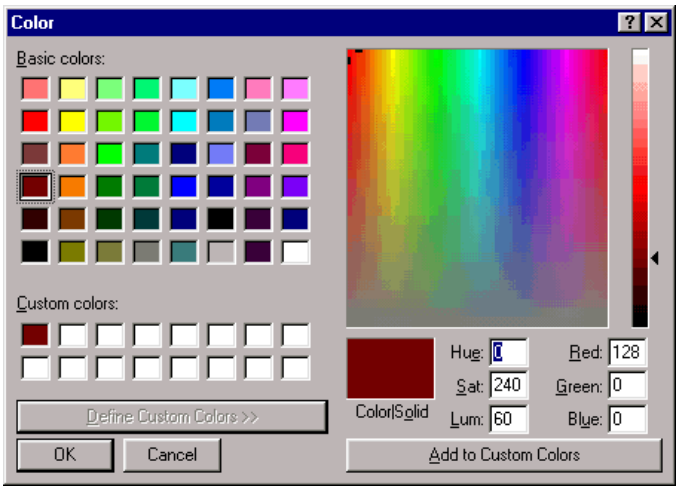
To change the color for any specific element, select the element name and then click the **Edit Color** button. The **Color** dialog box opens. Select any color from the **Basic colors** grid and click **OK** to use this color. Alternatively, click **Define Custom Colors** to set a custom color.

Custom Colors

To define a custom color, click the **Define Custom Colors** button and the dialog box will expand to include a palette for choosing custom colors.

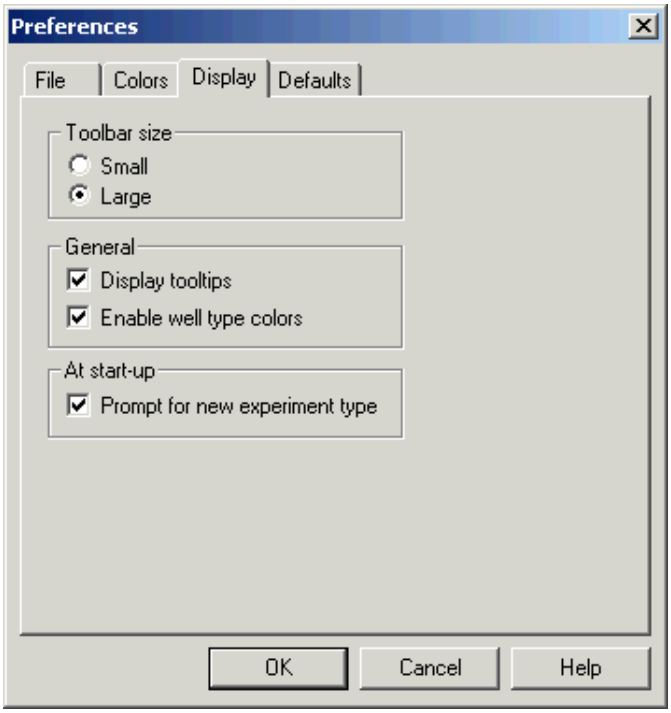
Custom colors can be defined by clicking and dragging the mouse within the color matrix (the large rectangle with colors blending together). The color that the cursor is selecting will appear in the **Color Solid** box. The color's brightness can be adjusted by using the dark/light vertical adjustment slider on the right-hand side of the dialog box. Custom colors may also be defined by entering numerical values directly in the **Hue**, **Sat**, and **Lum** boxes or in the **Red, Green** and **Blue** boxes.

To add the color that appears in the **Color Solid** box to the available custom colors, click **Add to Custom Colors**.



Preferences - Display

The **Display** tabbed page of the **Preferences** dialog box allows you to modify the display properties of several Mx3000P software features. To access the dialog box, select **Preferences** from the **Options** menu and then click the **Display** tab.

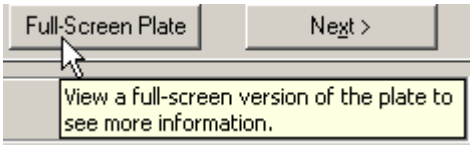


Toolbar Size

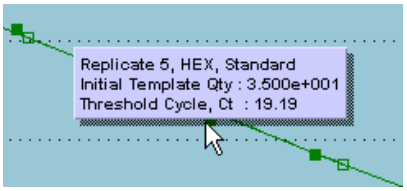
The size of the buttons displayed on the toolbar may be modified by selecting the appropriate radio button under **Toolbar size**. Sizes of other software features are not affected.

Display Tooltips

When **Display tooltips** is selected, tips summarizing the function of a variety of software features appear on-screen when the mouse cursor rests over the feature icon. See below for an example of a displayed tooltip.

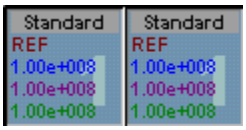


When viewing **Results** screens with **Display tooltips** selected, you may view the information associated with a specific data point by placing the cursor over the point of interest.

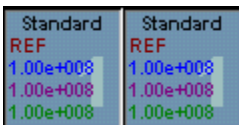


Enable Well Type Colors

When **Enable well type colors** is selected, a horizontal color bar is displayed across the top of each well. The color of this bar corresponds to the well type selected to allow a quick identification of well types. Well type colors can be changed in the **Colors** tabbed page of the **Preferences** dialog box. When **Enable well type colors** is selected, wells appear as shown below.

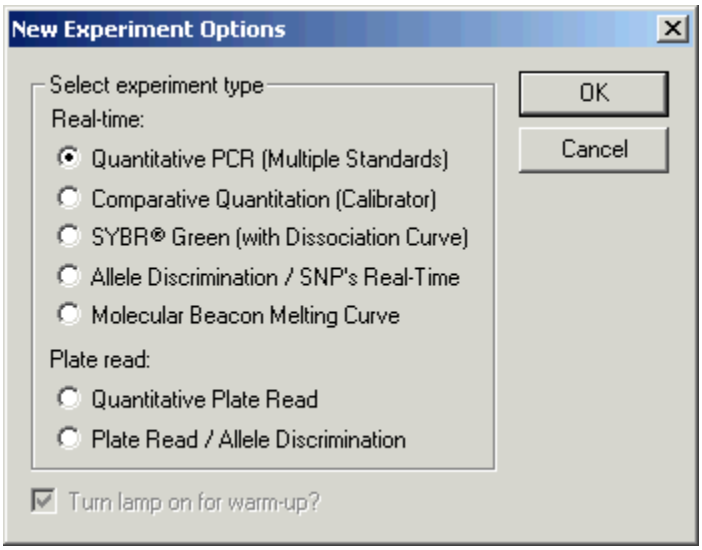


The same cells are shown below with **Enable well type colors** cleared.



Prompt for New Experiment Type at Start-up

When **Prompt for new experiment type** is selected, the **New Experiment Options** dialog box will open each time the Mx3000P software is started.

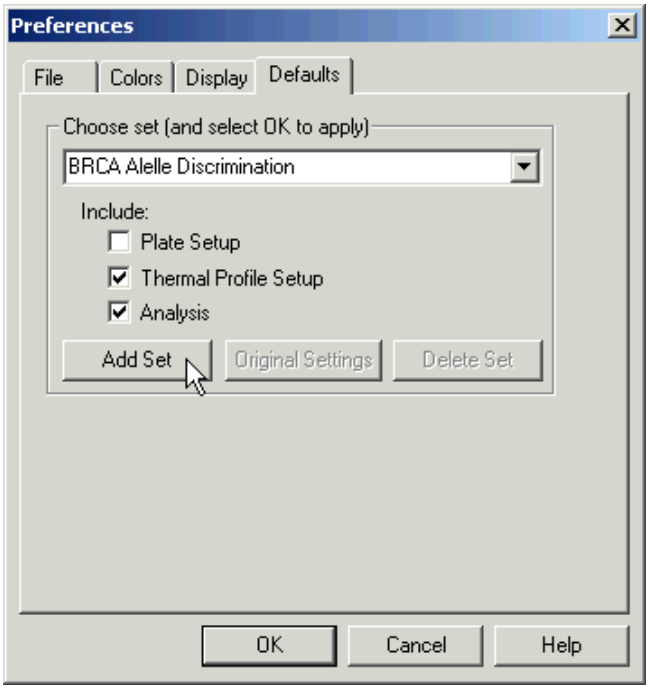


Preferences - Defaults

The Mx3000P system will store a variety of user-defined default settings in addition to the software's **Original Settings**. For example, if different users of the Mx3000P system wish to maintain differentiated settings over the course of multiple experiments, this may be facilitated by creating a different default set for each user. Once a new default set has been created, it may be used for the setup and analysis of new experiments, expediting the application of uniform customized settings across experiments.

The active default set dictates the default settings with which each new experiment is opened. These settings may include well definitions in **Plate Setup**, the properties of the default thermal profile used for **Thermal Profile Setup**, and the well selection and analysis settings in **Analysis Selection and Setup**. Any of the settings contributed by the default set in a new experiment may be modified during the course of experimental setup or analysis. Conversely, a default set containing established experimental setup and analysis parameters may be created for a routine assay, and runs may be performed with no further modifications to the default settings.

To create and manage default sets for use by the Mx3000P software, open the **Defaults** tabbed page of the **Preferences** dialog box (accessed through the **Options** menu).



Creating a New User-Defined Default Set

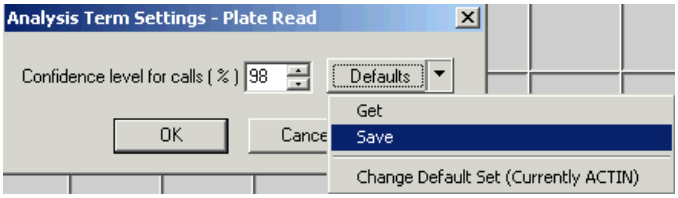
To create a new user-defined set of defaults, open an experiment that was set up and analyzed using the desired settings. Open the **Defaults** page of the **Preferences** dialog box. Expand the pulldown menu under **Choose set** and select **<ADD SET>**. Type the name under which the new the default set should be stored (e.g. *BRCA Allele Discrimination* in the example above). Next, define the segments of the open experiment (**Plate Setup**, **Thermal Profile Setup**, and **Analysis** settings) that should be included in the default set by selecting the appropriate checkboxes under **Include**. For any segments that are not selected, the settings comprising the original software

settings will be used in new experiments. In the example shown above, the new default set *BRCA Allele Discrimination* will contain the thermal profile and the analysis settings specified in the open experiment, but will contain a generic (blank) plate setup segment. When finished naming and defining the segments to be included in the new default set, click **Add Set**.

Click **OK** to establish the new default set as the active default set used for new experiments. Click **Cancel** to restore the previous default set as the active set.

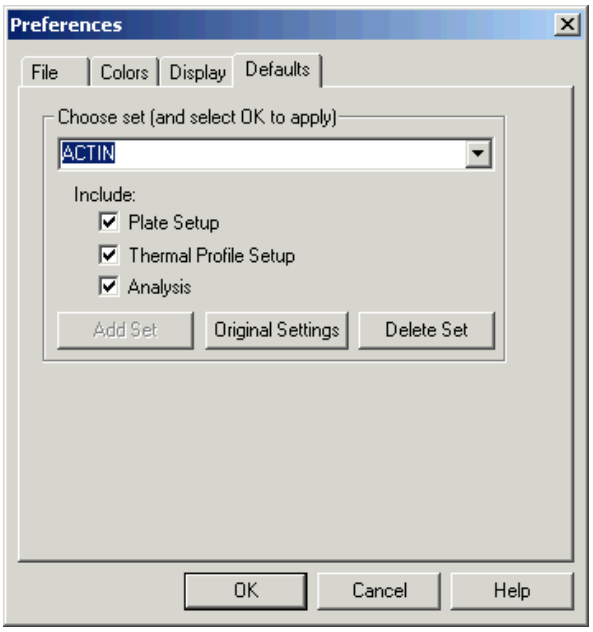
Modifying Settings Stored in a User-Defined Default Set

Once a default set has been created, the individual settings that comprise the set may be changed from the appropriate software view. For example, to change the confidence level value stored in a default set, access the **Analysis Term Settings-Plate Read** dialog box. Enter the desired value in the **Confidence level for calls** box. To change the default set setting to this value, click the **Defaults** menu arrow, then select **Save**. The default set that the new value will be saved into is indicated in the last entry of the **Default** menu, **Change Default set (Currently <set name>)**.



Viewing the Active Default Set

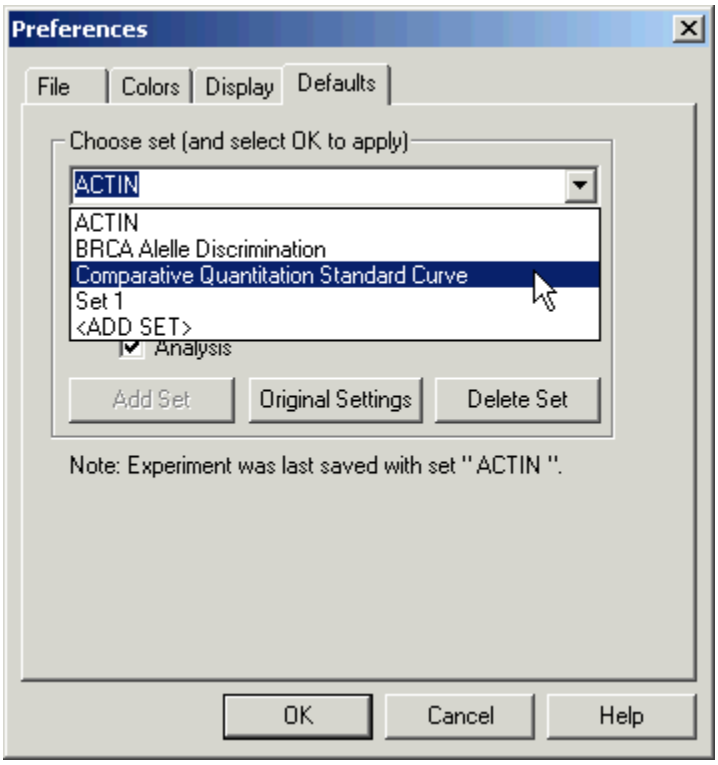
To view the identity of the active default set, open the **Defaults** tabbed page of the **Preferences** dialog box (accessed through the **Options** menu). When this dialog box is first opened, the active default set appears in the **Choose set** menu.



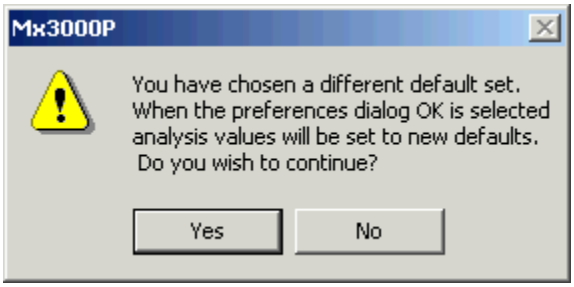
The active default set may also be identified from any of the software sections containing a default set (**Defaults**) menu, by clicking the **Defaults** arrow. The active default set is indicated in the final entry of this menu: **Change Default Set (Currently <set name>)**.

Changing the Default Set

To change the default set used in the open experiment, open the **Defaults** page of the **Preferences** dialog box (accessed from the **Options** menu). Select the name of the desired default set from the **Choose set** menu. Click **OK** to apply the selected default set to the open experiment and to use the selected default set when creating new experiments.

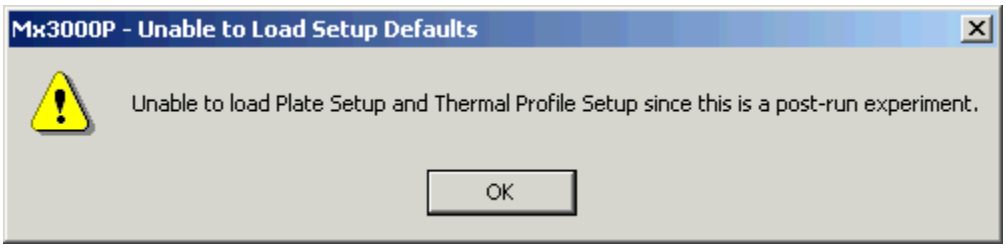


Changing the default set causes the software to apply all of the settings stored in the default set to the open experiment, as relayed by the warning dialog box that opens when a new default set is selected. Click **Yes** in the warning dialog box to continue with changing the setup and analysis properties of the open experiment, or click **No** to return to the **Defaults** page without changing default sets or changing any experimental settings.



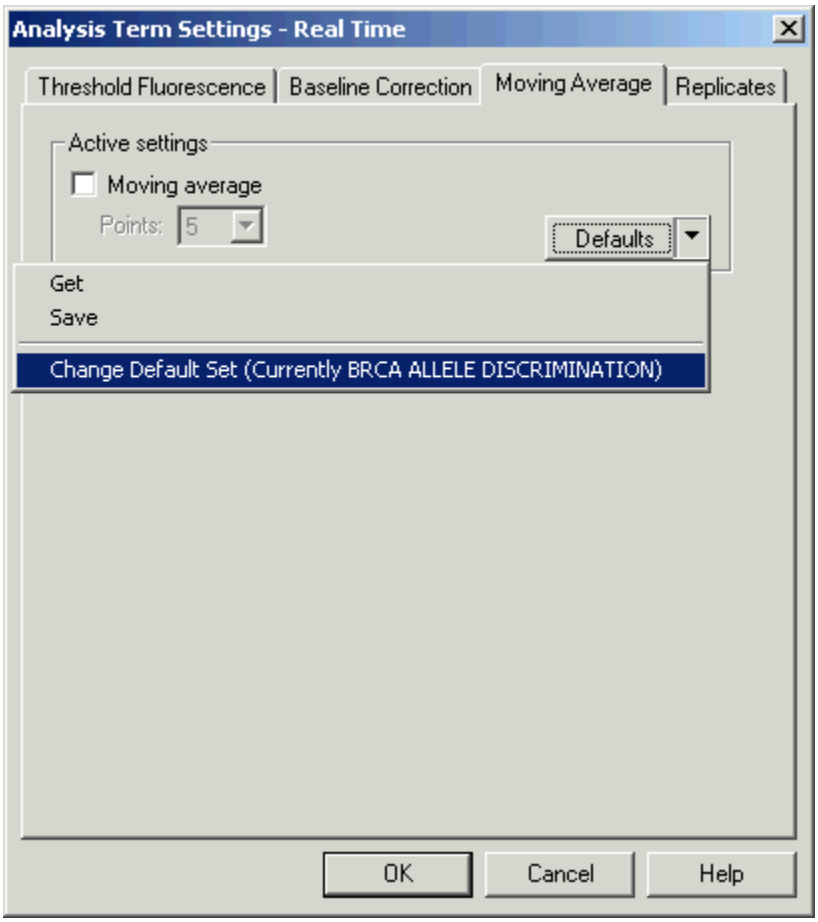
Changing the Default Set in a Post-Run Experiment

When changing the default set in a post-run experiment, **Plate Setup** and **Thermal Profile Setup** information cannot be applied to the open experiment, and a warning dialog box like the one shown below will appear. Click **OK** to change the active default set and apply only the default set analysis settings to the open post-run experiment.



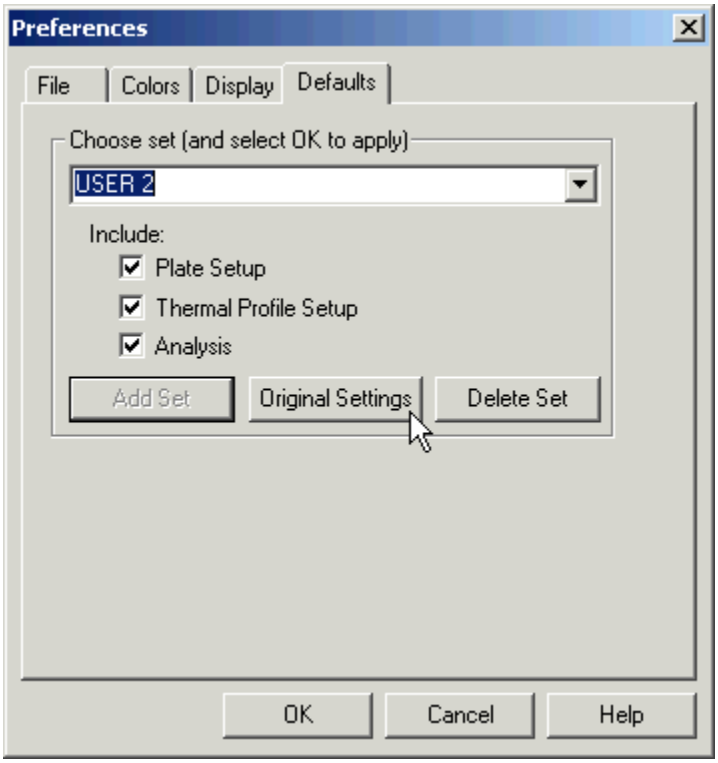
Changing the Default Set from Other Software Screens

Changing the default set may be initiated from any of the command panels or dialog boxes that contain settings stored in a default set. One example is shown below. From any of the software sections containing a default set settings menu, click the **Defaults** arrow and then select **Change Default Set (Currently <set name>)**. The **Preferences** dialog box will open to the **Defaults** page, allowing you to select a different default set from the **Choose set** menu.



Reinstating the Software's Original Settings

The original software setup and analysis settings (**Original Settings**) that were in place when the software was first installed have been designed to provide generally-applicable settings for the new user and to maximize experimental flexibility. These settings are stored separately from the default sets, and unlike the default sets, may not be modified. This ensures the ability to reinstate these settings at any time, after any number of modifications have been made to default sets. Reinstating the software setup and analysis **Original Settings** may be initiated from the **Defaults** tabbed page of the **Preferences** dialog box.

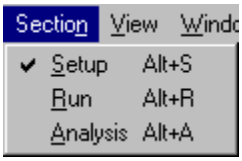


Clicking the **Original Settings** button in this dialog box will reset the active default set to the original software settings. If you wish to reinstate the original software settings without altering any existing default sets, first create a new default set, and then click **Original Settings** to apply the original software settings to the new default set. Click **OK** to activate the new default set (containing the original software settings) in the open experiment.

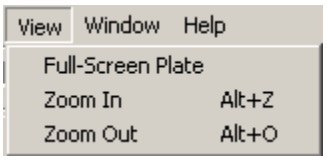
The original color settings for the software may be accessed through the **Colors** tab of the **Preferences** dialog box.

Section Menu

Use the **Section** menu commands to move between the **Setup**, **Run**, and **Analysis** areas of the application. These commands mirror the **Setup**, **Run**, and **Analysis** selection buttons in the upper right-hand corner of the screen.



View Menu



Full-Screen Plate

The **Full-Screen Plate** view command changes the display on the screen to a full-screen view of the **Plate Setup** for the open experiment.

Zoom In/Zoom Out

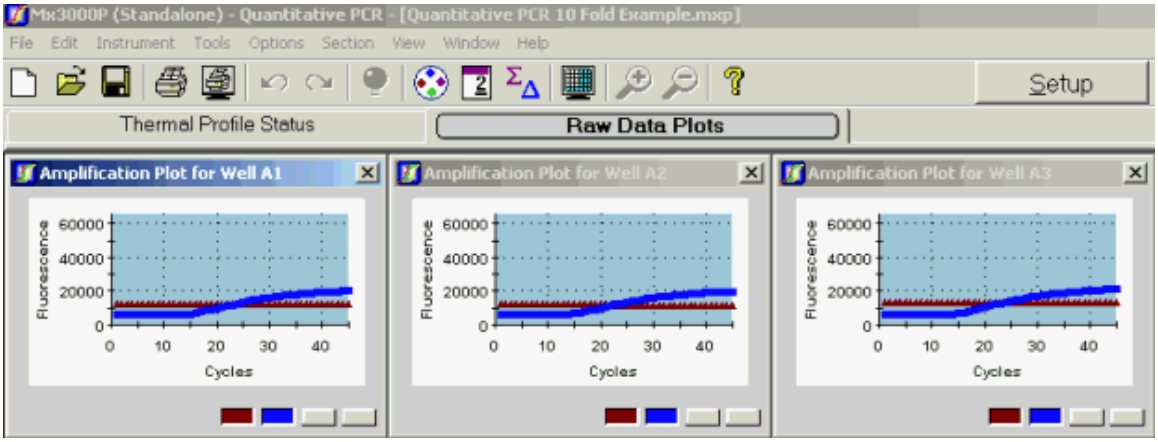
Use the **Zoom In** command to magnify a rectangular portion of a chart or plot from a **Results** screen. To use this feature, click **Zoom In**. The cursor will change to cross-hairs when it is positioned over a chart or graph. Click at one corner of the rectangle to be enlarged and drag the mouse until the area to be enlarged is enclosed by the rectangle. Release the mouse button and the rectangle contents will be enlarged. The resulting expanded chart can be further enlarged multiple times by repeated use of the **Zoom In** command. Clicking **Zoom Out** returns a chart or plot that has been enlarged to its normal view.

Window Menu

The **Window** menu commands **Tile Plots** and **Cascade Plots** control the format of expanded amplification plots displayed when viewing **Raw Data Plots** in the **Run** section of the software. (These commands are disabled on all other software screens.)

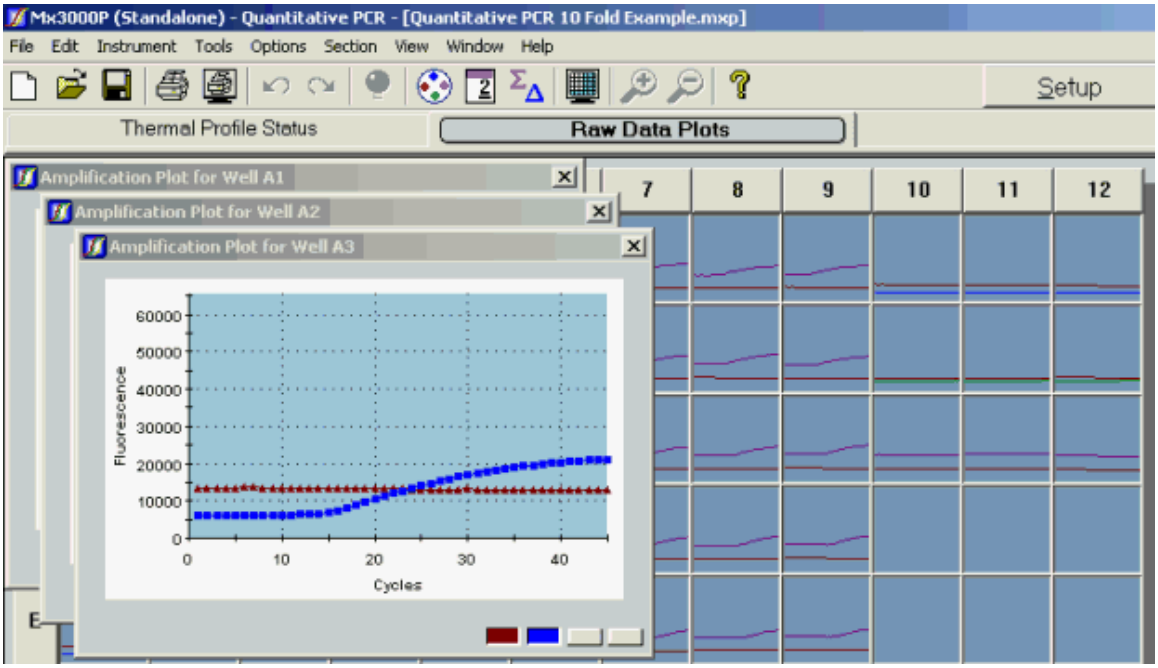
Tile Plots

Clicking **Tile Plots** causes the plots for individual wells to be displayed in a tile format, with plots lined up in rows and columns.



Cascade Plots

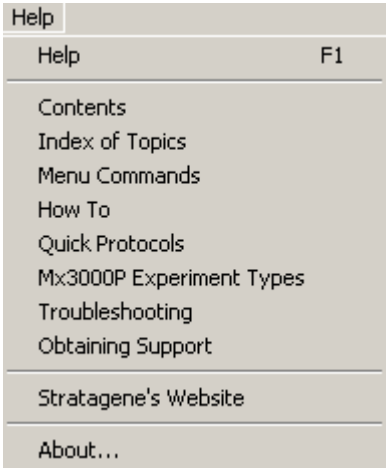
Clicking **Cascade Plots** causes the plots to be layered one on top of the other.



Help Menu

The **Help** menu accesses the Mx3000P software help system. Selecting the first command, **Mx3000P Help**, causes a help topic appropriate to the current screen to be displayed. This feature can also be accessed from anywhere within the program by pressing the **F1** key.

Contents and **Index of Topics** provide access to the help system in a broad manner to allow browsing or searching for help on any topic. Selecting one of the remaining options opens the Mx3000P help system to a specific, applicable area.



Selecting **Stratagene's Website** will open the Stratagene home page, www.stratagene.com if the computer is connected to the World Wide Web.

Selecting **About** will open a dialog box which lists the software version of the Mx3000P system interface and the firmware version of the instrument software.

Toolbar

A toolbar containing shortcut buttons provides convenient access to many of the frequently-used functions of the Mx3000P system. The buttons on the toolbar perform the same function as the similarly-named commands in dropdown menus or dialog boxes.

New

The **New** button  allows you to create a new experiment. Pressing the **New** button opens the **New Experiment Options** dialog box.

Open

Use the **Open** button  to open an existing experiment. Pressing this toolbar button causes the **Open** dialog box to appear.

Save

To save an experiment, click the **Save** button in the toolbar  . If the experiment has already been saved, no dialog box will be displayed and the experiment will be saved with any modifications under its current name. If the experiment has not yet been saved, the **Save As** dialog box will open.

Print

To print any view within the Mx3000P software, click the **Print** button  on the toolbar. The printer driver dialog box opens to allow printing configuration and execution.

Print Screen

Use the **Print Screen** button  to print any screen in the Mx3000P software. The image printed using the **Print Screen** command can be sized from 25% to 100% by using the **Print Page Setup** dialog box (accessed by selecting **Page Setup** from the **File** menu).

Undo

Undo allows you to undo multiple actions when working on **Plate Setup** and **Thermal Profile Setup** screens. Click the **Undo** button  to effect each undo action.

Redo

Click the **Redo** button  to reinstate the last action undone by the **Undo** command. The **Redo** function is available on **Plate Setup** and **Thermal Profile Setup** screens.

Lamp On/Off

Click the **Lamp On/Off** button  to switch the lamp on or off. The warm-up time for the lamp is 20 minutes. Stratagene recommends that no data be taken during this period. Turning the lamp off when it is not needed extends the life of the bulb.

The button is also an indicator of lamp status. If the lamp is off, the bulb shown in the button will be red. 

If the lamp is warming up, the bulb will be yellow. 

If the lamp is warmed up and ready, the bulb will be green. 

Optics Configuration

Click the **Optics Configuration** button  to open the **Optics Configuration** dialog box.

Replicate Wells Treatment Setting

The **Replicate Wells Treatment Setting** button is a shortcut for changing the analysis algorithm used for the open experiment such that wells defined as replicates are treated either collectively or individually during analysis. When the button appears with the replicate symbol underlined  , the replicate wells are treated collectively. When the replicate symbol appears without an underline  , the replicate wells are treated individually. Clicking on this button will switch the treatment of the replicate wells from individually to collectively or from collectively to individually. See *Treatment of Replicates* for more information.

Term Settings

The **Term Settings** button  opens the **Analysis Term Settings** dialog box, where you can modify several data analysis settings. Click here for more information on **Analysis Term Settings Real-Time** or here for more information on **Analysis Term Settings Plate Read**.

Full-Screen Plate View

The **Full-Screen Plate View** button  changes the view displayed on the screen to a full-screen view of the **Plate-Setup** for the open experiment.

Zoom In

Use the **Zoom In** button  to magnify a rectangular portion of a chart or plot from a **Results** screen. To use this feature, click the **Zoom In** button. The cursor will change to cross-hairs when it is positioned over a chart or graph. Click at one corner of the rectangle to be enlarged and drag the mouse until the area to be enlarged is enclosed by the rectangle. Release the mouse button and the enclosed rectangle will be enlarged. The resulting expanded chart can be further enlarged multiple times by repeating the **Zoom In** function.

Zoom Out

Clicking the **Zoom Out** button  returns a chart or plot that has been enlarged to its normal view.

Help

The **Help** button  opens the Mx3000P help system, giving access to help by contents, index, or searching.

In addition, context-sensitive help is available at any time by pressing the **F1** key. The appropriate help screen will automatically open.

Shortcut Keys

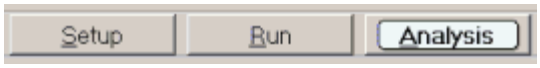
Shortcut keys allow rapid execution of common commands and facilitate movement between the different sections of the Mx3000P software.

Section Movement Shortcuts

Alt+S takes you to the Setup section.

Alt+R takes you to the Run section.

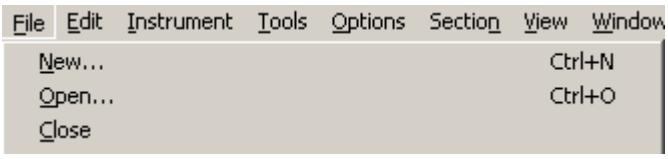
Alt+A takes you to the Analysis section.



Ctrl+<tab> takes you to the next tab within a section. For example, when on the Plate Setup screen of the Setup section, pressing **Ctrl+<tab>** takes you to the Thermal Profile screen**.**

Menu Commands Shortcuts

Menu commands may be executed by using keystroke shortcuts. Once a menu has been expanded (caused to drop down) by using a keystroke shortcut (**Alt** + the underlined letter in the menu name), the individual menu choices can be executed by pressing the key for the underlined letter within the command. For example, when the **File** menu is expanded by pressing **Alt + F**, pressing the **C** key will cause the experiment to close.



The keystroke combinations listed below will execute their associated command even if the menu has not been expanded.

File Menu

New experiment -- **Ctrl+N**

Open an existing experiment -- **Ctrl+O**

Save an experiment -- **Ctrl+S**

Save as-- Ctrl+A

Print -- Ctrl+P

Print Screen -- Alt+P

Export to PowerPoint -- Ctrl+W

Export to Image File -- Ctrl+I

Edit Menu

Undo -- Ctrl+Z

Redo -- Ctrl+Y

Copy -- Ctrl+C

Paste -- Ctrl+V

View Menu

Zoom In -- Alt+Z

Zoom Out -- Alt+O

Quick Protocols

Plate Setup Quick Protocol

Plate setup is performed by selecting a well or wells and then assigning well information for the selected well(s) using the command panel at the right of the screen. The command panel should generally be used in a top-to-bottom order, beginning with **Well type** assignment.

1. Select the well or wells to be configured.
2. Define the well type using the **Well type** menu. Available well types are specific to the type of experiment.
3. Using the **Collect fluorescence data** check boxes, choose the dyes for which data should be collected in the selected well(s).
4. If one of the dyes selected is a passive reference dye used for normalization, choose the dye from the **Reference dye** menu. (If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.)
5. If the well is a **Standard**, select the name of the standard dye and then enter the known amount of template in **Standard quantity.** Select the units from the **Standard units** menu. The **Auto-Increment** feature expedites assigning quantities to **Standards** set up using a dilution series.
6. Where appropriate, designate replicates by choosing the appropriate replicate set number from the **Replicate symbol** menu. It is often beneficial to define all wells on the plate according to steps 1–5 prior to replicate assignment. This facilitates using the **Auto-Increment** feature to automatically assign sequential replicate numbers to the replicate sets on the plate.

When **Plate Setup** is complete, for real-time experiments, click on the **Next** button to proceed to **Thermal Profile** setup. Plate read experiments may be initiated from the **Plate Setup** screen.

Time-Saving Tips

The Mx3000P software features several plate setup shortcuts including options for the **Import** of an existing setup, for the use a **Default** setup, and for rapidly specifying collection of data for all dyes in all wells by using **Quick Setup**.

Within a given experiment, information from groups of wells may be copied from one set of wells and pasted into another set of wells by using the **Copy** and **Paste** commands on the **Edit** menu or using the corresponding shortcut keys (**Ctrl+C** and **Ctrl+V**).

Thermal Profile Setup Quick Protocol

When a new **Thermal Profile** screen is opened, a default thermal profile appropriate to the experiment type appears on screen. Modify the default thermal profile as required for the new experiment.

Adding and Modifying Plateaus

To add a plateau in a segment, first select the plateau that will be positioned just before the new plateau. Next, under **Add** on the control panel, click **Plateau with Ramp**. A plateau will be added after the selected plateau, with a default temperature of 25° C and duration of 30 seconds.

To add a plateau to the beginning of a segment, select the whole segment then click **Plateau with Ramp**.

Adjust the duration and temperature of a plateau by double-clicking on the plateau to open the **Plateau Properties** dialog box. Enter the **Duration** and the **Temperature** for the plateau in the **First cycle** section of the dialog box. If all cycles should have the same **Duration** and the **Temperature** as the **First cycle**, enter null values in the **Cycle increments** section of the dialog box. To incrementally change the **Duration** or the **Temperature** for the plateau at each successive cycle, enter the appropriate increment or decrement in the **Cycle increments** section of the dialog box.

The plateau temperature may also be adjusted by grabbing the plateau with the mouse and dragging it up or down.

Adding and Modifying Segments

To add a segment, first select the segment that will be positioned just before the new segment, and then click **Normal Segment**. A new segment will be added with a default plateau temperature of 25°C and duration of 30 seconds.

Adjust the number of cycles for a segment by double-clicking in the segment to open the **Segment Properties** dialog box, and then entering the **Number of Cycles**.

Adding and Modifying Data Collection Markers

To add a data collection marker, grab the **Endpoints data collection marker** 

or the **All points data collection marker** 

and drag the icon to the appropriate plateau or ramp on the thermal profile. To remove a data collection marker, drag the marker icon from the thermal profile to any other part of the screen. The number of collection points appears at the upper right of the marker, and can be changed by clicking on the number to open a spin control.

When **Thermal Profile Setup** is complete, click **Start Run** to begin thermal cycling and data collection.

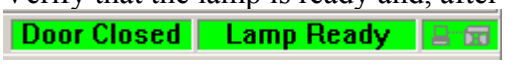
Time-Saving Tip

The Mx3000P software features thermal profile setup shortcuts including the ability to **Import** the profile from of an existing experiment, and to store and retrieve user-defined **Default** thermal profiles.

Running the Experiment Quick Protocol

Real-Time Experiments

Verify that the lamp is ready and, after loading the samples, that the instrument door is closed.



Start the run from the **Thermal Profile Setup** screen by clicking the **Start Run** button. When the **Run Status** dialog box opens, click **Start**.

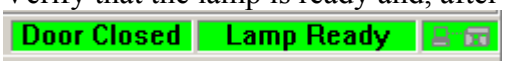
The **Run Status** dialog box remains open for the duration of the run, reporting the run status. During thermal cycling, the status of the run can also be monitored on the **Thermal Profile Status** screen. The vertical green marker tracks the progress through the thermal cycling profile.

To view accumulated fluorescence data during the run, click on the **Raw Data Plots** tab. On the **Raw Data Plots** screen, amplification plots for each dye in each well are displayed. The amplification plots can be expanded by selecting wells of interest and clicking **Show Plots**.

When the thermal profile is complete, the software will automatically proceed to the **Analysis** section to analyze the data.

Plate-Read Experiments

Verify that the lamp is ready and, after loading the samples, that the instrument door is closed.



On the **Plate Setup** screen, click **Set Read Properties**, and then enter the temperature and number of reads for the run.

Start the run by clicking the **Start Run** button on the **Plate Setup** screen.

When the **Run Status** dialog box opens, initiate collection of the appropriate set of data by clicking either **Pre-Read** or **Post-Read.**

Single-Reading Experiments

For single-read experiments, either **Pre-Read** or **Post-Read** may be used. Click here for more information on how this selection affects the reporting of results.

Pre- and Post-Read Experiments

For an experiment that utilizes pre-read and post-read data, complete the **Pre-Read** run and then exit and save the experiment. When ready to complete the post-read run, open the saved experiment, click **Start Run** from the **Plate Setup** screen, and then click the **Post-Read** button in the **Run Status** dialog box.

During data collection, the status of the run is reported in the **Run Status** dialog box, which remains open for the duration of the run. The run can also be monitored on the **Raw Data Plots** screen.

When the read is complete, the software will automatically proceed to the **Analysis** section to analyze the data.

Quantitative PCR Analysis Quick Protocol

After the thermal profile has run, the **Analysis Selection/Setup** screen will appear.

1. Select the wells to be analyzed.
2. Select the data set to be analyzed from the list under **Select data collection ramp/plateau**.

   Only ramps or plateaus associated with a collection marker  may be selected.
3. Select preferences for treatment of **Replicates** and for the use of **Algorithm enhancements**: **Amplification-based threshold**, **Adaptive baseline** and **Moving average**. If you wish to review or modify other settings that will be used for analysis, click **Analysis Term Settings** or **Adv. Algorithm Settings**.
4. Select the **Results** tab.
5. Select the type of data analysis to be performed under **Area to analyze:**

   The **Amplification plots** screen shows a plot of cycles versus fluorescence for each well and dye for which data was gathered. Plots of baseline-corrected fluorescence (**dR** or **dRn**) allow the **threshold fluorescence** (used to determine Ct values) to be displayed and adjusted.

   The **Plate sample values** screen displays **Fluorescence** or **Ct** values for the sampled wells in a plate format.

   The **Standard curve** screen shows a plot of the log of the initial template quantity versus **Ct** values for the **Standard** wells. Standard curves are used to determine (by interpolation) the initial template quantities for **Unknown** wells.

   The **Initial template quantity** screen displays interpolated quantities of template in **Unknown** wells before thermal cycling. The interpolation is based on the standard curve.

   The **Dual color scatter plot** screen displays a scatter plot of **Fluorescence** or **Ct** for any two dyes assigned to the same wells. This plot helps in assessing the suitability of a specific control target by displaying any correlation between amplification of a gene of interest and amplification of the control target.

   The **Text report** screen shows the run data in text format. The columns included in the report are specified using the **Column** check boxes.

Comparative Quantitation Analysis Quick Protocol

After the thermal profile has run, the **Analysis Selection/Setup** screen will appear.

1. Select the wells to be analyzed.
2. Select the segment (**Amplification** or **Dissociation**) to be analyzed by clicking the corresponding button on the command panel. From the dialog box that opens, select the data set to be analyzed from the list. Only ramps or plateaus associated with a collection marker  may be selected.
3. Select preferences for treatment of **Replicates** and for the use of **Algorithm enhancements**: **Amplification-based threshold**, **Adaptive baseline** and **Moving average**. If you wish to review or modify other settings that will be used for analysis, click **Analysis Term Settings** or **Adv. Algorithm Settings**.
4. Select the **Results** tab.
5. Select the type of data analysis to be performed under **Area to analyze:**

   The **Amplification plots** screen shows a plot of cycles versus fluorescence for each well and dye for which data was gathered. Plots of baseline-corrected fluorescence (**dR** or **dRn**) allow the **threshold fluorescence** (used to determine Ct values) to be displayed and adjusted.

   The **Dissociation curve** screen displays the fluorescence data collected during a dissociation segment of the run as a function of temperature. When performing SYBR Green dye-based detection, these data are used to verify that the predominant PCR products are amplicons of the gene of interest or the **Normalizer** targets.

   The **Plate sample values** screen displays **Fluorescence** or **Ct** values for the sampled wells in a plate format.

   The **Standard curve** screen shows a plot of the log of the initial template quantity versus **Ct** values for the **Standard** wells. The slope of the standard curve is used to calculate amplification efficiency for both the gene(s) of interest and the **Normalizer** targets.

   The **Relative quantity chart** screen displays a graphical representation of the amount of target present in **Unknowns** relative to the **Calibrator** (after correction using the **Normalizer** target). Results may be displayed as either a bar graph (default) or a scatter plot.

   The **Relative quantity plate** screen shows, in plate format, the relative quantities for each well and dye corresponding to a gene of interest (the **Normalizer** and **Reference** dyes are not quantified). **Calibrators** are defined as 1.0 and values for all **Unknowns** are relative to this benchmark.

   The **Text report** screen shows the run data in text format. The columns included in the report are specified using the **Column** check boxes.

SYBR Green Analysis Quick Protocol

After the thermal profile has run, the **Analysis Selection/Setup** screen will appear.

1. Select the wells to be analyzed.
2. Select the segment (**Amplification** or **Dissociation**) to be analyzed by clicking the corresponding button on the command panel. From the dialog box that opens, select the data set to be analyzed from the list. Only ramps or plateaus associated with a collection marker  may be selected.
3. Select preferences for treatment of **Replicates** and for the use of **Algorithm enhancements**: **Amplification-based threshold**, **Adaptive baseline** and **Moving average**. If you wish to review or modify other settings that will be used for analysis, click **Analysis Term Settings** or **Adv. Algorithm Settings**.
4. Select the **Results** tab.
5. Select the type of data analysis to be performed under **Area to analyze:**

   The **Amplification plots** screen shows a plot of cycles versus fluorescence for each well and dye for which data was gathered. Plots of baseline-corrected fluorescence (**dR** or **dRn**) allow the **threshold fluorescence** (used to determine Ct values) to be displayed and adjusted.

   The **Dissociation curve** screen displays the fluorescence data collected during the dissociation segment of the run as a function of temperature. These data are used to verify that the predominant PCR products are amplicons of the gene of interest. Plotting the temperature versus the first derivative of the fluorescence (**–R´(T)** or **–Rn´(T)**) generates a dissociation profile with peaks that identify the melting temperatures of each of the major product populations in the reaction mixture.

   The **Plate sample values** screen displays **Fluorescence** or **Ct** values for the sampled wells in a plate format.

   The **Standard curve** screen shows a plot of the log of the initial template quantity versus **Ct** values for the **Standard** wells. Standard curves are used to determine (by interpolation) the initial template quantities for **Unknown** wells.

   The **Initial template quantity** screen displays interpolated quantities of template in **Unknown** wells before thermal cycling. The interpolation is based on the standard curve.

   The **Text Report** screen shows the run data in text format. The columns included in the report are specified using the **Column** check boxes.

Allele Discrimination Real-Time Analysis Quick Protocol

After the thermal profile has run, the **Analysis Selection/Setup** screen will appear.

1. Select the wells to be analyzed.
2. Select the data set to be analyzed from the list under **Select data collection ramp/plateau**. Only ramps or plateaus associated with a collection marker  may be selected.
3. Select preferences for treatment of **Replicates** and for the use of **Algorithm enhancements**: **Amplification-based threshold**, **Adaptive baseline** and **Moving average**. If you wish to review or modify other settings that will be used for analysis, click **Analysis Term Settings** or **Adv. Algorithm Settings**.
4. Select the **Results** tab.
5. Select the type of data analysis to be performed under **Area to analyze:**

   The **Amplification plots** screen shows a plot of cycles versus fluorescence for each well and dye for which data was gathered. Plots of baseline-corrected fluorescence (**dR** or **dRn**) allow the **threshold fluorescence** (used to determine Ct values) to be displayed and adjusted.

   The **Plate sample values** screen displays **Fluorescence** or **Ct** values for the sampled wells in a plate format.

   The **Dual color scatter plot** screen displays a scatter plot of **Fluorescence** or **Ct** for any two dyes assigned to the same wells. Each point represents the coordinates of the **Fluorescence** or **Ct** for the two dyes in a single well. The plot provides a simple method for grouping the sample wells according to the amplification events indicated by either single dye (i.e. homozygous for one of two alleles) or by both dyes (i.e. heterozygous for the two alleles).

   The **Final call results** screen provides a simple depiction of whether product was accumulated for each dye in each well. A plus sign (**+**) signifies accumulation of product, while a minus sign (**–**) signifies that product accumulation was not detected. Calling is based on Ct, where **+** is returned if the Ct is less than the last cycle for which data was collected.

   The **Text report** screen shows the run data in text format. The columns included in the report are specified using the **Column** check boxes.

Molecular Beacon Melting Curve Analysis Quick Protocol

After the thermal profile has run, the **Analysis Selection/Setup** screen will appear.

1. Select the wells to be analyzed.
2. Select the data set to be analyzed from the list under **Select data collection ramp/plateau**.

   Only ramps or plateaus associated with a collection marker  may be selected.
3. Select preferences for treatment of **Replicates**. If you wish to review or modify the read statistic that will be used for analysis, click **Adv. Algorithm Settings**.
4. Select the **Results** tab.
5. Select the type of data analysis to be performed under **Area to analyze:**

   The **Annealing range** screen displays the optimal annealing range for the beacon(s) tested. The optimal range is the temperature range in which the molecular beacon plus perfectly- matched oligo (**MBO**) has a greater fluorescence than the beacon plus mismatched oligo (**MBMO**) and in which the background fluorescence is low.

   The **Melting curves/temperature** screen calculates and displays the optimal melting temperature for the beacon(s) tested.

Quantitative Plate Read Analysis Quick Protocol

After the read is complete, the **Analysis Selection/Setup** screen appears.

1. Select the wells to be analyzed.
2. Select preferences for treatment of **Replicates**. If you wish to review or modify other settings that will be used for analysis, click **Analysis Term Settings** or **Adv. Algorithm Settings**.
3. Select the **Results** tab.
4. Select the type of data analysis to be performed under **Area to analyze**.

   The **Plate sample values** screen displays **Fluorescence** values for the sampled wells in a plate format.

   The **Standard curve** screen shows a plot of the log of the initial template quantity versus **Fluorescence** values for the **Standard** wells. Standard curves are used to determine (by interpolation) the initial template quantities for **Unknown** wells.

   The **Initial template quantity** screen displays interpolated quantities of template in **Unknown** wells. The interpolation is based on the standard curve.

   The **Dual color scatter plot** screen displays a scatter plot of **Fluorescence** for any two dyes assigned to the same wells. This plot helps in assessing the suitability of a specific control target by displaying any correlation between amplification of a gene of interest and amplification of the control target.

   The **Fluorescence Intensity Values** screen displays a colorized image of final calls and fluorescence intensities. A plus sign (**+**) indicates a positive call and a minus sign (**–**) indicates a negative call. A spectrum of colors is used to indicate the relative fluorescence readings for each dye in each well.

   The **Final call results** screen provides a simple depiction of whether product was accumulated for each dye in each well. A plus sign (**+**) signifies accumulation of product, while a minus sign (**–**) signifies that product accumulation was not detected. Calling is based on fluorescence, where **+** is returned if the fluorescence value is statistically greater than the value measured for the control.

   The **Text report** screen shows the run data in text format. The columns included in the report are specified using the **Column** check boxes.

Plate Read/Allele Discrimination Analysis Quick Protocol

After the read is complete, the **Analysis Selection/Setup** screen appears.

1. Select the wells to be analyzed.
2. Select preferences for treatment of **Replicates**. If you wish to review or modify other settings that will be used for analysis, click **Analysis Term Settings** or **Adv. Algorithm Settings**.
3. Select the **Results** tab.
4. Select the type of data analysis to be performed under **Area to analyze:**

   The **Plate sample values** screen displays **Fluorescence** values for the sampled wells in a plate format.

   The **Dual color scatter plot** screen displays a scatter plot of fluorescence for any two dyes assigned to the same wells. Each point represents the coordinates of the **Fluorescence** for the two dyes in a single well. The plot provides a simple method for grouping the sample wells according to the amplification events indicated by either single dye (i.e. homozygous for one of two alleles) or by both dyes (i.e. heterozygous for the two alleles).

   The **Fluorescence intensity values** screen displays a colorized image of final calls and fluorescence intensities. A plus sign (**+**) indicates a positive call and a minus sign (**–**) indicates a negative call. A spectrum of colors is used to indicate the relative fluorescence readings for each dye in each well.

   The **Final call results** screen provides a simple depiction of whether product was accumulated for each dye in each well. A plus sign (**+**) signifies accumulation of product, while a minus sign (**–**) signifies that product accumulation was not detected. Calling is based on comparing the experimentally determined **p-value** (probability that a reading differs from the control readings) for each dye in each well to the user-defined **Confidence level for calls**.

   The **Text report** screen shows the run data in text format. The columns included in the report are specified using the **Column** check boxes.

How-To (Detailed Protocols)

How to Set Up a New Experiment

To set up an experiment on the Mx3000P real-time PCR system, choose the experiment type from the **New Experiment Options** dialog box (accessed by clicking the **New** button  on the toolbar).

The setup consists of two steps for a real-time experiment:

1. **Plate Setup**: In this step, the plate is configured including assigning well types and dyes to wells
2. **Thermal Profile Setup**: In this step, the thermal profile is configured for temperature cycling and data collection parameters

For plate read experiments, only **Plate Setup** is required.

Some aspects of the contents of the **Plate Setup** and **Thermal Profile Setup** screens for a new experiment are determined by the properties of the software's active default set. For example, user-defined default sets may be set up to include the plate configuration as well as the thermal profile and analysis settings. See *Preferences-Defaults* in the *Navigating in the Mx3000P Software* section for more information on creating and managing default sets.

If the experiment will be started within the next 20 minutes, and the lamp is off, turn the lamp on to allow it to warm to operating temperature by the time the experiment begins. To turn on the lamp, click the **Lamp On/Off** button  on the toolbar.

Plate Setup

Introduction to Plate Setup

To access the **Plate Setup** window, click the **Setup** button on the toolbar and then click the **Plate Setup** tab. Use the **Plate Setup** window to provide the specifications for sample wells in the experiment.

Adding well information during plate setup consists of two steps:

1) Select the wells to be configured

2) Assign the appropriate characteristics to the selected wells

Alternatively, plate setup may be accomplished using one of three shortcuts: using **Import** plate setup, using a **Default** plate setup or using **Quick Setup.**

Most features of the plate setup can be modified before, during, and after a run.

Comments can be entered in the **Plate setup comments** text box at the lower right-hand corner of the screen and the comments will be saved along with the experiment.

After all plate setup functions are complete, for real-time experiments, click **Next** in the lower right-hand corner to proceed to the **Thermal Profile Setup** screen. For plate read experiments, you may start the run from the **Plate Setup** screen.

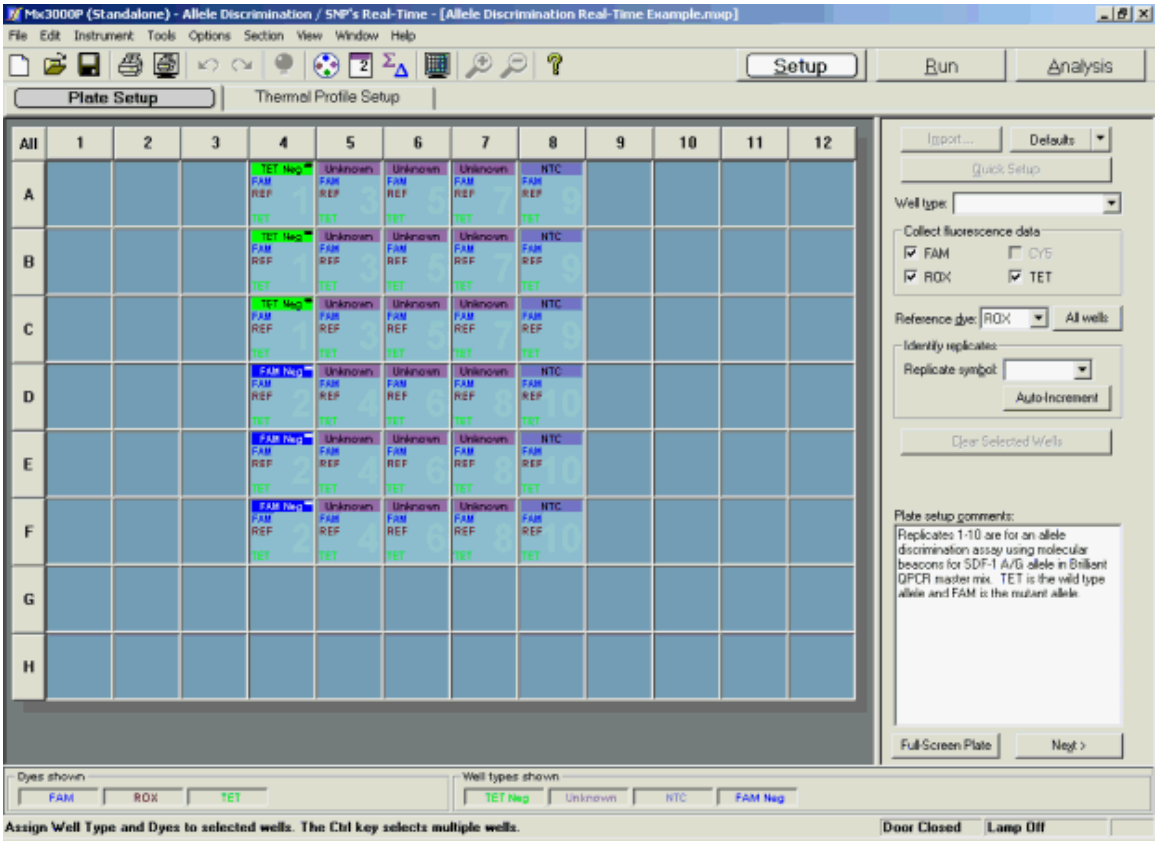


Plate Setup Display Options

A number of modifications to the appearance of the **Plate Setup** screen may be made to facilitate the plate setup process. Using any of these display modification features will not affect the data collected or the analysis available for the experiment.

Displaying or Hiding Dyes

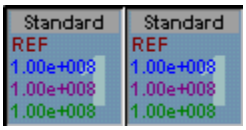
The dyes in use on the plate may be displayed or hidden by using the **Dyes shown** selection bar at the lower left-hand corner of the screen. When the button is depressed (like **FAM** in the picture below), the dye's well assignments are displayed, but when the button is released (like **ROX** in the picture below), the dye's well assignments are hidden from display. **Note that using this feature does not affect data collection**.

Displaying or Hiding Specific Well Types

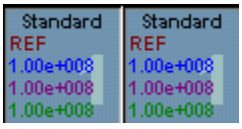
The well types that are displayed on the plate are controlled by using the buttons in the **Well types shown** selection bar. Any of the well type buttons may be depressed in order to display the information for wells of the indicated type or may be released to hide the information. For example, if you want to view only the **Unknown** wells on the plate, depress the **Unknown** button and release all other well type buttons (such as the **NTC** button in the example below). **Note that using this feature does not affect data collection.**

Color Coding Well Types

The **Enable well type colors** command, accessed on the **Display** tabbed page of the **Preferences** dialog box, causes a horizontal color bar to be displayed across the top of each well. The color of this bar corresponds to the well type selected to allow a quick identification of well types. Well type colors can be changed in the **Colors** tabbed page of the **Preferences** dialog box. When **Enable well type colors** is selected, wells appear as shown below.

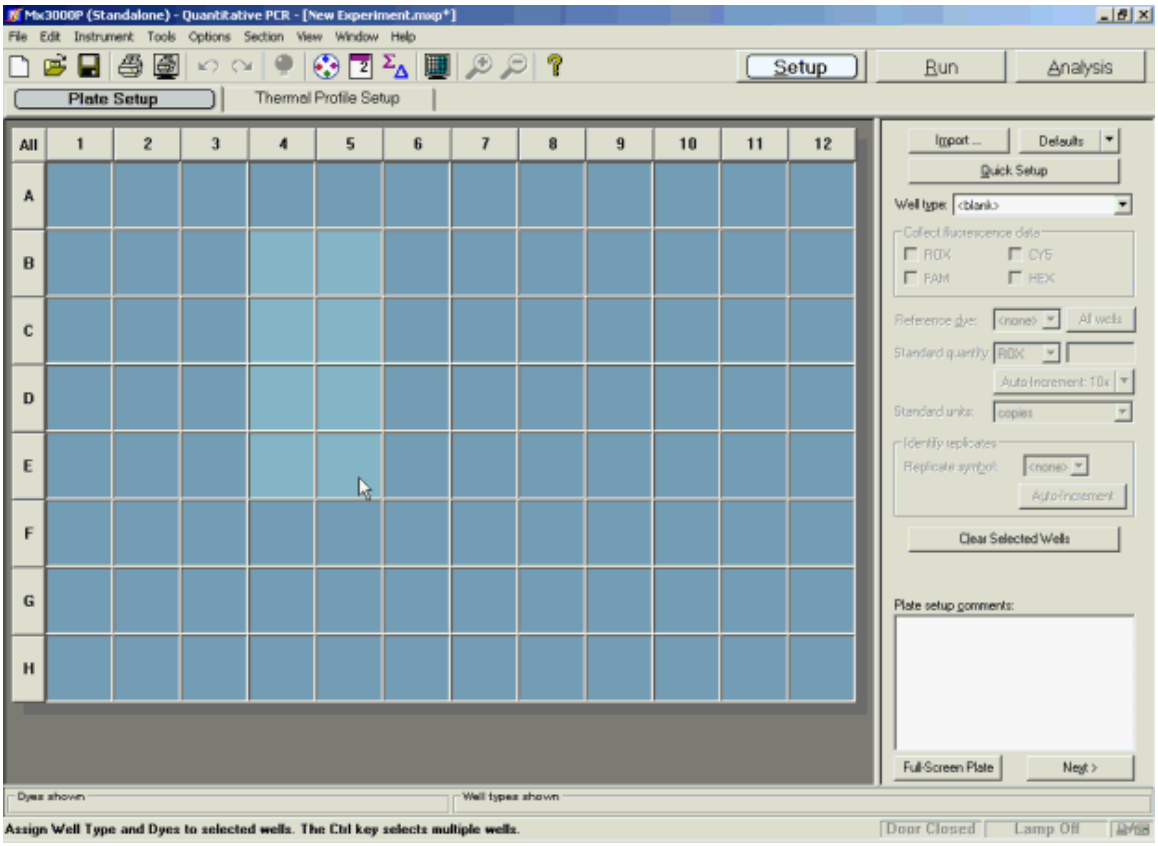


When **Enable well type colors** is cleared, the same wells appear as shown below.



Selecting Wells

The first step of **Plate Setup** is selecting the wells to which well information will be assigned. Wells must be selected/highlighted before well types and other information can be assigned.



To select an individual well, single click in that well.

To select an entire row or column, click on the row header or column header.

To select all wells in the plate, click **All** in the upper left-hand corner of the plate.

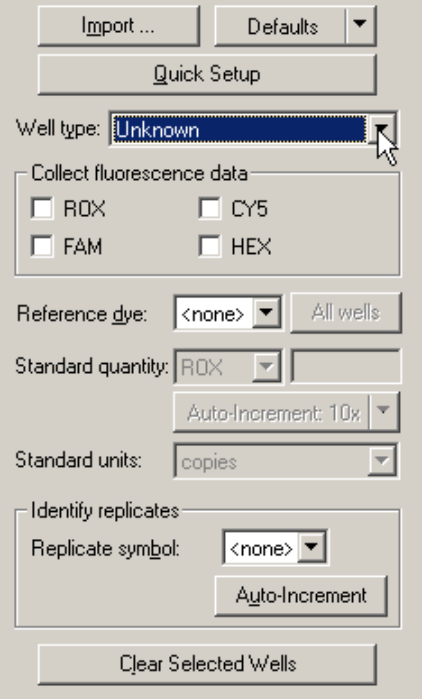
Select a contiguous block of wells by dragging across the range of wells with the mouse while holding the left mouse button down. A visible rectangle will appear. Release the left mouse button to select the cells included in the rectangle.

Select a group of non-contiguous wells by holding down the **Ctrl** button on the keyboard while selecting wells.

To deselect a well, hold down the **Ctrl** key and click on the well to be deselected. To rapidly deselect many wells, click the **All** button twice. This will select and then deselect all wells.

Assigning Well Types and Dyes for Data Collection

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.



Assigning the Well Type

After selecting one or more wells, assign the well type to the selected wells.

To assign the well type, click on the arrow in the **Well type** menu. A list of well types specific to the experiment type will appear. Select the well type to be assigned to the well(s).

Selecting the Dyes for Data Collection

Once a well type is assigned, select the dyes for which fluorescence data are to be collected for the well(s) using the **Collect fluorescence data** checkboxes. Data can be collected for up to four dyes. Fluorescence data are collected for **only** those dyes selected for each well.

Clearing Wells

To clear the information assigned to selected well(s), including well type, data collection specifications and replicate set designations, click the **Clear Selected Wells** button.

Use of Copy/Paste for Entering Well Information

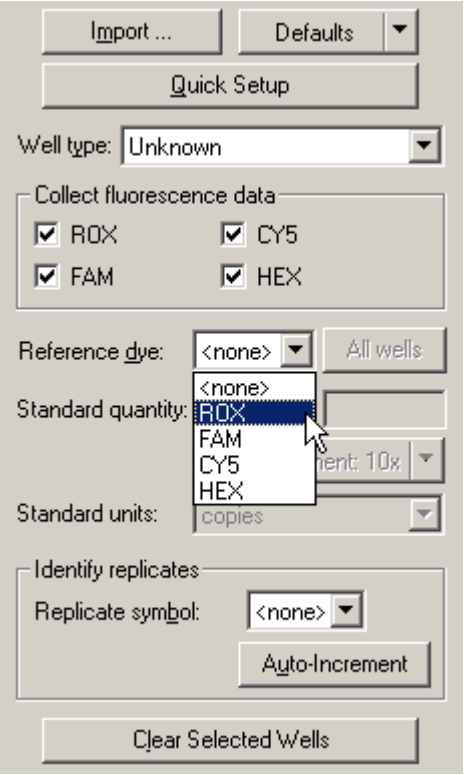
Within a given experiment, information from groups of wells may be copied from one set of wells and pasted into another set of wells by using the **Copy** and **Paste** commands on the **Edit** menu or using the corresponding shortcut keys (**Ctrl+C** and **Ctrl+V**).

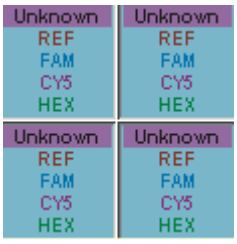
Assigning a Reference Dye

Passive reference dyes are used for normalization of the fluorescence signal, in order to compensate for non-PCR related variations in fluorescence. Typically, ROX is used as the reference dye. Only dyes assigned to optical paths in the **Optics Configuration** dialog box are available for assignment as reference dyes.

If a dye was added as a passive reference dye in the experiment, select the dye from the **Reference dye** menu. The assignment of that dye as a reference dye will be indicated in the selected well(s) by the presence of the text **REF** in the position of the dye in the well.

If all sample wells on the plate will contain the same passive reference dye, depress the **All Wells** button. This will cause the selected dye to be assigned as a reference dye for all sample wells on the plate.

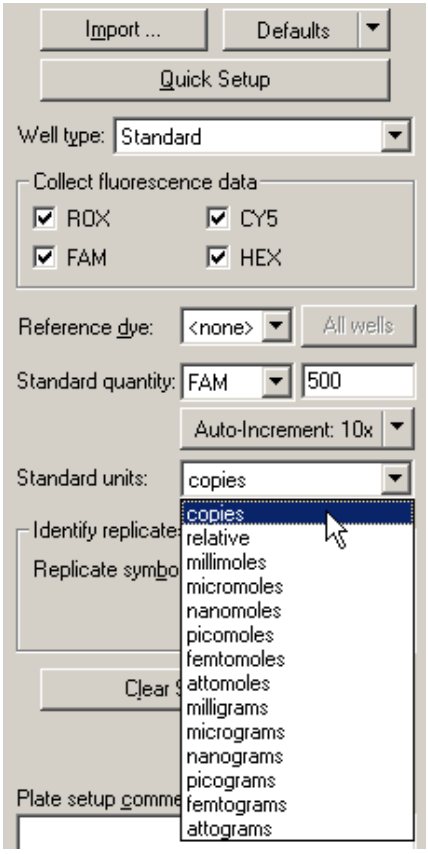




Assigning Quantities to Standard Wells

If a sample type of **Standard** is assigned to the selected well(s), specify the quantity of the standard added and the unit of measurement using the **Standard quantity** box and the **Standard units** menu. This section of the command panel is enabled only when wells assigned as **Standards** are selected.

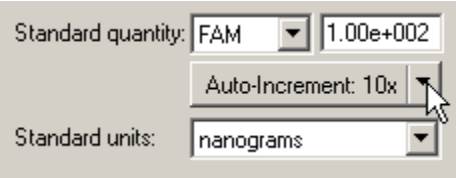
First, select the dye associated with the standard from the pulldown menu following **Standard quantity**. Next, enter the quantity of the standard added to the selected well(s) in the rightmost box following **Standard quantity** as a decimal number (for example, 500 or 1.234). Specify the units for the quantity in the **Standard units** menu. The standard quantity value will be converted to scientific notation and will appear in the selected wells in the position of the designated dye. When using a dilution series to generate a standard curve, the **Auto-Increment** utility can be used to expedite the process of assigning quantities to **Standard** wells. See below for more information on the use of this feature.



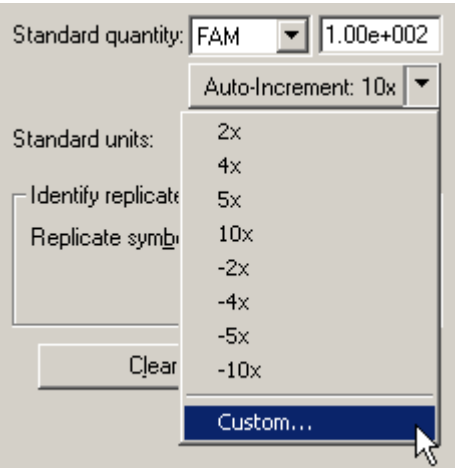
Time-Saving Tip - Using the Auto-Increment Feature

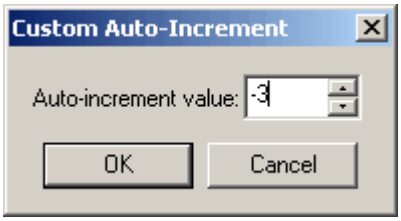
When using a dilution series to generate a standard curve, the **Auto-Increment** utility may be used to expedite standard quantity assignments during plate setup. When using this feature, given one quantity in the dilution series, the software will automatically assign standard quantities to sequential sets of **Standard** wells according to the specified dilution factor.

To assign **Standard quantities** using **Auto-Increment**, first complete the well type and data collection assignments for all of the **Standard** wells in the dilution series. When finished, select all replicates of the first set of **Standard** wells in the dilution series. Select the dye associated with the target, and then enter the quantity of target in the box to the right of the dye. Specify the standard quantity units for this dilution series from the **Standard units** menu.



After the assignment of the first replicate set in the dilution series is complete, click the **Auto-Increment: 10x** menu to expand the menu. Select the appropriate description of the dilution series from the menu. Note that if you begin the process with the wells containing the lowest concentration of target in the dilution series, a positive increment (e.g. 10× or 2×) should be selected such that the subsequent wells will be assigned with multiples of the dilution factor. Conversely, if you begin the process with wells containing the highest concentration of target, a negative increment (e.g. –10× or –2×) should be selected. If the dilution factor used in the current experiment is not listed in the menu, select **Custom** to access the **Custom Auto-Increment** dialog box, in which user-defined increment values may be entered. Note that positive custom increment values should be entered when starting assignments with the lowest concentration in the series and negative custom increment values should be entered when starting assignments with the highest concentration in the series.





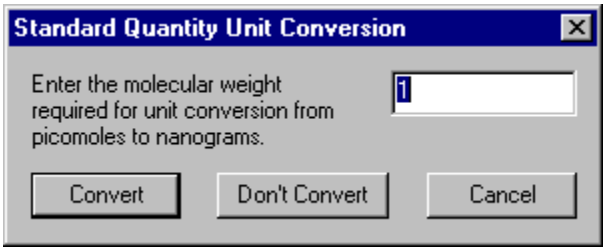
When the cursor is moved to the plate area on the screen, it will change to a hand symbol containing the incrementally-adjusted standard quantity value.

Using the hand-cursor, select the range of cells corresponding to the next set of **Standards** in the dilution series. When the mouse button is released, the quantity that had shown in the cursor will appear in the wells that had been selected and the number showing in the cursor will change incrementally.

Repeat the standard quantity assignment process using the hand-cursor until all members of the dilution series have been assigned, and then click on the **Auto-Increment: 10x** button again to restore the standard cursor.

Converting Standard Quantity Units

To convert standard quantity units in assigned wells, select a **Standard** well that contains the original standard quantity defined in the original units and then select the new unit description from the **Standard units** menu. When the basic unit type is the same (e.g. milligrams to micrograms) the conversion will occur automatically. When the units describe different properties of the sample (e.g. moles to grams), the **Standard Quantity Unit Conversion** dialog box will open and prompt for the molecular weight.



To complete the conversion, which will change the numerical value in the **Standard** wells in addition to changing the units, enter the molecular weight and then click **Convert**. The conversion will be applied to all **Standard** wells on the plate.

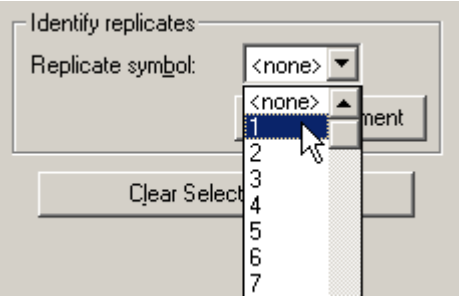
To change the units while retaining the numerical values assigned to **Standards** (e.g. to simply correct erroneous units for a correct numerical value) click **Don't Convert**. To return to **Plate Setup** without changing any numerical values or unit designations, click **Cancel**.

Assigning Replicates

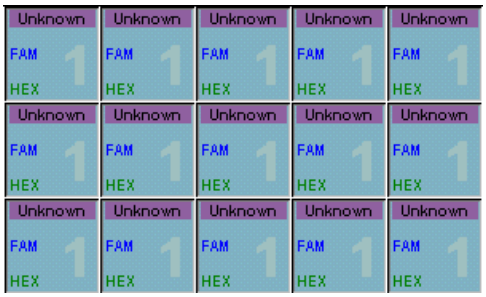
Identify replicate wells on the plate by assigning the same replicate symbol to the group of wells that make up a replicate set. Assigning replicates allows the option of treating the wells collectively during analysis. See *Treatment of Replicates* for more information on the effects of treating replicates individually or collectively during analysis. Note that although replicates are assigned on the **Plate Setup** screen, you can choose whether or not to use replicates during data analysis on the **Analysis Selection/Setup** screen.

Identifying Samples as Replicates

To specify replicates during **Plate Setup**, select those wells which contain the replicated samples and then select the appropriate replicate set number from the **Replicate symbol** menu.



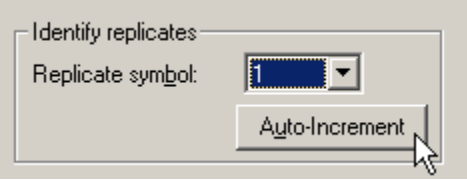
Replicates are assigned to the selected wells as soon as the replicate number is selected.



Time-Saving Tip - Using the Auto-Increment Feature

The **Auto-Increment** utility may be used to expedite replicate set assignments during plate setup. This utility is used to sequentially assign a series of different replicate sets with increasing replicate symbol numbers using an automatic counting mechanism.

To assign replicates using **Auto-Increment**, first assign well types and dye collection attributes to all of the wells that will be assigned with replicate symbols. When finished, click on any sample well to enable the **Auto-Increment** feature. Click the **Auto-Increment** button in the **Identify replicates** section of the command panel.

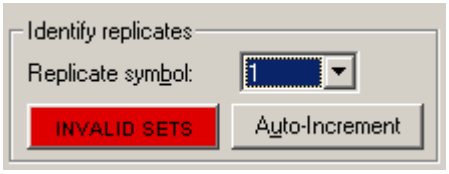


When the cursor is moved to the plate area on the screen, it will change to a hand symbol containing the next available replicate set number  . Using the hand-cursor, select a range of cells to be grouped as replicates. When the mouse button is released, the replicate symbol that had shown in the cursor will appear in the wells and the number in the cursor will increase incrementally. 

Select the next set of replicate wells with the hand-cursor to assign the new replicate symbol to those wells. Repeat this process until all replicates have been assigned. When finished making assignments, click on the **Auto-Increment** button again to restore the standard cursor.

Invalid Replicate Sets

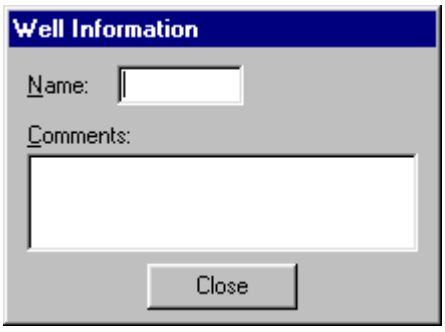
Only identically assigned wells should be designated as replicates. The software uses a number of methods to prevent incompatible wells from being assigned as replicates. If the warning below appears, non-identical wells have been assigned the same replicate number.



Clicking the **Invalid Sets** button opens a dialog box that indicates which replicate sets have a conflict. To resolve these conflicts, reassign replicates such that only wells with the same attributes are grouped.

Entering Custom Well Information

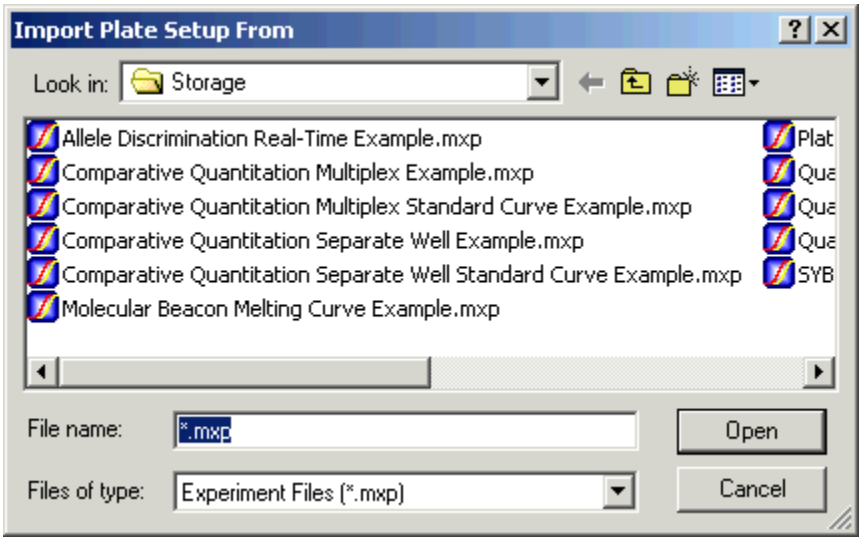
Each well in **Plate Setup** has a comment box available for entering a well name (48-character maximum) and well-specific comments. The **Well Information** dialog box is accessed by double-clicking on the well while in **Plate Setup**. The well name is visible in the well in the **Full-Screen Plate** view (first 10–14 characters) and may be included in the **Text Report** (all characters).



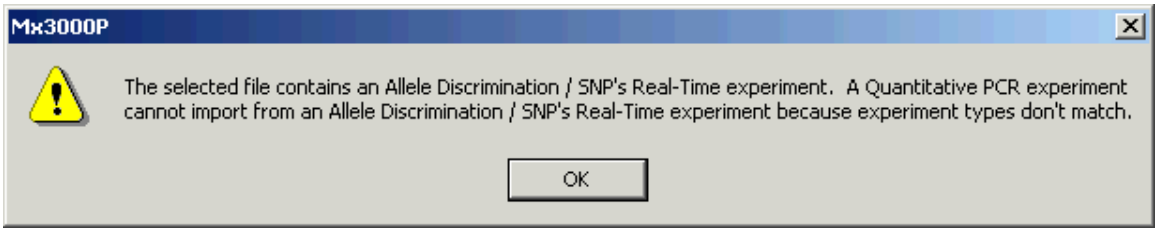
Using Import Plate Setup

The **Import Plate Setup** function allows you to recall a plate setup from an existing experiment. Clicking **Import Plate Setup** on the **Plate Setup** screen causes the software to display the **Import Plate Setup From** dialog box.

The **Import Plate Setup From** dialog box lists Mx3000P experiments stored in the Mx3000P data storage folder. Select the experiment that includes the desired plate setup information, then click **Open**.



If the experiment selected is not compatible with the experiment being set up, a warning dialog box appears as shown below. If this warning appears, click the **OK** button and select another experiment of the same type as the one being set up.

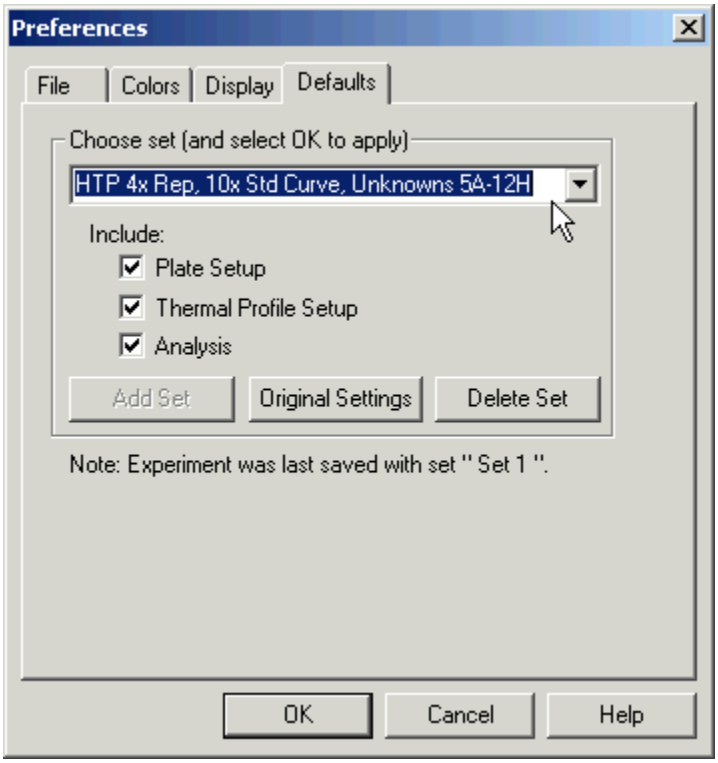


Using a Default Plate Setup

Plate setup may be completed by incorporating a user-defined default plate configuration into the **Plate Setup** for new experiments.

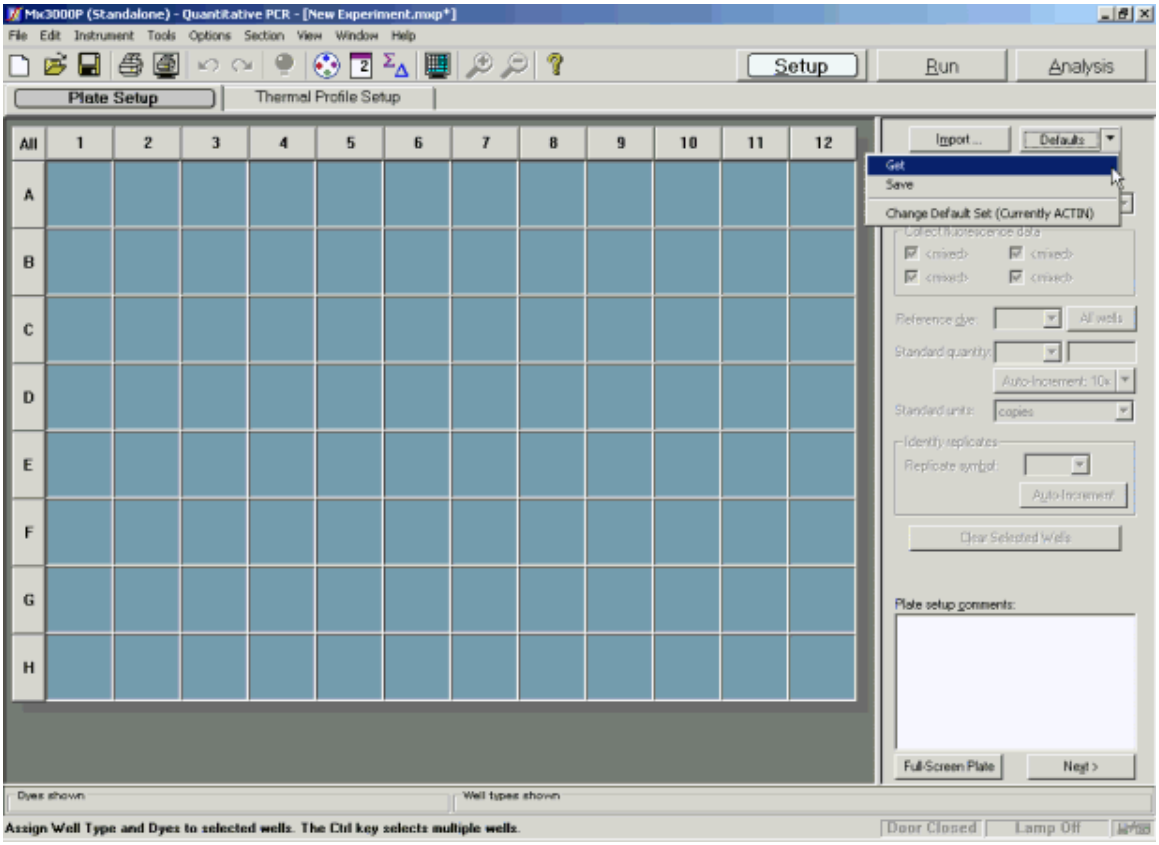
Using a default plate setup requires creating a default set containing the desired plate configuration. Note that the **Plate Setup** checkbox under **Include** must be selected when the default set is created in order for **Plate Setup** information to be included in the default set.

Once the appropriate default set is created, select this default set as the active default set in the **Choose set** menu of the **Preferences-Defaults** dialog box and then click **OK**. The plate configuration stored in the active default set, including all well assignments, will automatically be applied to the open experiment and to all new experiments, until the active default set is changed again.



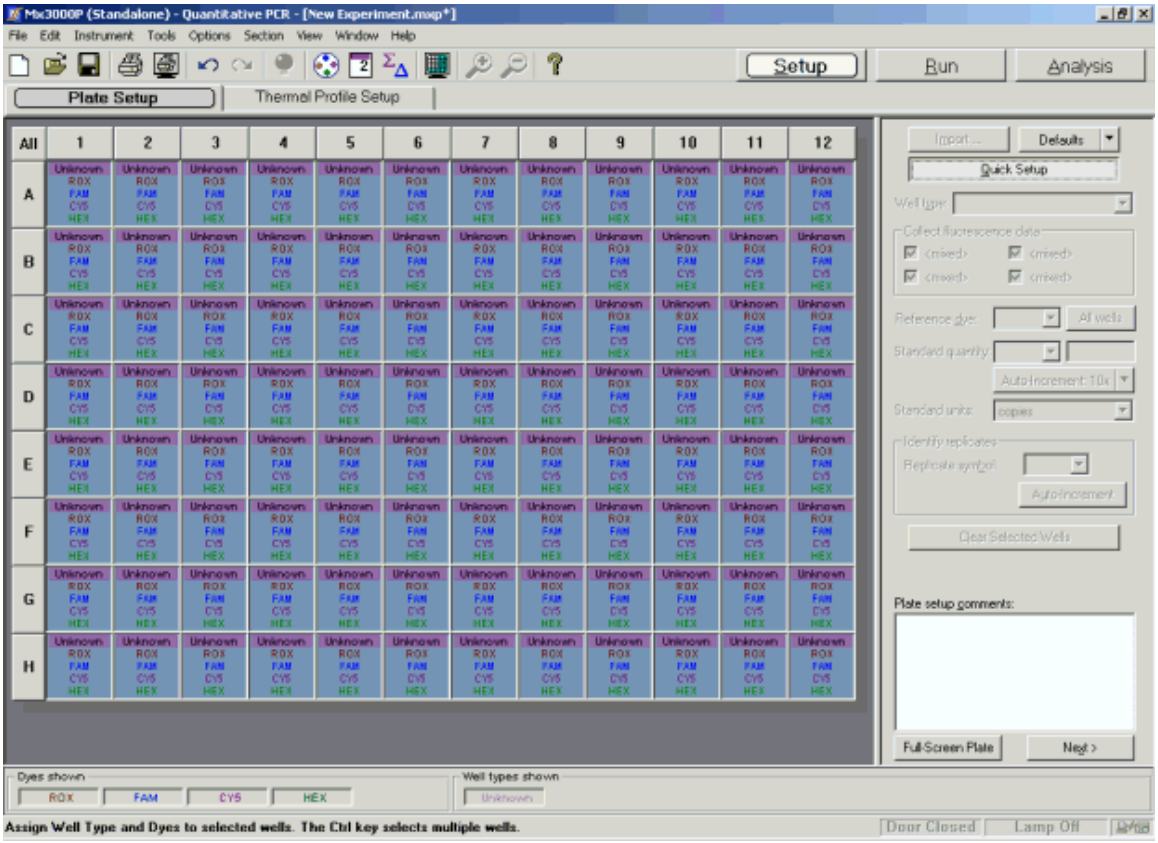
The plate configuration in an existing default set may also be applied to the plate setup in an open experiment by using the **Defaults** menu on the control panel of the **Plate Setup** screen. To retrieve the plate configuration from the active defaults set, click **Defaults** to expand the menu, and then select **Get**.

See *Preferences-Defaults* for more information about creating and using default sets to streamline experimental setup and analysis.



Using Quick Setup

Clicking the **Quick Setup** button on the Plate Setup screen automatically enters well information for all wells, such that fluorescence data are collected for all four dyes in all 96 wells. This feature saves up-front setup time. Because all other parts of **Plate Setup** can be modified during and after a run, using **Quick Setup** allows you to collect all dye data for all wells and then configure the plate after the run.



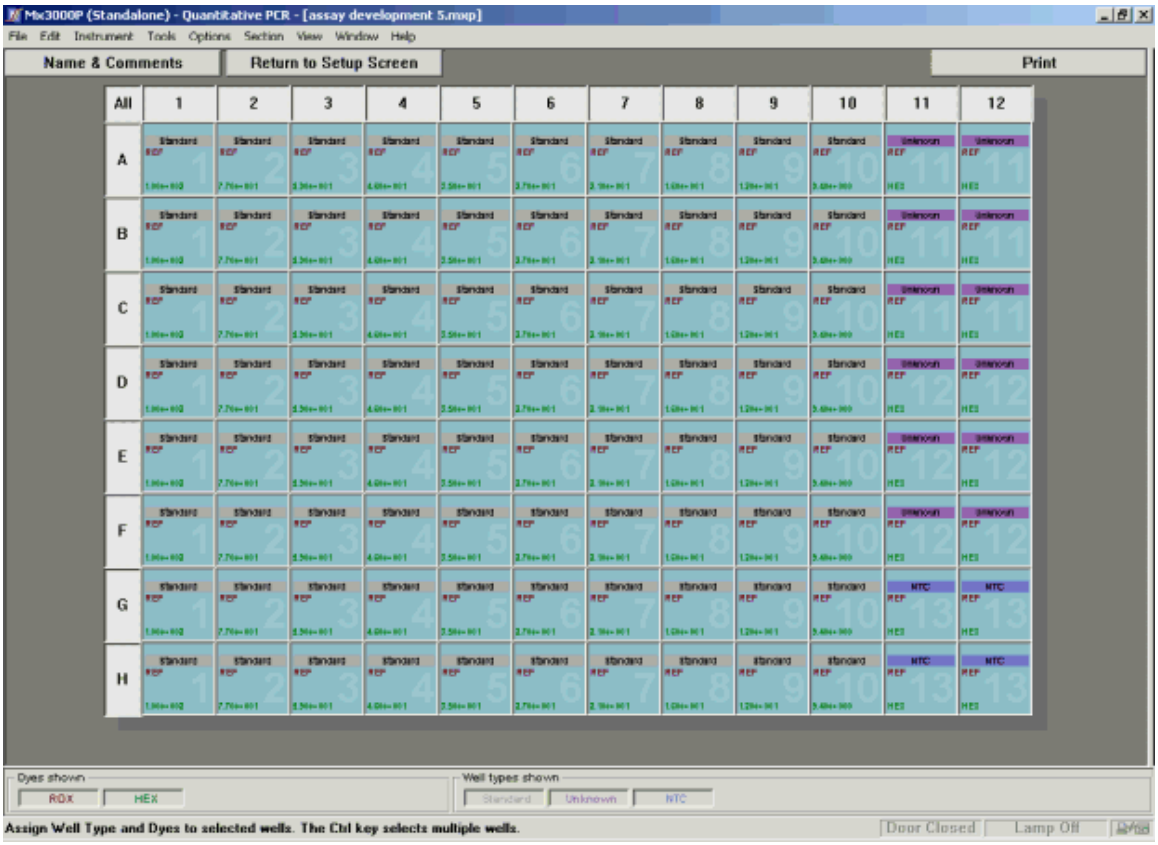
Using the Full-Screen Plate View

To enlarge the view of the plate to cover the full screen, click the **Full-Screen Plate** button at the bottom of the command panel (lower right-hand corner of the **Plate Setup** screen). No modifications to the plate setup parameters can be performed in the full-screen view.

To print the plate from full-screen plate view, click **Print** in the upper right of the screen. If you want to print all contents of the screen, including title bars and toolbars, select **Print Screen** from the **File** menu. The size of either type of printed image can be altered by selecting **Page Setup** from the **File** menu, which opens the **Print Page Setup** dialog box. The valid size range is from 25% to 100%.

If a **Name** was assigned to a well under **Well Information**, it will be visible in **Full-Screen Plate** view. To see or edit **Comments** recorded in the **Well Information** dialog box, select the well of interest, and then click **Name & Comments**. Alternatively, double-click on the well of interest to access the **Well Information** dialog box for that well.

To return to **Plate Setup** in order to continue with experimental setup, click the **Return to Setup Screen** button.



Thermal Profile Setup

Thermal Profile Introduction

The Mx3000P real-time PCR system thermal profile is a visual representation of the temperature cycling program that directs the instrument to incubate samples at specific temperatures at specific times. The programmed temperatures represent the desired fluid temperatures of the reaction samples. The Mx3000P system thermal profile consists of **plateaus**, **ramps** and **segments**. Thermal profiles are used only in real-time experiments; in plate read experiments, samples are held at a single temperature.

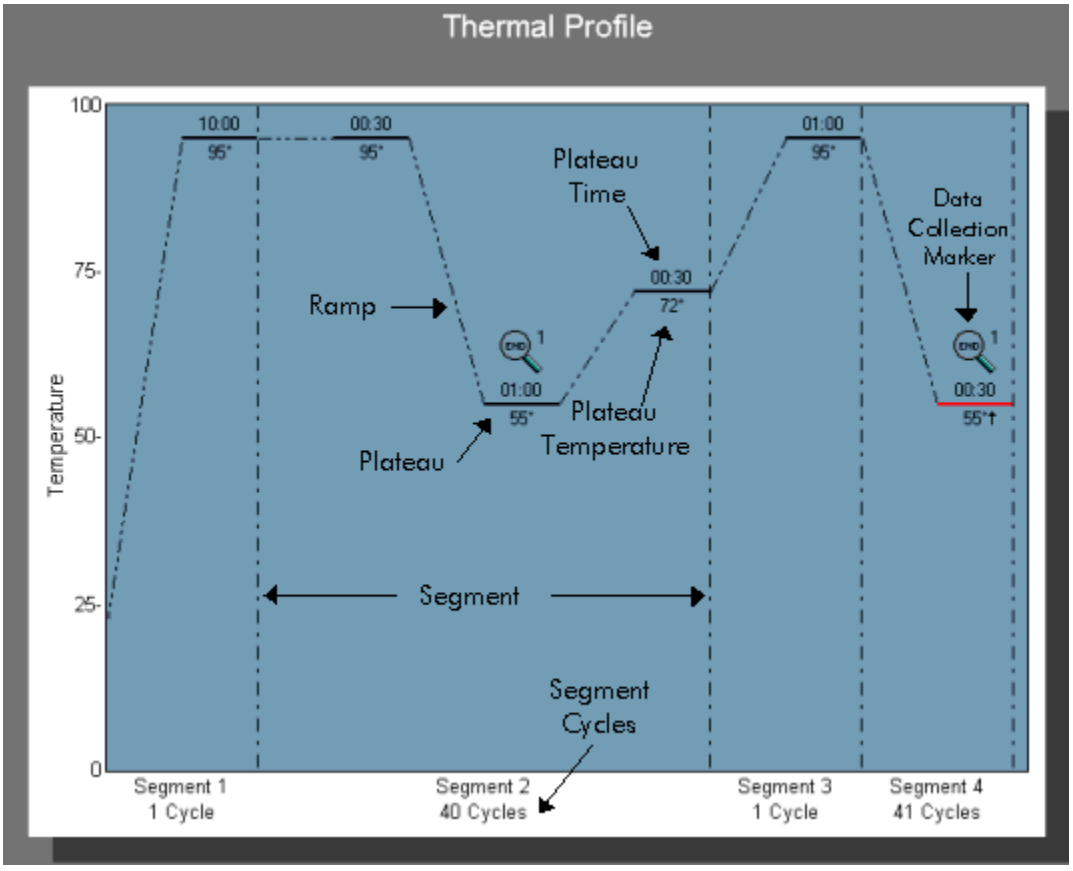
Elements of the Mx3000P Thermal Profile

A **plateau** is a temperature held over a period of time. It is represented with a solid horizontal line in a thermal profile. The valid temperature range is 25° (ambient) to 99°C. The valid range for a plateau duration is 1 second to greater than 18 hours.

A **ramp** is the transition between two plateaus.

A **segment** is a group of 1 to 127 plateaus and the intervening ramps that has been set to cycle at least one time. Segments are delineated in the thermal profile by vertical dotted lines.

A **cycle** is one pass through a segment. Each segment can be set to cycle up to 255 times.



Data Collection During Thermal Cycling

The Mx3000P system can collect data during the **plateaus** and **ramps** of a thermal profile.

Two types of data collection are available: **Endpoints** and **All Points**. See *Data Collection* for more detailed information on how readings are taken and analyzed using these collection settings.

Data collection markers show the points designated for data collection on the **Thermal Profile** display.

Endpoint collection, indicated by the **Endpoint data collection marker** 

causes the system to take readings at the end of a **plateau** or **ramp**.

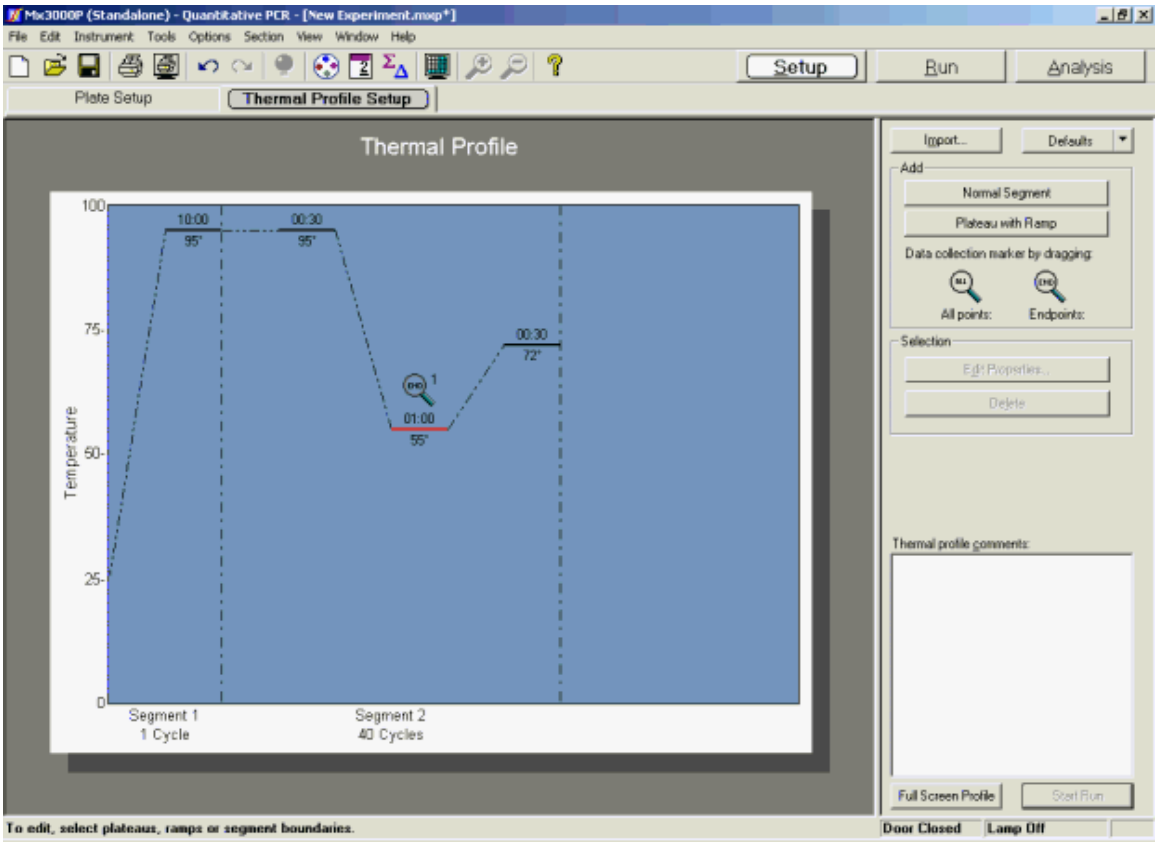
All points collection, indicated by the **All points data collection marker** 

causes the system to take readings as often as possible during a **plateau** or **ramp**.

Thermal Profile Setup Procedures

When the **Thermal Profile Setup** tab is selected in a new experiment, a default thermal profile appropriate to the experiment type appears. The default thermal profile offers a starting point for creating the appropriate thermal profile for the experiment. Instructions for modifying the default thermal profile by adding or changing segments, plateaus and ramps are provided below.

For information on importing a thermal profile from another experiment, see *Import Thermal Profile Setup*. For more background information on the nomenclature and functions of the thermal profile components, see *Thermal Profile Introduction*.



Adding a Normal Segment

To add a normal segment, go to the **Add** section of the command panel and click the **Normal Segment** button. The segment added will have one plateau with a default temperature of 25°C and a default cycle number of 1 cycle. See *Adding a Plateau to a Segment*, below, for information on adding or modifying plateaus in the new segment.

Position of the Added Segment

If no segment is selected when the **Normal Segment** button is clicked, the new segment will be added to the beginning of the profile. If an entire segment is selected when the **Normal Segment** button is clicked, the new segment will be added after the selected segment.

Properties of the Added Segment

To adjust the number of cycles for the segment, double-click in the segment to open the **Segment Properties** dialog box, and then enter the **Number of Cycles**. Alternatively, click on the cycle number text below the segment to change the value.

If you want to add a descriptive label for the segment, double-click in the segment to open the **Segment Properties** dialog box, and then enter the desired text in the **Label** box.

For more information, see *Segment Properties Dialog Box*.

Adding a Plateau to a Segment

To add a plateau, go to the **Add** section of the command panel and click the **Plateau with ramp** button. When a plateau is added, ramps leading to and from the new plateau are also added by the software.

Position of the Added Plateau

If no segment or plateau is selected when the **Plateau with ramp** button is clicked, the new plateau will be added at the beginning of the first segment of the profile. If an entire segment is selected when the **Plateau with ramp** button is clicked the plateau will be added to the beginning of the segment. If a plateau is selected when the **Plateau with ramp** button is clicked, the new plateau will be added after the selected plateau within the same segment.

Properties of the Added Plateau

The default temperature for a new plateau is 25°C and the default duration is 30 seconds.

The plateau temperature and duration may be modified by any of the following methods:

- Grab the plateau with the mouse and drag it up or down to change the temperature
- Double-click on the plateau to open the **Plateau Properties** dialog box
- Click on the temperature or duration value associated with the plateau on the **Thermal Profile** screen; this action opens a spin box in which the value may be changed

A **data collection marker** may be added to the plateau by using the **Plateau Properties** dialog box or by dragging the appropriate collection marker 

from the command panel to the plateau on the **Thermal Profile** screen.

For more information, see *Plateau Properties Dialog Box*.

Modifying Ramps

When a plateau is added, ramps leading to and from the new plateau are automatically added. Likewise, when a segment or plateau is deleted, the associated ramps are deleted or modified as necessary to connect adjacent plateaus.

A data collection marker may be added to the ramp by using the **Ramp Properties** dialog box or by dragging the appropriate collection marker  from the command panel to the ramp on the **Thermal Profile** screen.

For more information, see *Ramp Properties Dialog Box*.

Deleting a Plateau or Segment

A plateau or segment can be deleted by selecting the plateau or segment and then clicking the **Delete** button in the **Selection** section of the command panel. If a plateau is deleted, the associated ramps are also deleted or modified as necessary to connect adjacent plateaus.

Modifying Data Collection Settings

Data collection events are shown on the **Thermal Profile** screen as either **Endpoint**  or **All points**  **data collection markers.** A data collection marker may be added to any ramp or plateau, by dragging the appropriate collection marker from the command panel to the plateau on the **Thermal Profile** screen. See *Data Collection* for a detailed description of data collection options.

Adding Comments

Information about the thermal profile can be entered in the **Thermal profile comments** text box at the lower right-hand corner of the screen. The comments are saved as part of the experiment. Note that the **Thermal profile comments** are distinct from any comments entered on the **Plate Setup** screen.

Displaying a Full-Screen Thermal Profile

If the profile becomes too wide to fit on the screen, a horizontal scroll bar will appear below the profile. The entire profile can be seen on the screen by clicking on the **Full Screen Profile** button in the lower right-hand corner of the screen. In full-screen view, the profile cannot be edited. To edit the profile, press the **Return to Thermal Profile** button in the lower right-hand corner.

Starting the Run

When finished setting up the **Thermal Profile**, click on the **Start Run** button at the lower right-hand corner of the screen. This will save the experiment and start the run. If the experiment has not already been saved, the **Save As** dialog box will appear.

Thermal Profiles for Individual Experiment Types

Additional information on **Thermal Profile Setup** specific to the individual experiment types is found in the individual experiment sections:

Quantitative PCR Thermal Profile Setup

Comparative Quantitation Thermal Profile Setup

SYBR Green Thermal Profile Setup

Allele Discrimination/SNP's Real-Time Thermal Profile Setup

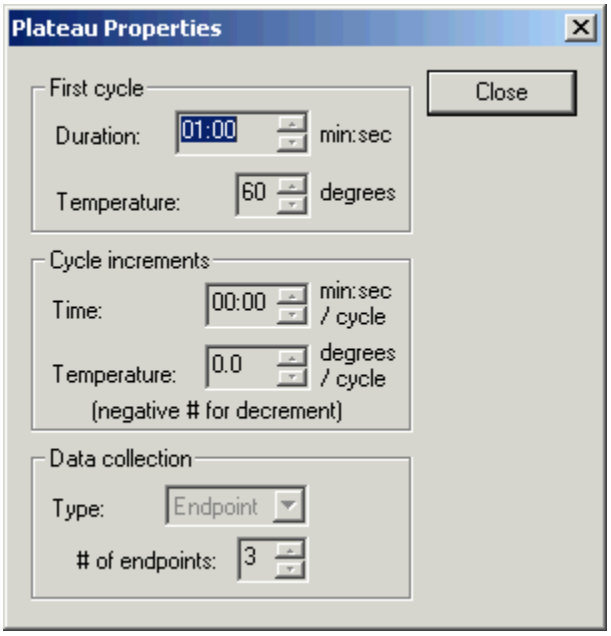
Molecular Beacons Melting Curve Thermal Profile Setup

Plate read experiments do not use a thermal profile; the temperature is constant during the read. See Running a Plate-Read Experiment.

Plateau Properties Dialog Box

The **Plateau Properties** dialog box is used to modify the properties of a plateau, including temperature, duration, and data collection. To access this dialog box, do one of the following:

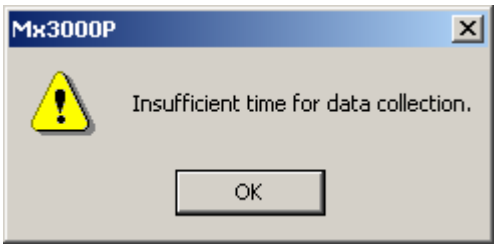
- Double-click on the plateau.
- Select the plateau. Go to the **Selection** section of the command panel and click **Edit Properties**.



Temperature and Duration (First Cycle)

Set the **Temperature** and **Duration** of the first cycle in the **First cycle** section of the dialog box. The values listed under **First cycle** will be used for all cycles unless the **Cycle increments** default null values are modified. **Duration** and **Temperature** values may be typed directly or may be selected by using the spin controls.

When data are collected for a plateau, the plateau has a minimum duration based on the time required for data collection. The amount of time required for data collection depends both on the configuration of wells from which data are being collected and on the number of data collection points specified. If the duration specified for the plateau is not sufficient to allow the specified data collection routine, a dialog box similar to the one shown below will appear. Adjust either the plateau **Duration** or the **Data collection** settings accordingly.



Cycle Increments

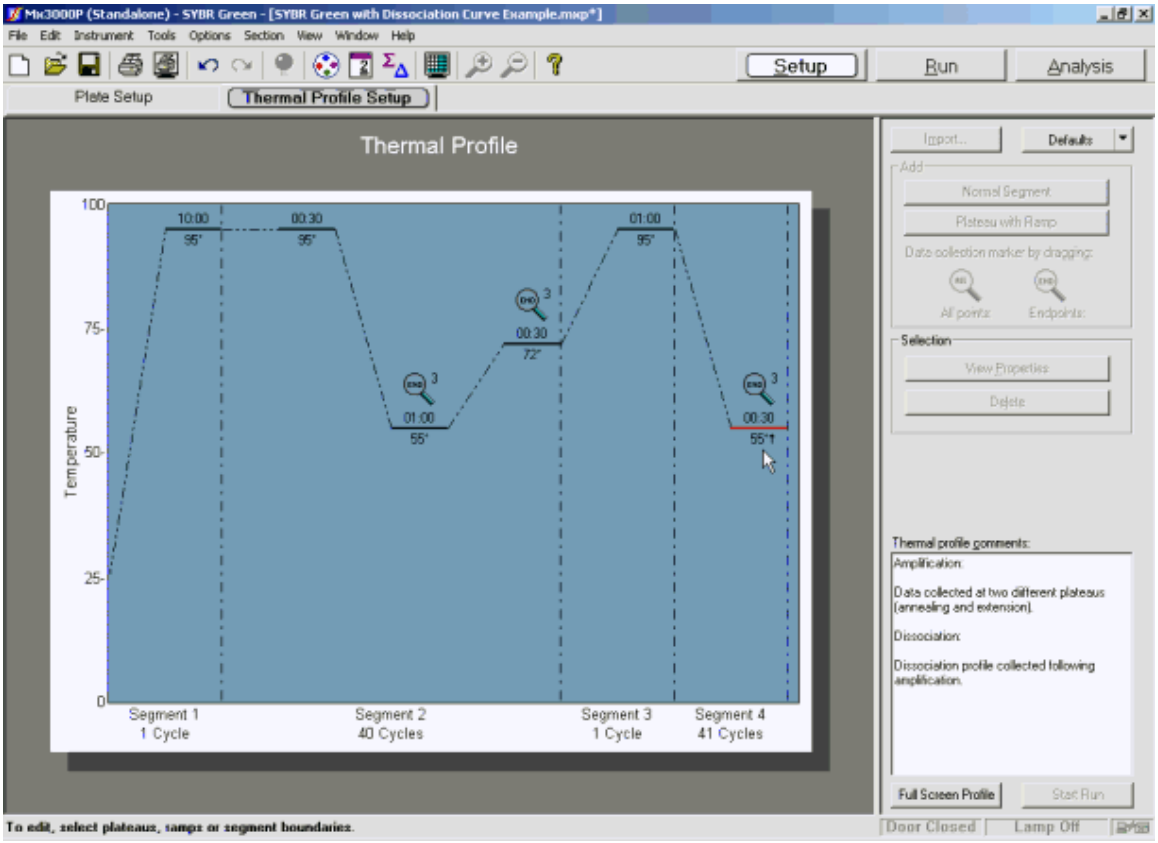
The **Cycle Increments** controls allow you to set the instrument to incrementally increase or decrease the temperature or duration of a plateau with each cycle within the segment.

Setting **Time** to a positive number will cause the duration of the plateau to increase by the specified amount of time with each cycle. Setting **Time** to a negative number will cause the plateau duration to be shortened by the specified amount of time each cycle.

Similarly, setting **Temperature** to a positive number will cause the temperature to increase by the specified number of degrees with each cycle and setting the **Temperature** to a negative number will cause the temperature to decrease by the specified number of degrees with each cycle. This setting can be adjusted in 0.1-degree increments.

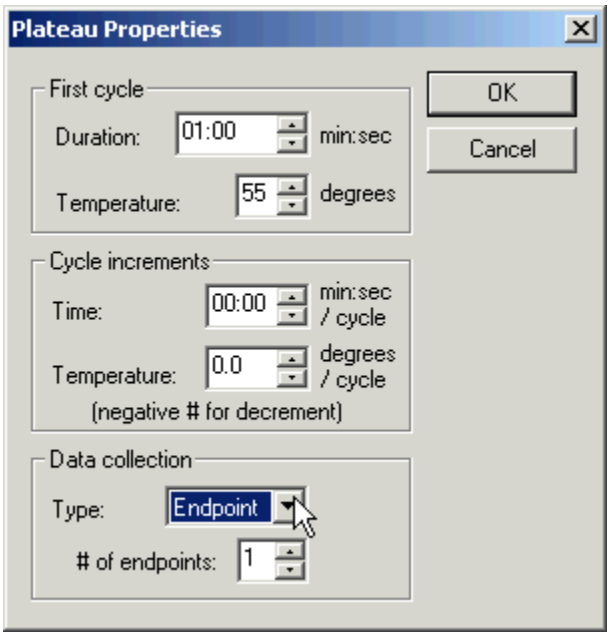
Example of Thermal Profile using Cycle Increments

An important use of cycle increments is in performing a dissociation curve. A thermal profile for a **SYBR Green** experiment is shown below and includes a **Dissociation** segment that is set up with a 1.0 degree **Cycle increment**. On the **Thermal Profile**, the incremental change in plateau temperature is indicated by the upward-arrow adjacent to the plateau temperature text. In this segment of this experiment, the temperature of the plateau in the first cycle will be 55°C and then will increase by 1.0°C at every cycle.



Data Collection Settings in Plateau Properties

In the **Plateau Properties** dialog box, each plateau will have one of the following **Data collection** settings: **None, Endpoints** or **All Points.**



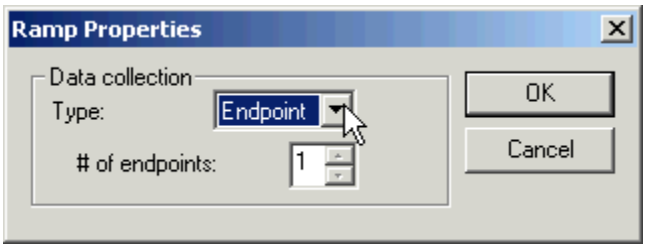
To set the instrument to collect data for the plateau, select either **Endpoints** or **All Points** from the **Type** menu. Selecting **Endpoints** will enable the **#of endpoints** selection box. Type or select the number of reads to be taken at the end of the plateau period in each cycle.

See the *Data Collection* section for a detailed discussion of how the **Data collection** settings options affect data collection and analysis.

Ramp Properties Dialog Box

The **Ramp Properties** dialog box is used to modify the properties of a ramp, including whether and how data are collected during the ramp. To access this dialog box from the **Thermal Profile** screen, do one of the following:

- Double-click on the ramp.
- Select the ramp. Go to the **Selection** section of the command panel and click **Edit Properties**.



Data Collection Settings in Ramp Properties

In the **Ramp Properties** dialog box, each ramp will have one of the following **Data collection** settings: **None, Endpoints** or **All Points.**

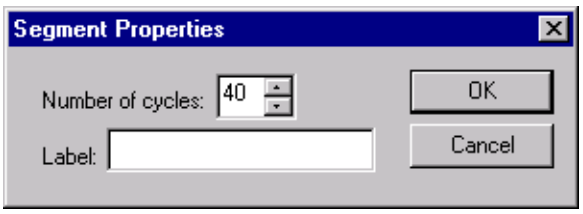
To set the instrument to collect data for the ramp, select either **Endpoints** or **All Points** from the **Type** menu. Selecting **Endpoints** will enable the **#of endpoints** selection box. Type or select the number of reads to be taken at the end of the ramping period in each cycle.

See the *Data Collection* section for a detailed discussion of how the **Data collection** settings options affect data collection and analysis.

Segment Properties Dialog Box

The **Segment Properties** dialog box is used to modify the properties of a **segment**. To access this dialog box, do one of the following:

- Double-click on the segment (but not directly on a ramp or plateau).
- Select the plateau. Go to the **Selection** section of the command panel and click **Edit Properties**.



Number of Cycles

Enter or select the number of times that the segment should be repeated (cycled) in the **Number of cycles** box. The acceptable range is 1–255.

Label

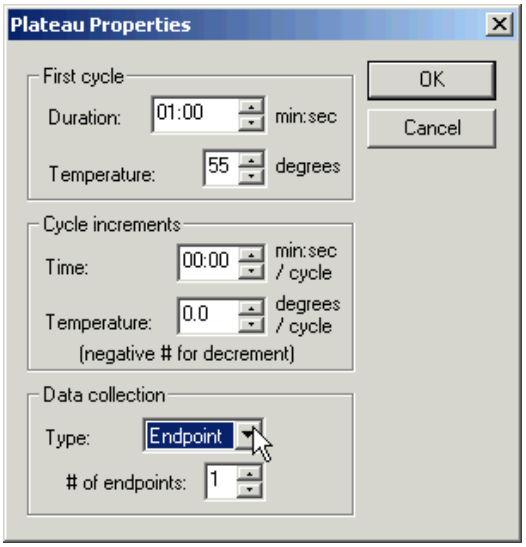
To add a descriptive label to the segment, which will appear below the number of cycles on the **Thermal Profile**, type the desired text in the **Label** box. The maximum length is 22 characters.

Data Collection

The points at which data should be collected by the Mx3000P instrument during thermal cycling are specified on the **Thermal Profile**. Two types of data collection are available: **Endpoints** or **All Points**.

Data collection markers  show the points designated for data collection on the **Thermal Profile** display.

In the **Plateau Properties** or **Ramp Properties** dialog box, each plateau or ramp will have a **Data collection** setting of **None, Endpoint** or **All Points** in the **Type** menu.



Endpoint Data Collection

Endpoint data collection causes the system to take readings at the end of a plateau or ramp. Setting the number of data collection points (the range is 1–31) specifies how many data points will be collected. The system calculates the time necessary to read the plate the specified number of times and then begins reading when that amount of time remains in the plateau or ramp. For example, if reading a plate takes 7 seconds and the specified number of data collection points is 3, the system will begin collecting data when 21 seconds remain in the plateau or ramp. How the multiple endpoint readings are handled during analysis is specified using the **Advanced Algorithm Settings** dialog box during **Analysis Selection/Setup**.

To include an **Endpoint** data collection step in a ramp or plateau, in the **Plateau Properties** or **Ramp Properties** dialog box (accessed by double-clicking the ramp or plateau) select **Endpoint** from the **Type** menu. Enter the desired number of data collection points in the **# of endpoints** spin box.

Identical results are achieved on the **Thermal Profile** display screen by dragging an **Endpoints data collection marker**  from the command panel to the desired **plateau** or **ramp**. Once the marker is in place, the number of collection points appears at the upper right of the marker, and can be changed by clicking on the number to open a spin control.

All Points Data Collection

All Points data collection causes the system to take readings as often as possible during a plateau or ramp. How the multiple readings for each cycle are handled during analysis is specified using the **Advanced Algorithm Settings** dialog box during **Analysis Selection/Setup**.

To include an **All Points** data collection step in a ramp or plateau, in the **Plateau Properties** or **Ramp Properties** dialog box (accessed by double-clicking the ramp or plateau) select **All Points** from the **Type** menu. Identical results are achieved on the **Thermal Profile** display screen by dragging an **All points data collection marker**  from the panel on right-hand side of the screen to the desired **plateau** or **ramp**.

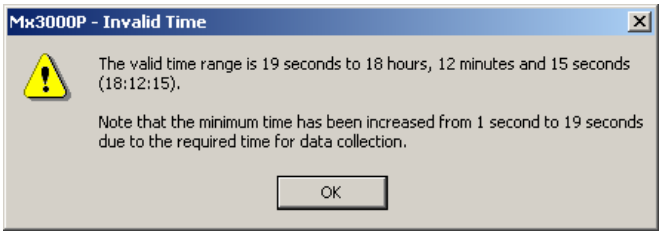
No Data Collection

In the **Plateau Properties** or **Ramp Properties** dialog box (accessed by double-clicking the ramp or plateau) a setting of **None** in the **Type** menu causes no data to be collected during the plateau or ramp. This is the default setting for new plateaus or ramps added to a thermal profile.

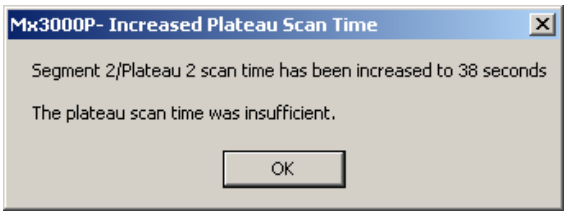
Plateaus or ramps that currently have data collection settings of **Endpoint** or **All Points** may be changed to **None** by selecting **None** from the **Type** menu or by dragging the appropriate **data collection marker** off of the **Thermal Profile** display area.

Data Collection-Based Constraints on the Thermal Profile

A plateau has a minimum duration needed to acquire data. If the duration specified is not sufficient for the indicated data collection on the plateau, a dialog box similar to the one shown below will appear. Adjust either the plateau **Duration** or the **Data collection** settings accordingly.



The plateau minimum duration for acquiring data varies based on the **Plate Setup** configuration. The configuration determines the minimum duration by specifying the number of well rows collected and the amount of data collected per well. Consequently, if changes are made to the **Plate Setup** such that the duration of a plateau becomes insufficient to allow the specified data collection settings, a dialog box similar to the one shown below will appear upon exiting the **Plate Setup** screen. The plateau duration will automatically be increased to the minimum required time.

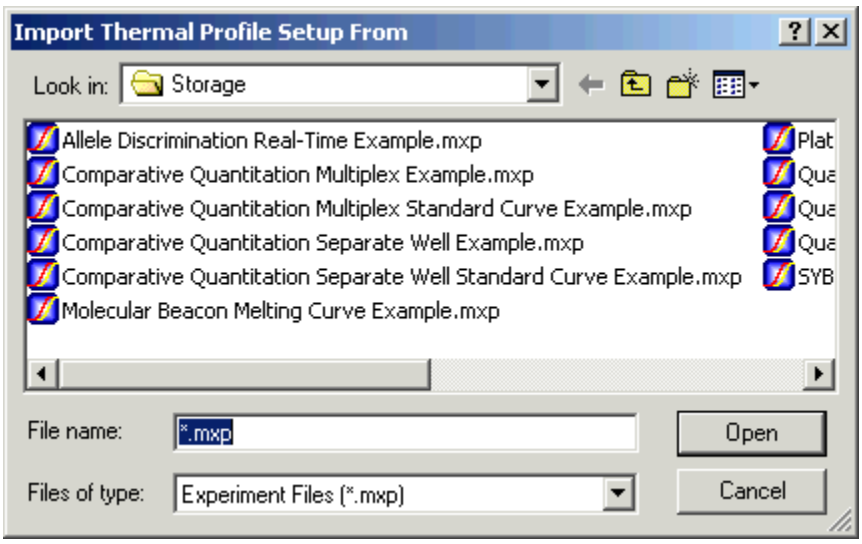


Using Import Thermal Profile Setup

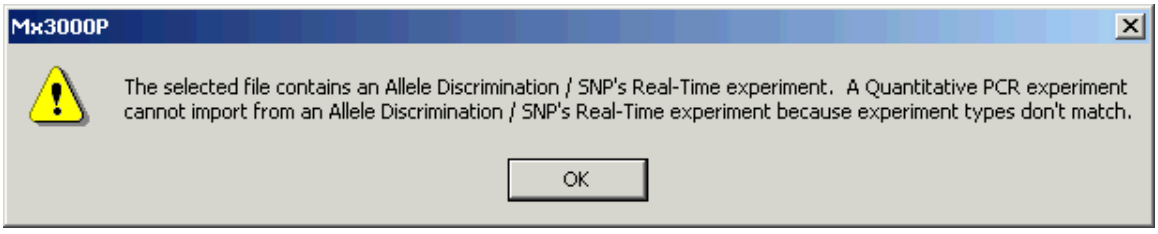
The **Import** function of **Thermal Profile Setup** allows you to recall the thermal profile from an existing experiment when setting up a new experiment.

To use an existing thermal profile for the new experiment, click the **Import** button on the command panel. **The Import Thermal Profile Setup From** dialog box will appear.

This dialog box lists Mx3000P experiments stored in the Mx3000P data storage folder. Select the experiment that includes the desired thermal profile setup information, and then click the **Open** button.



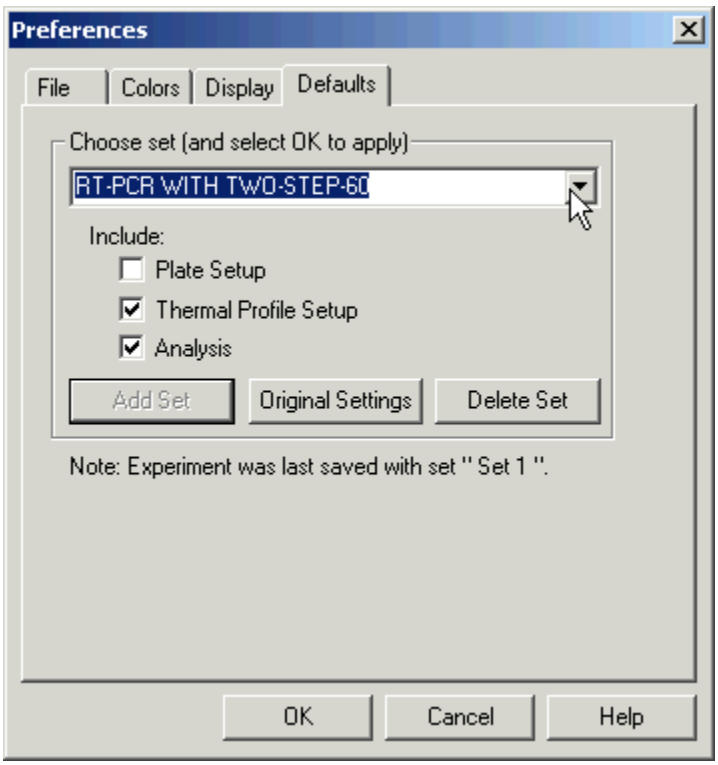
If the experiment you selected is not compatible with the experiment being set up, a warning box will appear. If this warning appears, click the **OK** button and select another experiment of the same type as the one being set up.



Using a Default Thermal Profile Setup

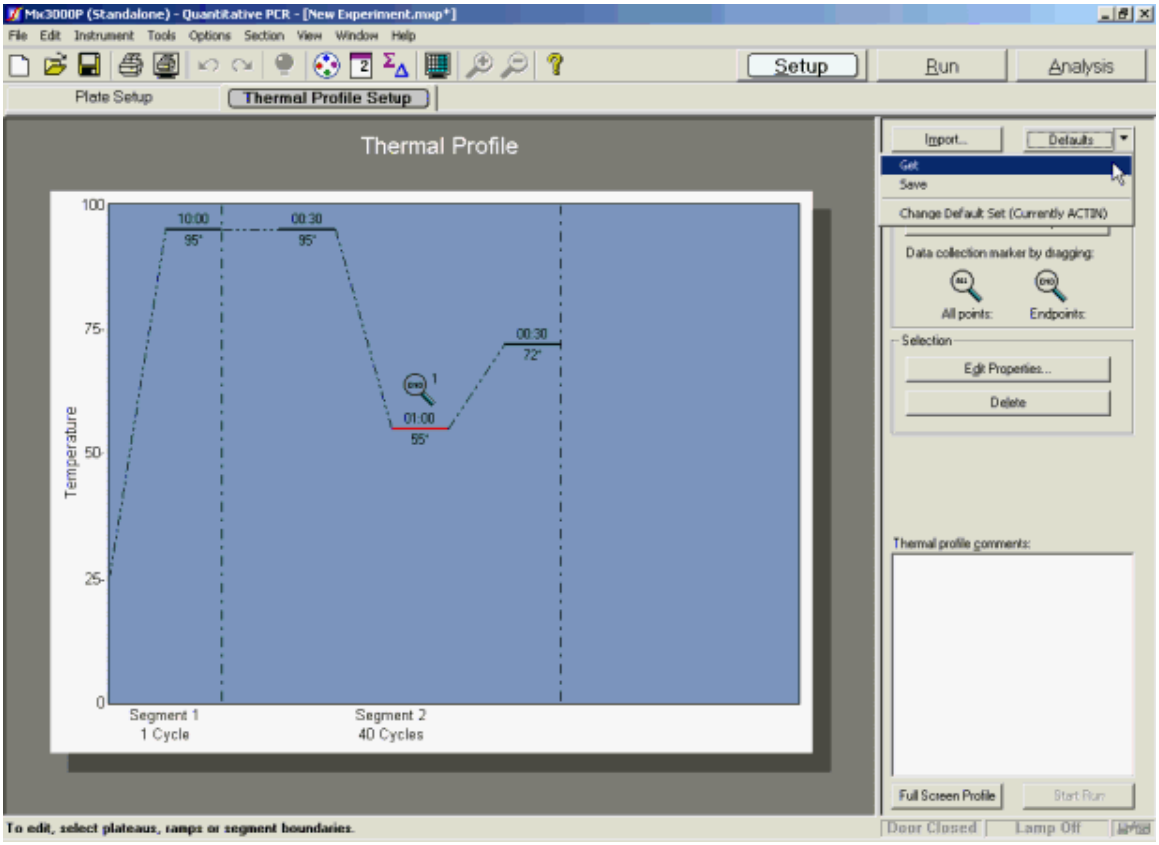
In addition to the pre-configured default thermal profiles supplied by the Mx3000P software (as part of the **Original Settings**), user-defined default thermal profiles may be created and retrieved for setting up new experiments.

Using a user-defined default thermal profile for **Thermal Profile Setup** requires creating a default set containing the desired thermal profile. Once the appropriate default set is created, select this default set as the active default set in the **Choose set** menu of the **Preferences** dialog box and then click **OK**. The thermal profile stored in the active default set will automatically be applied to the open experiment and to all new experiments, until the active default set is changed again.



The thermal profile in an existing default set may also be applied to the thermal profile setup in an open experiment by using the **Defaults** menu on the **Thermal Profile Setup** screen. To retrieve the thermal profile from the active defaults set, click **Defaults** to expand the menu, and then select **Get**.

See *Preferences-Defaults* for more information about creating and using default sets to streamline experimental setup and analysis.

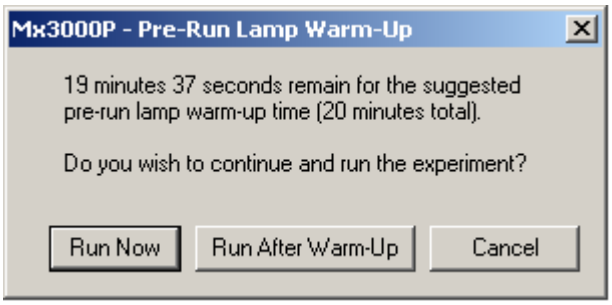


How to Run a Real-Time Experiment

Preparing for the Run

Either open an existing experiment or set up a new experiment. Complete both Plate Setup and Thermal Profile Setup steps. In preparation for running the experiment, verify that the lamp is ready. Place the samples in the instrument and close the instrument door. The indicators at the bottom of the screen should appear as shown below.

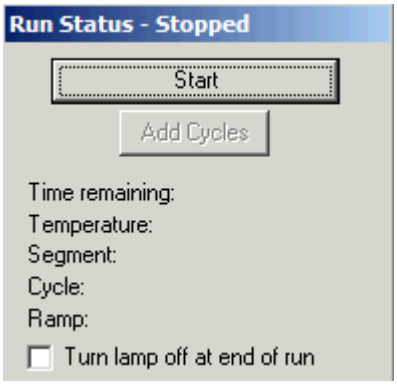
The warm up time for the lamp is 20 minutes. Stratagene recommends that no data be taken during this period. If a run is started before the warm-up time is complete, a dialog box appears displaying the remaining warm-up time. Click **Run After Warm-Up** to automatically start the run at the end of the warm-up period. Click **Run Now** to proceed with the experiment before the lamp is warmed-up (not recommended).



Starting the Run

Initiate the run from the **Thermal Profile Setup** screen by clicking the **Start Run** button.

The **Run Status** dialog box will appear. If you want to **Turn the lamp off at end of run** select the checkbox at the bottom of the dialog box. Click **Start** to begin the run.

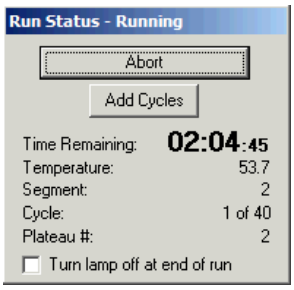


Note that a run can only be started once for any given experiment to ensure that data are not mistakenly overwritten.

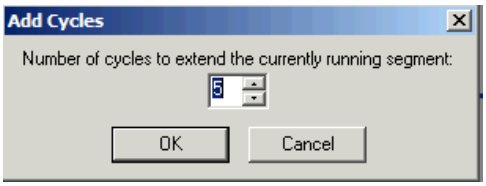
Monitoring the Run

Run Status Dialog Box

The **Run Status** dialog box is displayed throughout the run, reporting the **Time remaining** in the run, the **Temperature** (estimated current temperature of the sample reactions within the tubes) and the current cycling status of the run (the **Segment**, **Cycle**, and **Plateau** in progress). The dialog box may be moved to a different position on the screen by dragging the box from the title bar.

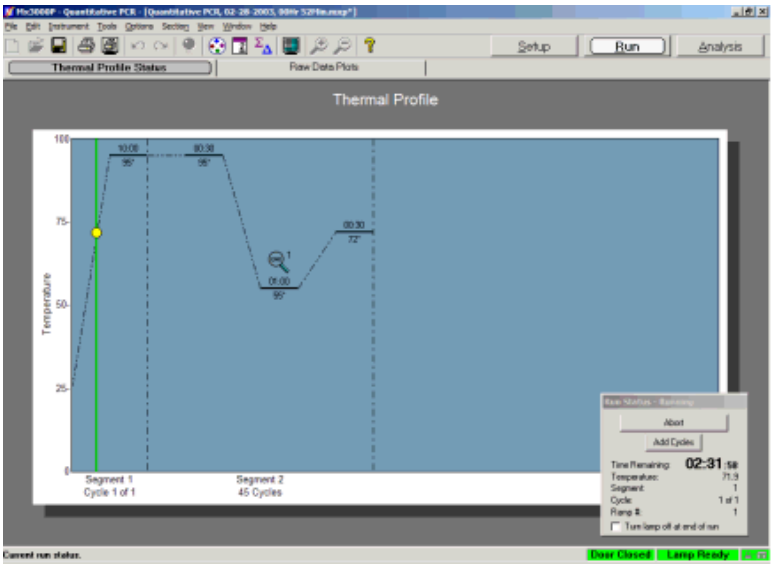


If desired, additional cycles may be added to the currently-running segment of the thermal profile by clicking the **Add Cycles** button. The **Add Cycles** dialog box will appear. Use the spin controls to specify the number of cycles to be added to the currently-running segment and then click **OK**.



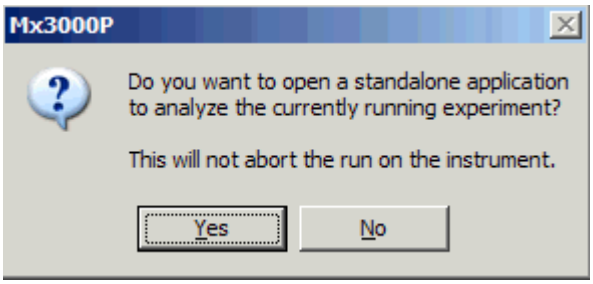
Thermal Profile Status

By default, the **Thermal Profile Status** screen is displayed during the run to provide a graphical representation of the progress of the thermal cycling. (The view may also be switched to **Raw Data Plots** during the run.)



Analyzing Data During the Run

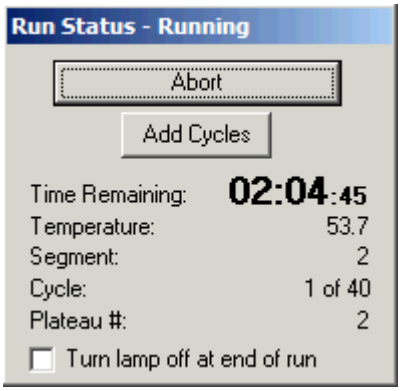
To analyze data during a run, click the **Analysis** tab. The following dialog box will appear.



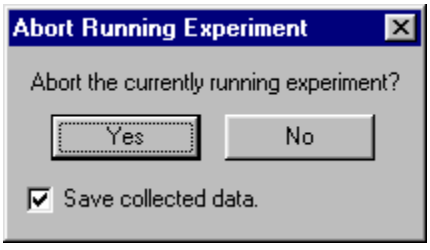
If you choose to open a standalone Mx3000P application, a second copy that does not have communications with the instrument, the data collected from the beginning of the run to that point will be available for analysis. Unless you abort the run, data collection will continue and the complete data set will be saved at the end of the experiment.

Aborting the Run

To stop a running experiment, click **Abort** in the **Run Status** dialog box.



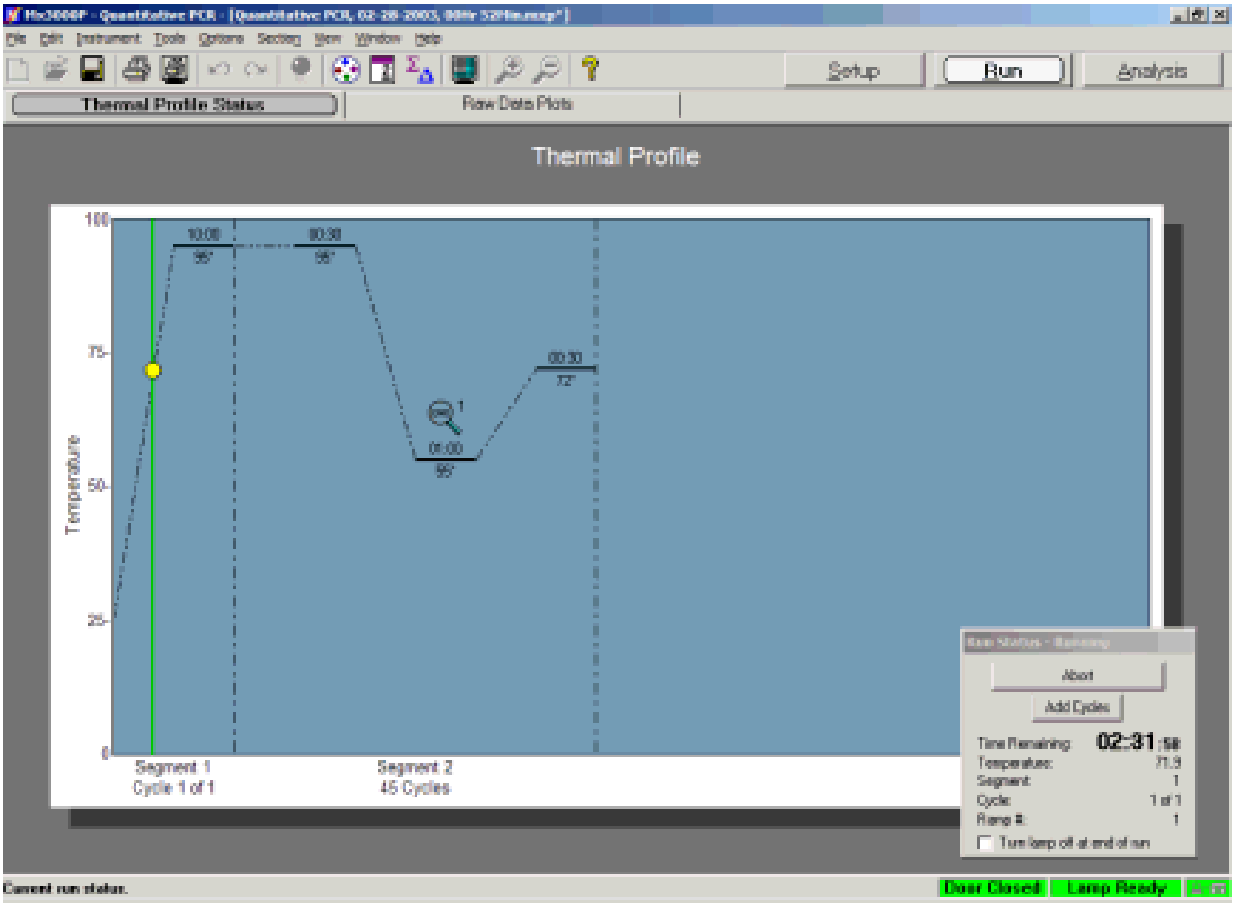
The **Abort Running Experiment** dialog box will appear. Click **Yes** to stop the experiment. To save the already-collected raw data from the run, select the **Save collected data** checkbox before clicking **Yes**. To allow the run to continue, click **No**.



If the real-time experiment is aborted very early, then in some cases it may be possible to begin again, cycling the samples and generating useful data. In many cases, however, the samples will no longer be useful for generating data. For example, if some rounds of amplification have already occurred when a run is aborted, it is not possible to generate legitimate standard curves or determine initial template quantities from the samples in a new run.

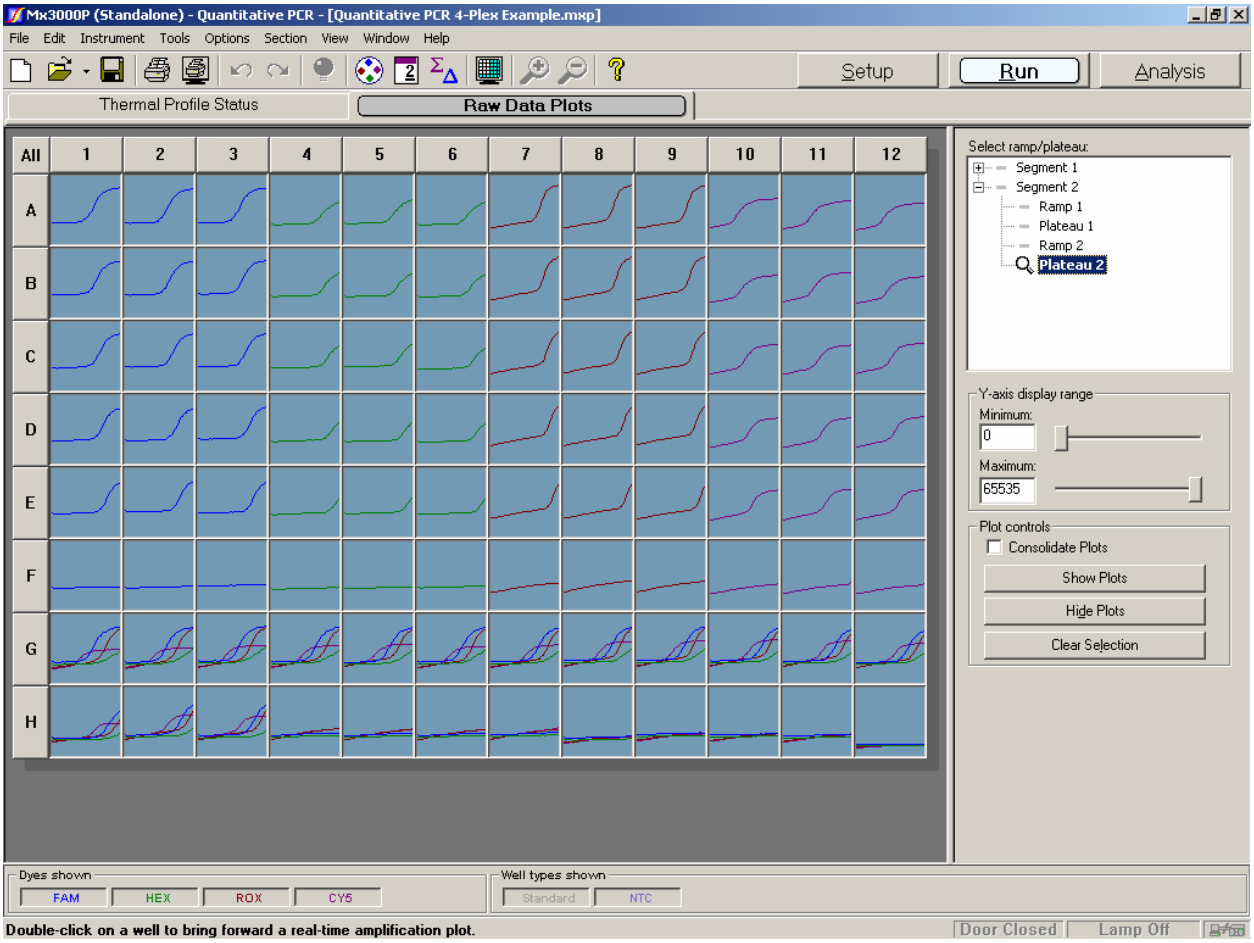
Viewing the Thermal Profile Status

By default, the **Thermal Profile Status** screen is displayed during the run to provide a graphical representation of the progress of the thermal cycling. During the run, a yellow dot and green status line are displayed on the thermal profile image to indicate the current status of the run with respect to the profile. If, instead, you want to monitor product accumulation during the run, click the **Raw Data Plots** tab. Likewise, clicking the **Thermal Profile Status** tab returns the view to the **Thermal Profile Status** screen.



Viewing Raw Data Plots

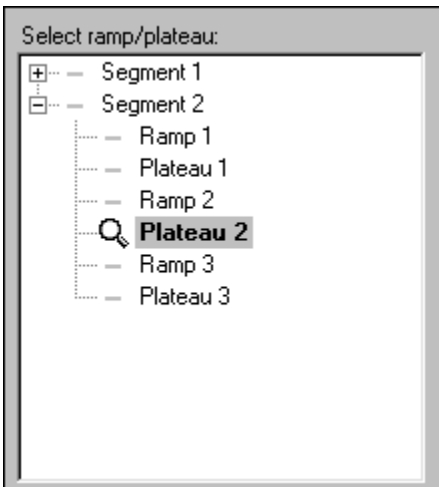
During the run, raw data plots can be viewed to give an immediate indication of product accumulation. To view the full-screen raw data amplification plots while the thermal profile is running, click on the **Raw Data Plots** tab. (Raw data plots may also be viewed after the run by clicking the **Run** section button, and then clicking the **Raw Data Plots** tab.) The plots shown are raw fluorescence (R) on the Y-axis versus cycle number on the X-axis. For a more detailed view of the plot in an individual well, double-click on the well to open the well's expanded amplification plot.



Display Options for Raw Data Plots

Selecting the Ramp or Plateau to Display

After the run, it is possible to view raw data amplification plots for each ramp and plateau for which data was collected. Select the ramp or plateau of interest from the list under **Select ramp/plateau**. A magnifying glass indicates that the ramp or plateau has had data collected; a gray dash indicates that the ramp or plateau was not selected for collection, so there is no data to display.



Displaying or Hiding Dyes

The dyes in use on the plate may be displayed or hidden by using the **Dyes shown** selection bar at the lower left-hand corner of the screen. When the button is depressed (like **FAM** in the picture below), the dye's well assignments are displayed, but when the button is released (like **ROX** in the picture below), the dye's well assignments are hidden from display. **Note that using this feature does not affect data collection**.

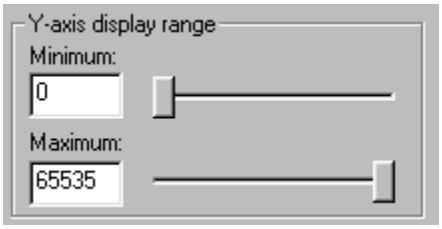
Displaying or Hiding Well Type Assignments

The well types that are displayed on the plate are controlled by using the buttons in the **Well Types Shown** selection bar. Any of the well type buttons may be depressed in order to display the information for wells of the indicated type or may released to hide the information. For example, if you want to view only the **Unknown** wells on the plate, depress the **Unknown** button and release all other well type buttons (such as the **NTC** button in the example below). **Note that using this feature does not affect data collection.**

Setting the Range of the Y-axis

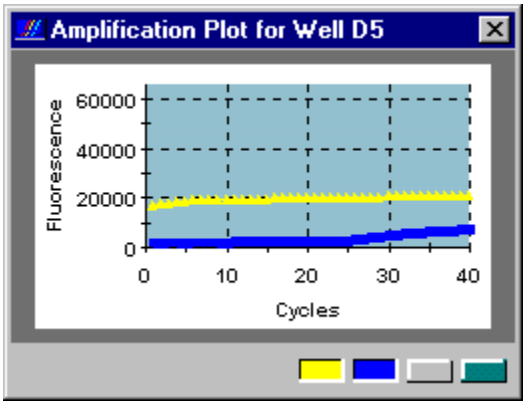
The minimum and maximum Y-axis values for the plots can be set using the **Y-axis display range** slider controls on the command panel. By adjusting the Y-axis range, the scale can be expanded or contracted to show better differentiation between curves. The minimum value is 0 and the maximum value is 65535.

The X-axis value is fixed to include the number of cycles that are being collected.



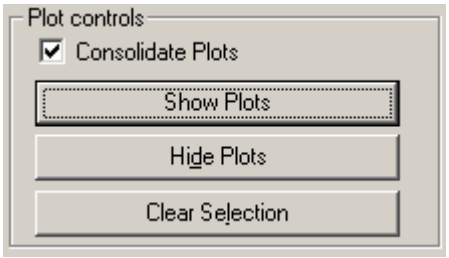
Viewing Expanded Amplification Plots (Real-Time Experiments)

When viewing **Raw Data Plots** for a real-time experiment, an individual well's plot can be expanded for a clearer picture of product accumulation in that well. To expand a well's plot, double-click on the well. The plot will appear as shown:

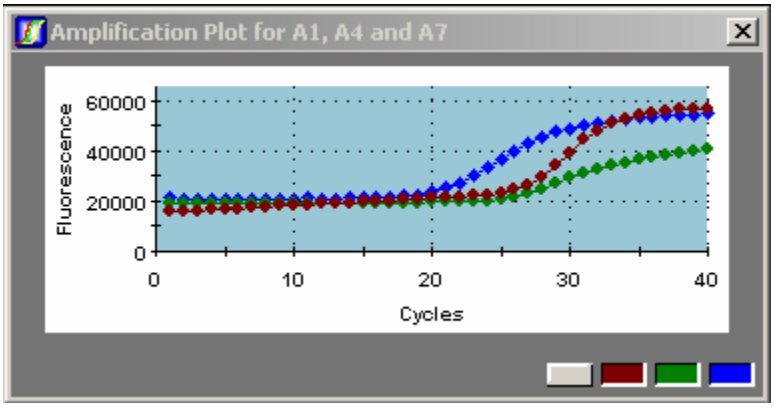


Display Options for Expanded Amplification Plots

Multiple wells can be shown (up to a maximum of 9 wells) by selecting a group of wells and then clicking on the **Show Plots** button on the command panel.



When **Consolidate Plots** is selected, the selected plots appear together in a single window.



When **Consolidate Plots** is cleared, the selected plots appear in individual windows, in a tiled format by default. To change the arrangement of plots to a cascade format, select **Cascade Plots** from the **Window** menu.

A plot can be resized by dragging any side or corner with the cursor.

A plot can be moved by pressing and holding the left mouse button down in the title bar of the plot box and dragging the plot to a different location.

Using the panel of colored buttons below the plot, an individual dye can be shown in the plot (by depressing the corresponding color button) or hidden (by releasing the corresponding color button).

The Y-axis range displayed for the expanded plots can be adjusted using the **Y-axis display range** slider controls on the command panel. The minimum value is 0 and the maximum value is 65535.

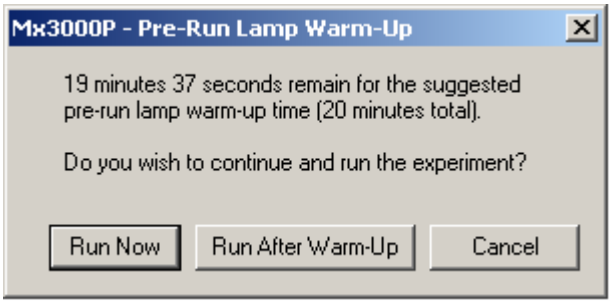
To hide a single displayed plot, click the plot's **Close** button . To hide all plots, click **Hide Plots** on the command panel. Clicking **Clear Selection** will clear (deselect) any highlighted wells.

How to Run a Plate-Read Experiment

Preparing for the Run

Either open an existing experiment or set up a new experiment. Complete the Plate Setup. In preparation for running the experiment, verify that the lamp is ready. Place the samples in the instrument and close the instrument door. The indicators at the bottom of the screen should appear as shown below.

The warm up time for the lamp is 20 minutes. Stratagene recommends that no data be taken during this period. If a run is started before the warm-up time is complete, a dialog box appears displaying the remaining warm-up time. Click **Run After Warm-Up** to automatically start the run at the end of the warm-up period. Click **Run Now** to proceed with the experiment before the lamp is warmed-up (not recommended).

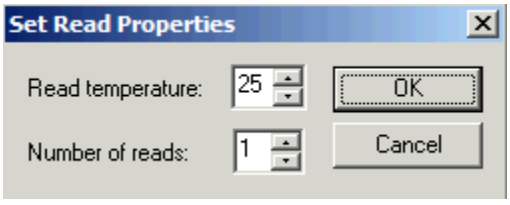


Starting the Run

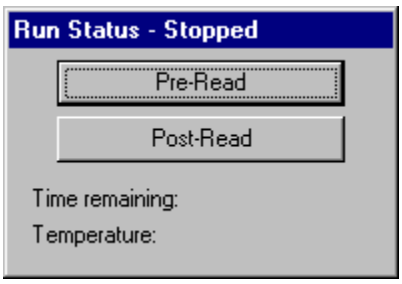
Starting a plate-read run requires the following steps, each of which are detailed below:

1. Set the **Read Properties** (temperature and number of reads)
2. Start the run using the **Start Run** button on the **Plate Setup** screen
3. Initiate data collection and specify whether the run is a Pre-read or Post-Read in the Run Status dialog box

Step 1: Specify the read properties by clicking the **Set Read Properties** button on the **Plate Setup** screen. The **Set Read Properties** dialog box opens, allowing you to set the temperature and number of readings to be taken.



Step 2: Click the **Start Run** button at the bottom of the **Plate Setup** screen. The **Run Status** dialog box opens when the **Start Run** button is clicked, allowing you to initiate the plate read run.



Step 3: Click either the **Pre-Read** or the **Post-Read** button in the **Run Status** dialog box to initiate data collection by the instrument.

A plate read run can be completed as either a one-time read or a pre- and post-read sequence.

One-time Plate Read Experiments

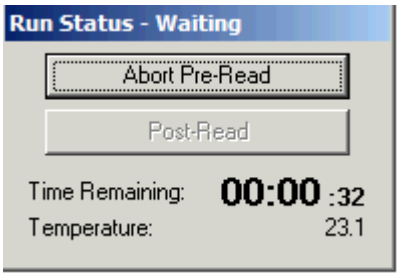
One-time plate readings may be initiated by clicking either the **Pre-Read** or the **Post-Read** button in the **Run Status** dialog box. Note that once the run is complete, fluorescence data type selections on **Results** screens are affected by the choice made in the **Run Status** dialog box. If the run was initiated using the **Pre-Read** button, select fluorescence data types of **Rpre** or **Rn,pre** on **Results** screens. If the run was initiated using the **Post-Read** button, select fluorescence data types of **Rpost** or **Rn,post** on **Results** screens.

Pre-Read and Post-Read Sequence Experiments

To run a pre-read/post-read sequence, start the pre-read segment by clicking the **Pre-Read** button. When the read is complete, exit and save the experiment. To run the post-read segment for the same samples after cycling, open the saved experiment (containing the pre-read data), click **Start Run** from the **Plate Setup** screen, and then click the **Post-Read** button in the **Run Status** dialog box.

Monitoring the Run

The **Run Status** dialog box is displayed throughout the run, reporting the **Time remaining** in the run and the **Temperature** (estimated current temperature of the sample reactions within the tubes).

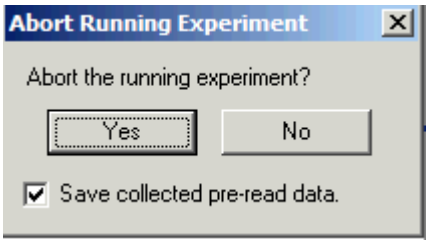


During the run, the **Raw Data Plots** screen is also displayed, showing readings for each well while data collection occurs.

Aborting the Run

To stop a running experiment, click **Abort Pre-Read** or **Abort Post-Read** in the **Run Status** dialog box.

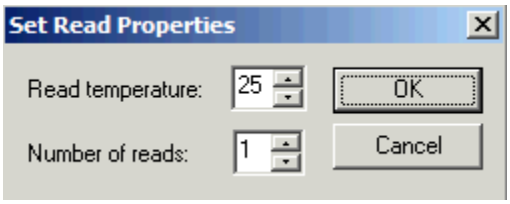
The **Abort Running Experiment** dialog box will appear. Click **Yes** to stop the experiment. To save the already-collected raw data from the run, place a check mark in the box next to **Save collected data** before clicking **Yes**. To allow the run to continue, click **No**.



Note that, for plate read experiments, if a run is aborted the read can generally be taken again.

Setting the Read Properties for a Plate-Read Experiment

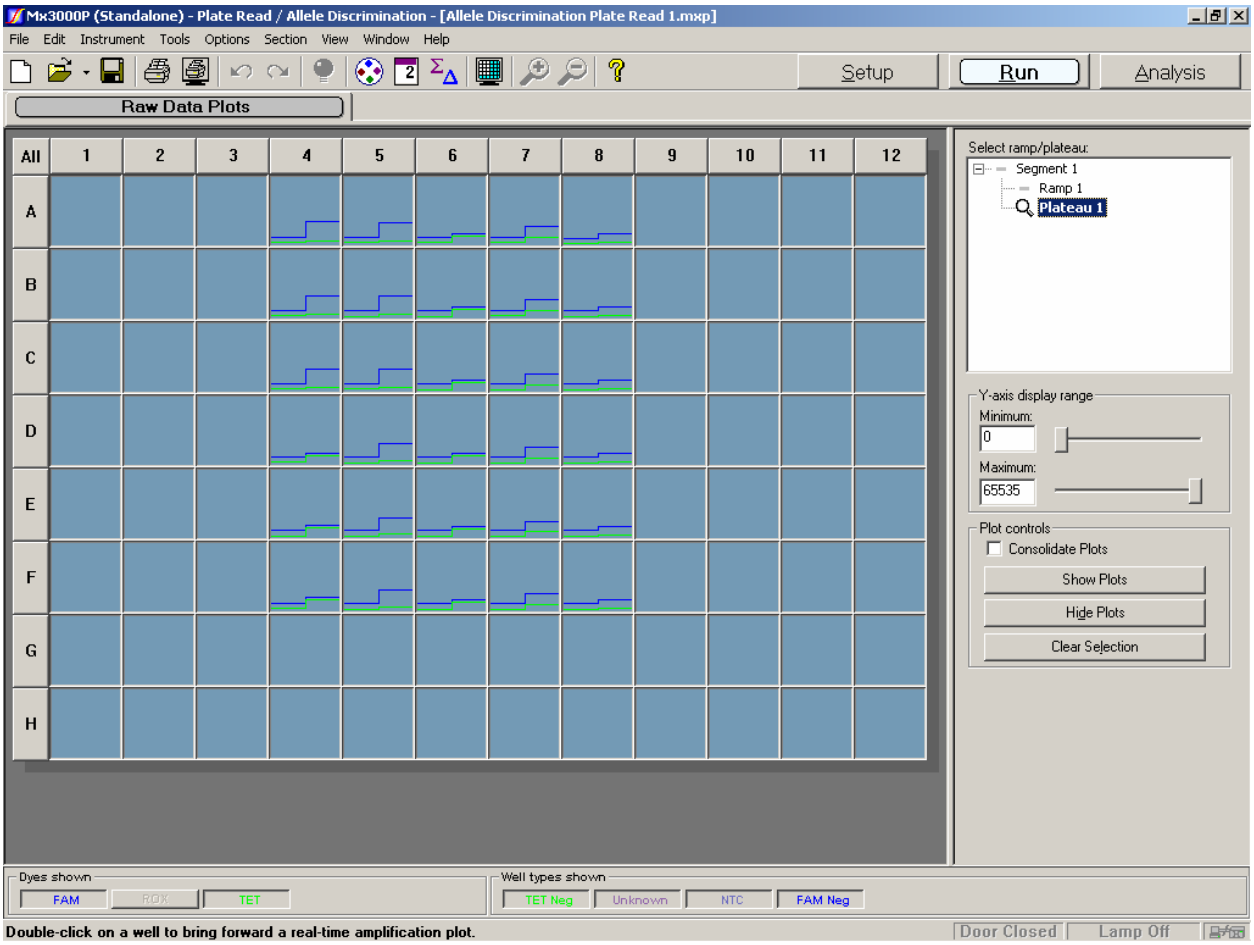
To access the **Set Read Properties** dialog box, click the **Set Read Properties** button on the command panel of the plate-read experiment **Plate Setup** screen.



Set the temperature (25–99°C) at which the samples should be held during the run by using the **Read temperature** spin controls. Set the number of times for the plate to be read by using the **Number of reads** spin controls. If the run includes multiple reads, you can specify whether the data should be reported as the average, the minimum value, the maximum value, or the endpoint of the read series by clicking the **Advanced Algorithm Settings** button on the **Analysis Selection/Setup** screen.

Viewing Raw Data Plots for Plate-Read Experiments

During the run, the **Raw Data Plots** are displayed to give immediate indication of fluorescence readings for the samples. (Raw data plots may also be viewed after the run by clicking the **Run** section button, which opens to the **Raw Data Plots** screen.) The plots shown are bar graphs of raw fluorescence (R) on the Y-axis versus pre-read and/or post-read data collection points on the X-axis. For a more detailed view of the plot in an individual well, double-click on the well to open an expanded plot for that well.



Display Options for Raw Data Plots

Displaying or Hiding Dyes

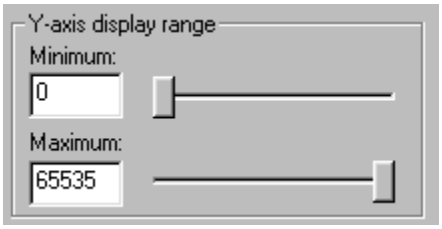
The dyes in use on the plate may be displayed or hidden by using the **Dyes shown** selection bar at the lower left-hand corner of the screen. When the button is depressed (like **FAM** in the picture below), the dye's well assignments are displayed, but when the button is released (like **ROX** in the picture below), the dye's well assignments are hidden from display. **Note that using this feature does not affect data collection**.

Displaying or Hiding Well Type Assignments

The well types that are displayed on the plate are controlled by using the buttons in the **Well Types Shown** selection bar. Any of the well type buttons may be depressed in order to display the information for wells of the indicated type or may released to hide the information. For example, if you want to view only the **Unknown** wells on the plate, depress the **Unknown** button and release all other well type buttons (such as the **NTC** button in the example below). **Note that using this feature does not affect data collection.**

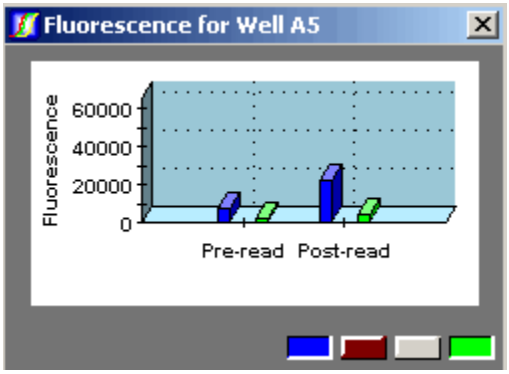
Setting the Range of the Y-axis

The minimum and maximum Y-axis values for the plots can be set using the **Y-axis display range** slider controls on the command panel. By adjusting the Y-axis range, the scale can be expanded or contracted to a scale more appropriate to the sample/dye of interest. The minimum value is 0 and the maximum value is 65535.



Viewing Expanded Raw Data Plots for Plate-Read Experiments

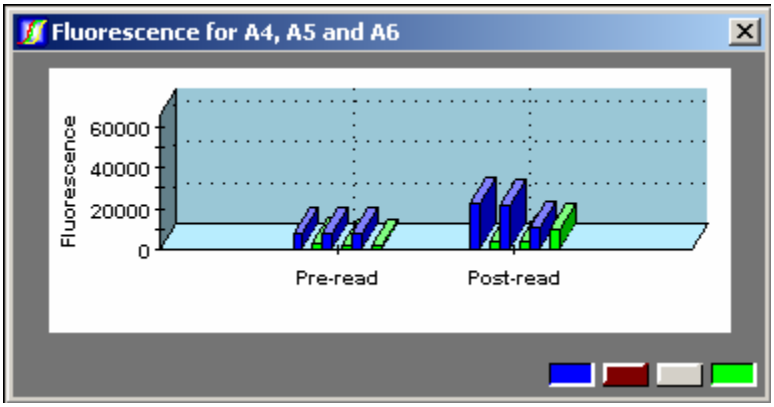
When viewing **Raw Data Plots** for a plate read experiment, an individual well's plot can be expanded for a clearer picture of fluorescence readings in that well. To expand a well's plot, double-click on the well. The plot will appear as shown:



Display Options for Expanded Plots

Multiple wells can be shown (up to a maximum of 9 wells) by selecting a group of wells and then clicking on the **Show Plots** button on the command panel.

When **Consolidate Plots** is selected, the selected plots appear together in a single window.



When **Consolidate Plots** is cleared, the selected plots appear in individual windows, in a tiled format by default. To change the arrangement of plots to a cascade format, select **Cascade Plots** from the **Window** menu.

A plot can be resized by dragging any side or corner with the cursor.

A plot can be moved by pressing and holding the left mouse button down in the title bar of the plot box and dragging the plot to a different location.

Using the panel of colored buttons below the plot, an individual dye can be shown in the plot (by depressing the corresponding color button) or hidden (by releasing the corresponding color button).

The Y-axis range displayed for the expanded plots can be adjusted using the **Y-axis display range** slider controls on the command panel. The minimum value is 0 and the maximum value is 65535.

To hide a single displayed plot, click the plot's **Close** button . To hide all plots, click **Hide Plots** on the command panel. Clicking **Clear Selection** will clear (deselect) any highlighted wells.

How to Analyze the Data

Analyzing the Data

To begin data analysis for an open experiment, click the **Analysis** tab or select **Analysis** from the **Section** menu.

The **Analysis** section includes the **Analysis Selection/Setup** screen, and a series of different **Results** screens that are appropriate to the type of experiment.

For general information on how to use the **Analysis** section of the Mx3000P software, see the following topics:

Analysis Selection and Setup

Treatment of Replicates

Positive and Negative Calling

Graphs and Graph Properties

Adjusting the Threshold Fluorescence Manually

More detailed information about the specialized aspects of **Analysis** for each of the experiment types is available in the experiment-specific sections:

Quantitative PCR

Comparative Quantitation

SYBR Green

Allele Discrimination/SNP's Real-Time

Molecular Beacon Melting Curve

Quantitative Plate Read

Plate Read/Allele Discrimination

Analysis Selection/Setup

The first step in data analysis is to use the **Analysis Selection/Setup** screen to make selections and provide settings that will be used for data analysis. These include:

- Selecting the wells for which collected data should be analyzed
- Choosing the ramp or plateau for which the collected data will be analyzed
- Specifying the settings that determine how data are analyzed

Note that if you wish to use the default analysis settings, you need only select the wells to analyze, choose the data collection ramp or plateau, and click on the **Results** tab. The specific default settings that are in place when the **Analysis Selection/Setup** screen is opened are determined by the contents of the active default set.

Selecting the Wells to Analyze

Note *Only those dyes and wells selected in the* **Analysis Selection/Setup** *screen are used by the analysis algorithms.*

To select an individual well, single click in that well.

An entire row or column of wells can be selected by clicking on the corresponding **row header** (A–H) or **column header** (1–12). All the wells in the plate can be selected by clicking on the **All** button in the upper left-hand corner of the plate.

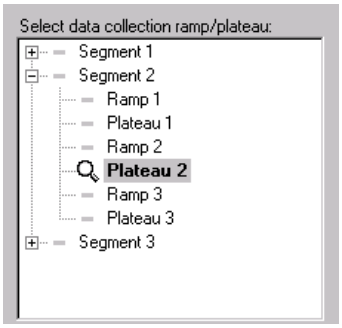
To select a range of adjacent wells, click and hold the left mouse button down and drag the cursor across the wells to be selected. A visible marking rectangle will appear. When all of the required wells are included in the rectangle, release the left mouse button and the range of wells will be selected.

To select a group of non-contiguous wells, hold down the **Control** key and click on each of the wells.

To deselect a well, hold down the **Control** key and click on the well to be deselected. To deselect a selected row or column, click on that row or column's header. To rapidly deselect all wells, click the **All** button twice. This will select and then deselect all wells.

Choosing the Data Collection Ramp or Plateau

Data may be viewed for any ramp or plateau that data was collected on. On the command panel, the **Select data collection ramp/plateau** box shows a summary of the thermal profile, with the data collection points indicated with the collection marker icon. (A gray dash indicates that the ramp or plateau was not selected for collection, so there is no data to display.) To choose a ramp or plateau for data analysis select it in the **Select data collection ramp/plateau** box.

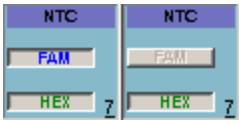


For **SYBR Green** and **Comparative Quantitation** data analysis, it is necessary to select data collection points to be used in two parts of the profile: one data set for the **Dissociation curve** (set by clicking the **Dissociation** button) and another data set for the rest of the data analysis options (set by clicking the **Amplification** button).

Excluding Individual Dyes from Analysis (Optional)

Exclude Dyes allows you to exclude individual dyes in specific wells from analysis.

Clicking the **Exclude Dyes** button will change the display of the wells to include independent buttons for each dye in each well, as shown below.



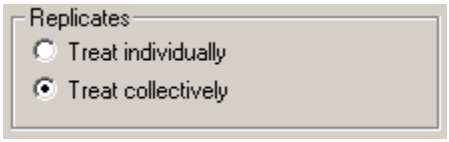
A dye button which is depressed (with colored lettering) signifies that data collected for that dye in that well will be used in analysis algorithms. A raised button (with grayed lettering) signifies that the well/dye data will not be included in the analysis. When the **Exclude Dyes** view is first accessed in an experiment, all buttons are depressed. To exclude a specific dye in a specific well from analysis, release the corresponding dye button in the appropriate well.

When all dye exclusions have been specified, click the **Exclude Dyes** button again to return to the normal **Analysis Selection/Setup** view.

Specifying Data Analysis Settings

Replicates

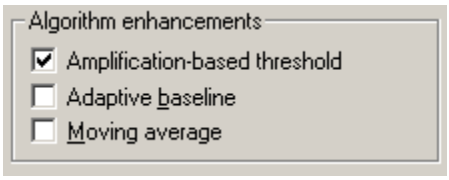
Wells identified as replicates in **Plate Setup** may be treated either individually or collectively during **Analysis**. Selecting **Treat Individually** under **Replicates** will cause the software to analyze each well independently and display or report results for each well separately. Selecting **Treat Collectively** will cause the software to average the data from all wells that make up a replicate set and to report a single result for the replicate set, effectively treating the measurements as if they came from the same well. See *Treatment of Replicates* in the *How-To (Detailed Protocols)* section for more details on how this selection affects the reporting of results.



Algorithm Enhancements

The **Algorithm enhancements** settings are used to specify whether or not the listed algorithm enhancements should be applied in the current round of analysis. **Algorithm enhancements** are not used in plate read or molecular beacon melting curve experiments.

Amplification-Based Threshold

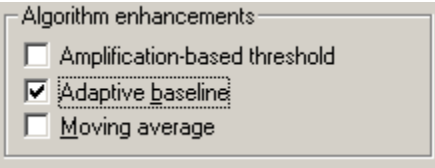


The **Amplification-based threshold** enhancement automatically calculates the threshold fluorescence for each dye, based on the amplification properties of the reactions in the specific experiment. See *Analysis Term Settings-Real Time-Threshold Fluorescence* for more information. To use this enhancement, select the **Amplification-based threshold** check box in the **Algorithm enhancements** section. The settings that affect this calculation may be viewed or modified in the **Analysis Term Settings-Threshold Fluorescence** dialog box.

Baseline-based threshold fluorescence is used for analysis when the **Amplification-based threshold** check box is cleared. This threshold calculation algorithm uses user-defined settings to determine the threshold fluorescence based on the background fluorescence in the experiment. The settings that affect this calculation may be viewed or modified in the **Analysis Term Settings-Threshold Fluorescence** dialog box.

The threshold fluorescence value may also be manually set or adjusted.

Adaptive Baseline

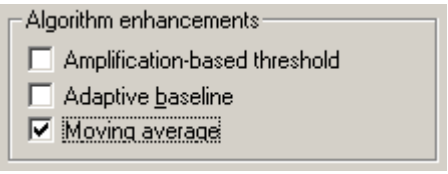


The **Adaptive baseline** enhancement automatically calculates the best baseline for each plot individually, thereby providing the most accurate Ct. **Adaptive baseline** is activated by checking the **Adaptive baseline** check box in the **Algorithm enhancements** section.

When uniform range baseline calculations are used for analysis (by clearing the **Adaptive baseline** check box), the range of cycles delimited for background correction is, by default, identical for all amplification plots. The starting and ending cycles for calculating the baseline function are specified in the **Active Settings** section of the **Analysis Term Settings-Baseline Correction** dialog box.

After using either baseline correction algorithm, the start and end cycle values can be adjusted manually for one or more amplification plots by using the **Resulting Cycle Range** section of the **Analysis Term Settings-Baseline Correction** dialog box.

Moving Average



Moving average applies curve-smoothing to help reduce **Ct** variation by decreasing the effect of signal noise. When this enhancement is selected, the fluorescence value for each data point in the amplification curve represents the average of the values for a specified range of cycles (original software settings=3 cycles) surrounding a given point.

To perform data analysis employing a moving average calculation, select the **Moving average** check box. The number of points averaged during **Moving average** curve-smoothing can be modified in the **Analysis Term Settings-Moving Average** dialog box.

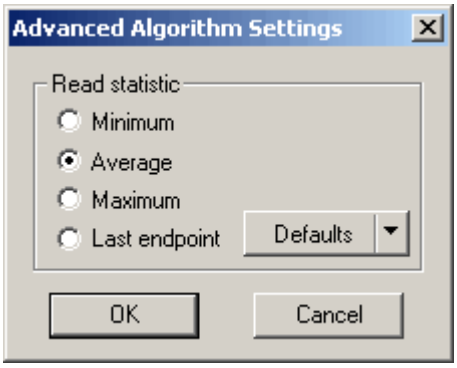
Applying a **Moving average** calculation reduces the resolution of the reported results. Using **Moving average** curve-smoothing also increases the Ct values for a given threshold fluorescence.

Analysis Term Settings

The **Analysis Term Settings** button opens the **Analysis Term Settings** dialog box, allowing adjustment of a variety of settings that influence the way data are analyzed. Most of the basic settings controlled by the **Analysis Term Settings** dialog box may be selected or deselected in the **Replicates** and **Algorithm Enhancements** sections of the **Analysis Selection/Setup** control panel. However, the **Analysis Term Settings** button provides convenient access to the dialog box in order to view or modify the parameters used in the calculations for a given setting selection.

Advanced Algorithm Settings

The **Adv. Algorithm Settings** button opens a dialog box that displays the **Read statistic** radio button selection box.



The Mx3000P instrument may be set up to take more than one fluorescence reading for each reported measurement at each cycle in a real-time experiment or at each plate read. The number of readings per collection point is specified during **Thermal Profile Setup** for real-time experiments and using the **Set Read Properties** dialog box for plate read experiments. The **Read statistic** setting allows specification of how the multiple data readings should be analyzed.

Selecting **Minimum** will cause the software to use the lowest value collected for each well and dye combination in each cycle or each plate read. Selecting **Average** will cause the software to use the average (mean) of the values collected for each well and dye combination in each cycle or each plate read. Selecting **Maximum** will cause the software to use the highest value collected for each well and dye combination in each cycle or each plate read. Selecting **Last endpoint** will cause the software to use the last data point collected each well and dye combination in each cycle or each plate read.

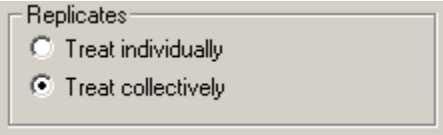
See *Preferences-Defaults* for information about the use of the **Defaults** menu and the application of defaults sets to the settings specified in this dialog box.

Adding Analysis Comments

The **Analysis comments** box allows you to enter comments to be saved with the experiment. When printing the **Analysis Selection/Setup** section, these comments will appear on the printout.

Treatment of Replicates

Replicate wells can be identified by assigning the same replicate symbol to a group of wells during **Plate Setup**. Wells that are identified as replicates can then be treated collectively during **Analysis**. When **Treat** (replicates) **collectively** is selected in the **Analysis Selection/Setup** screen, fluorescence values for all wells that are identified with the same replicate symbol are averaged, so that the results for all wells with the same replicate number will be identical. When **Treat** (replicates) **individually** is selected, fluorescence values for all wells are analyzed and reported independently, regardless of any replicate set assignments that had been made in **Plate Setup**.



Collective Treatment of Replicates in Real-Time Experiments

When the **Treat Collectively** option is selected in real-time experiments, fluorescence data from all wells with the same replicate number is averaged cycle-by-cycle. Hence, a single amplification plot or dissociation curve is produced for each replicate set. In addition, a single **Ct** value (and any other value derived from the fluorescence data) is reported for the replicate set on all of the analysis screens. These values are calculated from the averaged amplification plot.

Collective Treatment of Replicates in Plate Read Experiments

When the **Treat Collectively** option is selected in plate read experiments, the fluorescence data reading from all wells with the same replicate number is averaged for each dye. Hence, all members of the set will have the same value (the average value of all set members) reported on the **Plate Sample Values** screen and will have a single value reported in a **Text Report**.

On the **Final Call Results** screen, the averaged value for each **Unknown** replicate set is compared to the averaged value for the **Control** replicate set. If the average for an unknown set is statistically higher (based on p-values) than the average of the control wells, all members of the replicate set are called '+'. The user-specified confidence level in the **Analysis Term Settings** dialog box determines how high the **p-value** must be to yield a "+" final call.

Positive and Negative Calling

Real-Time Experiments

In real-time experiments, calling in **Final Call Results** is based on **Ct** values that are derived from either baseline-corrected raw fluorescence or baseline-corrected, normalized raw fluorescence. If the derived **Ct** is less than the last cycle for which data was collected, a positive call is returned. If the **Ct** is greater than or equal to the last cycle for which data was collected, a negative call is returned. A plus sign (**+**) signifies that product accumulation was detected, while a minus sign (**–**) signifies a failure to detect product accumulation.

Plate Read Experiments

In plate read experiments, calling in **Final Call Results** is based on comparing the experimentally determined **p-value** (probability value) for each dye in each well to the user-defined **Confidence level for calls**. The **Confidence level for calls** stipulates the minimum **p-value** required (the minimum confidence that amplification did occur in the well) for a positive call to be issued.

The **p-value** of a dye in a well is the probability that the amount of sample in that well differs from the amount of sample in the controls. For example, a **p-value** for an **Unknown** well of 99% means that 1% of the time, a measurement of a sample identical to the control wells will produce a value at least as great as the actual measurement collected for the **Unknown** well. In other words, the chance of a false positive is 1%. Thus, assuming that the only cause of false positives is measurement error, the confidence that amplification did occur is 99%.

Presuming the user-defined **Confidence level for calls** setting is 99%, a positive call (**+**) for an **Unknown** well means that at most 1% of the time a measurement of sample identical to the control wells will produce a value as great as the actual measurement collected for the **Unknown** well.

Conversely, a negative call (**–**) in an **Unknown** well (with a **Confidence level for calls** setting of 99%) means the chance that measurement of a sample identical to the control wells would produce a value at least as great as the actual measurement in the **Unknown** well is greater than 1%. Hence, there is less than 99% confidence that amplification occurred, assuming no other sources of experimental error.

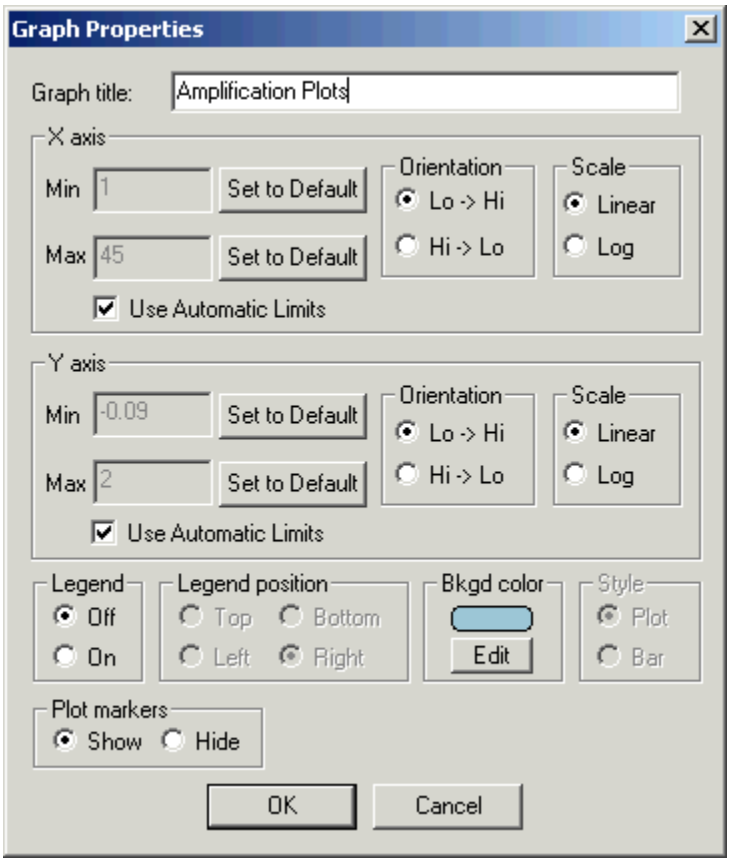
Set the confidence level for making final calls in the **Analysis Term Settings-Plate Read** dialog box.

The **Text Report** screen for plate-read experiments reports the **p-value** for each dye in each well, which is the probability that the actual measurement of the dye in the well differs from the measurements of the dye in the control wells. Other **Results** screens report only the **Final Call** (**+** or **–**).

Note *In order for the Mx3000P software to calculate p-values for positive or negative calling in* **Unknown** *wells, the plate read experiment must include at least three* **NTC** *or* **dye-specific negative control** *wells for each dye.*

Graphs and Graph Properties

The **Graph Properties** dialog box allows you to customize several of the features of graphs displayed on **Results** screens. To access this dialog box, double-click anywhere in the graph.



Graph Title

The **Graph Title** box shows the title displayed above the graph on the **Results** screen. To change the title displayed, type the desired title text in the box.

X-Axis and Y-Axis Controls

The **X-Axis** and **Y-Axis** sections of the dialog box allow you to customize the X- (horizontal) and Y- (vertical) axes of the graph. By default, the limits of both axes are determined automatically. To change the limits, clear the **Use Automatic Limits** check box, and then enter the desired **Min** and **Max** values for the axis. The **Orientation** and **Scale** of either axis may also be changed by using the radio buttons provided.

Background Color

To modify the background color for the graph, click **Edit** in the **Bkgd color** box. For Help with selecting or defining colors, see *Preferences-Color* in the *Options Menu* section.

Style

The **Style** radio buttons are available only from the **Relative Quantity Chart** screen of **Comparative Quantitation** experiments. When available, you can select a display graphic of either a **Bar** chart or a line **Plot** using the corresponding radio buttons.

Plot Markers

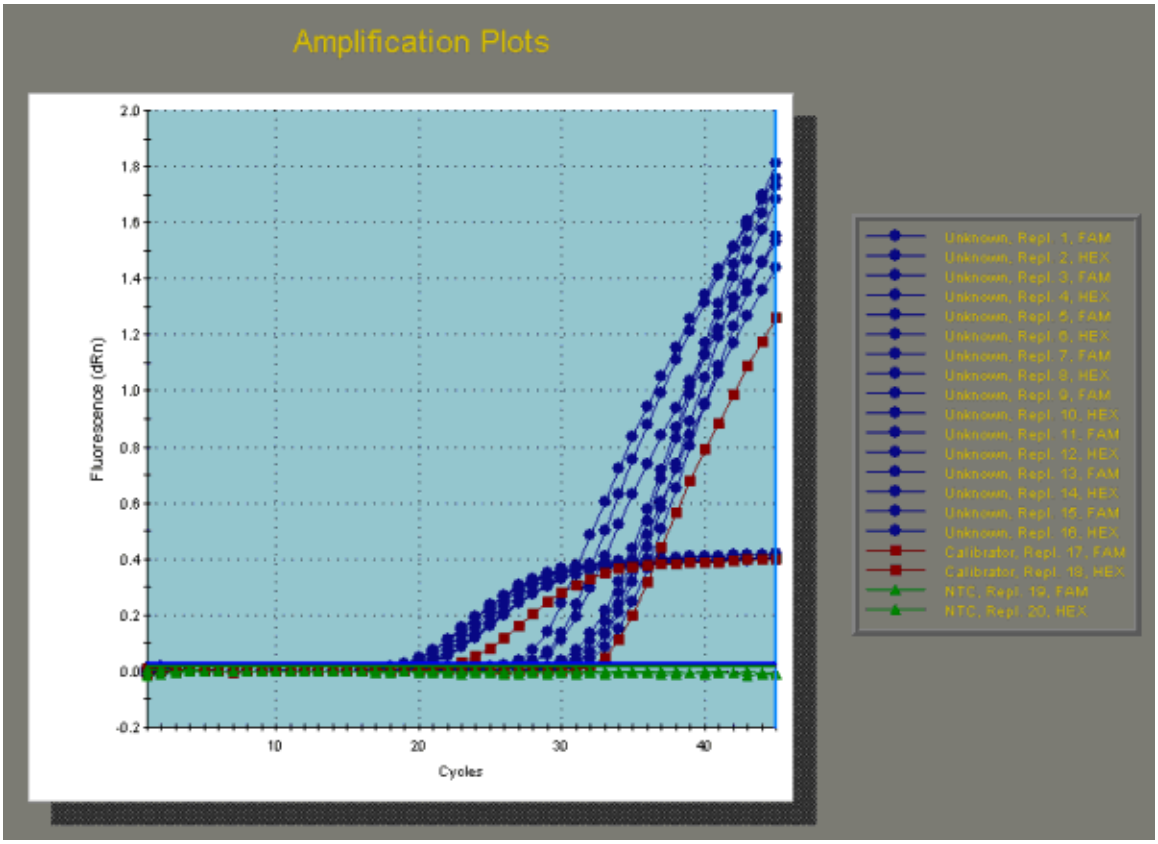
To view curves as solid lines without plot markers, select the **Hide** radio button under **Plot markers**. To restore the markers, select the **Show** radio button.

Legend

To show the legend for the graph, select the **On** radio button under **Legend**. Select the desired position of the legend, relative to the graph, by using the radio buttons under **Legend Position**. (If the legend is **Off**, **Legend Position** choices will not be available.)

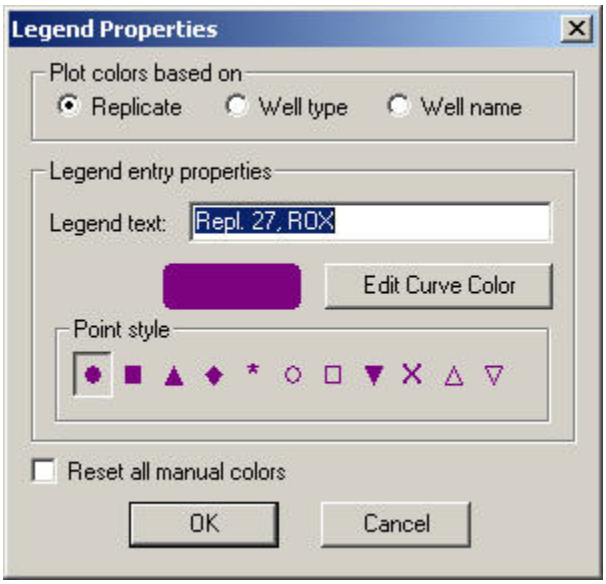
To hide the legend for the graph, select the **Off** radio button under **Legend**. This setting allows a full-sized display of the graph.

In the image below, the legend is turned **On** and positioned at **Right**. For information on changing the properties of elements listed in the legend, see *Legend Properties*, below.



Legend Properties

To modify the appearance of the elements listed in the graph legend, open the **Legend Properties** dialog box by double-clicking on an entry in the legend.



To change the basis used for assigning plot colors, use the **Plot colors based on** radio buttons. For example, if you want to distinguish between plots for different well types by color, select the **Well type** radio button. This will cause the curves for all wells with the same well type to be shown in the same color. In addition, making this selection will cause the **Well type** for each plot to be listed first in the identifying text in the legend.

To change the text used to identify the plot, enter the desired text in the **Legend text** box.

To change the curve color, click **Edit Curve Color.** The **Color** dialog box opens to allow specification of a new color.

To change the plot markers for the curve, select the desired marker shape under **Point style**.

To reset all curves to their original colors after color editing, select **Reset all manual colors** and then click **OK**.

Adjusting the Threshold Fluorescence Manually

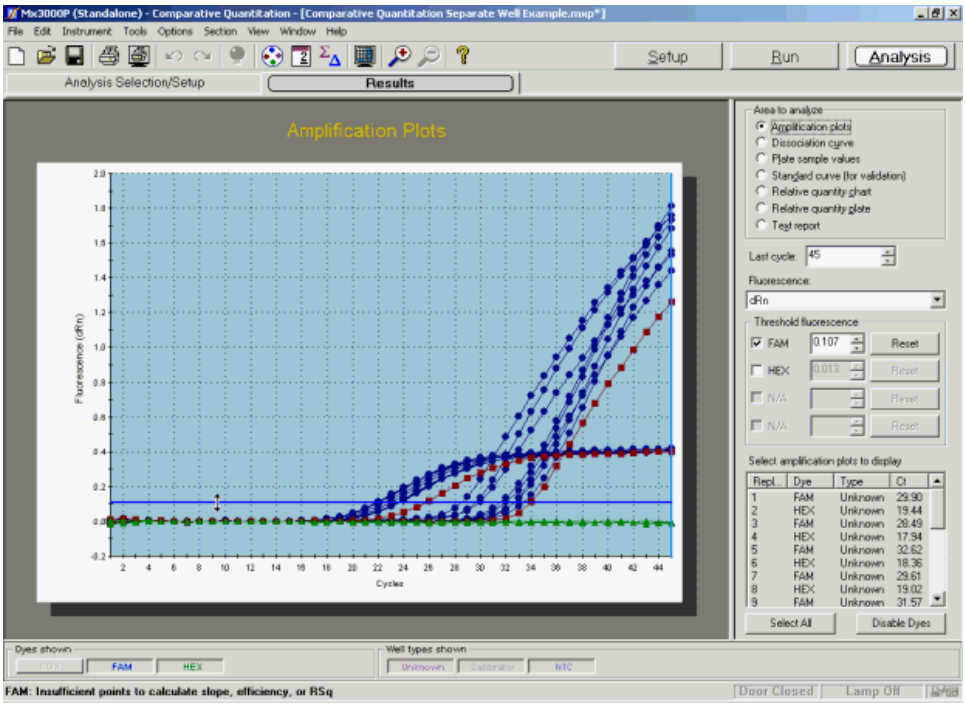
The threshold fluorescence level, used to derive Ct values, is automatically determined by the Mx3000P software using one of several algorithms based on selections made during **Analysis Selection/Setup**.

The Mx3000P software also allows you to manually select a threshold fluorescence value during **Analysis**. On an **Amplification Plot** screen, the **Threshold Fluorescence** value for each dye is listed on the command panel and is marked on the graph by a horizontal line. (Note that if a dye is hidden using the **Dyes shown** controls or if the check box next to the dye is cleared, the line corresponding to threshold fluorescence for that dye will not be shown on the graph.)

Either of the procedures listed below may be used to manually change the threshold fluorescence value used for Ct calculation:

- Position the mouse over the appropriate threshold fluorescence indicator line on the graph. The cursor becomes a vertical arrow. Click on the line, and holding down the left mouse button, drag the line to the desired position. The corresponding numerical value on the command panel will also change.
- Enter a new threshold fluorescence value in the box adjacent to the dye of interest in the command panel by typing the desired value or by using the spin controls to make incremental changes. The position of the corresponding horizontal line will change on the graph.

To reset the threshold fluorescence value for a dye to the value calculated automatically by the Mx3000P software, click the **Reset** button adjacent to the dye of interest. Threshold fluorescence for each dye is managed independently; to reset all dyes, click the **Reset** button for each dye.



Mx3000P System Experiments: Quantitative PCR

The Quantitative PCR Experiment Type

Note *The Mx3000P software includes the experiment type* **Quantitative PCR,** *which refers to a specific assay type in which fluorogenic oligonucleotide probes are used to quantitate target levels by way of a standard curve. Other specific experiments which fall under the general usage of the term* quantitative PCR, *using different detection chemistries or analysis methods, are featured in other experiment types.*

The **Quantitative PCR** experiment type uses a standard curve to accurately quantitate the amount of target present in **Unknown** samples using a fluorescence-labeled probe for detection. This method is sometimes referred to as absolute quantitation or as standard-curve quantitation in the literature.

In **Quantitative PCR** experiments, the Mx3000P instrument detects the fluorescence of one or more fluorophores during each cycle of the thermal cycling process and a **Fluorescence** value is reported for each fluorophore at each cycle. Fluorescence readings are generally acquired in the annealing stage of thermal cycling. Results are typically displayed in an **Amplification plot**, which reflects the change in fluorescence during cycling.

The initial copy numbers of RNA or DNA targets can be quantified using real-time PCR analysis based on threshold cycle (Ct) determinations. The Ct is defined as the cycle at which a statistically-significant increase in fluorescence (above background signal contributed by the fluorescence-labeled oligonucleotides within the PCR reaction) is detected. The threshold cycle is inversely proportional to the log of the initial copy number. In other words, the more template that is present initially, the fewer the number of cycles required for the fluorescence signal to be detectable above background. The Mx3000P offers both automatic and manual methods for determination of the **Threshold fluorescence** level that is used to determine **Ct** values.

Typical **Quantitative PCR** experiments use a **Standard curve** to quantitate the amount of target present in an **Unknown** sample. In this method, a series of **Standards**, containing a dilution series of a known amount of target, are amplified to generate a curve that relates the initial quantity of the specific target in the **Standard** sample to the Ct. The standard curve is then used to derive the **Initial template quantity** in **Unknown** wells based on their **Ct** values.

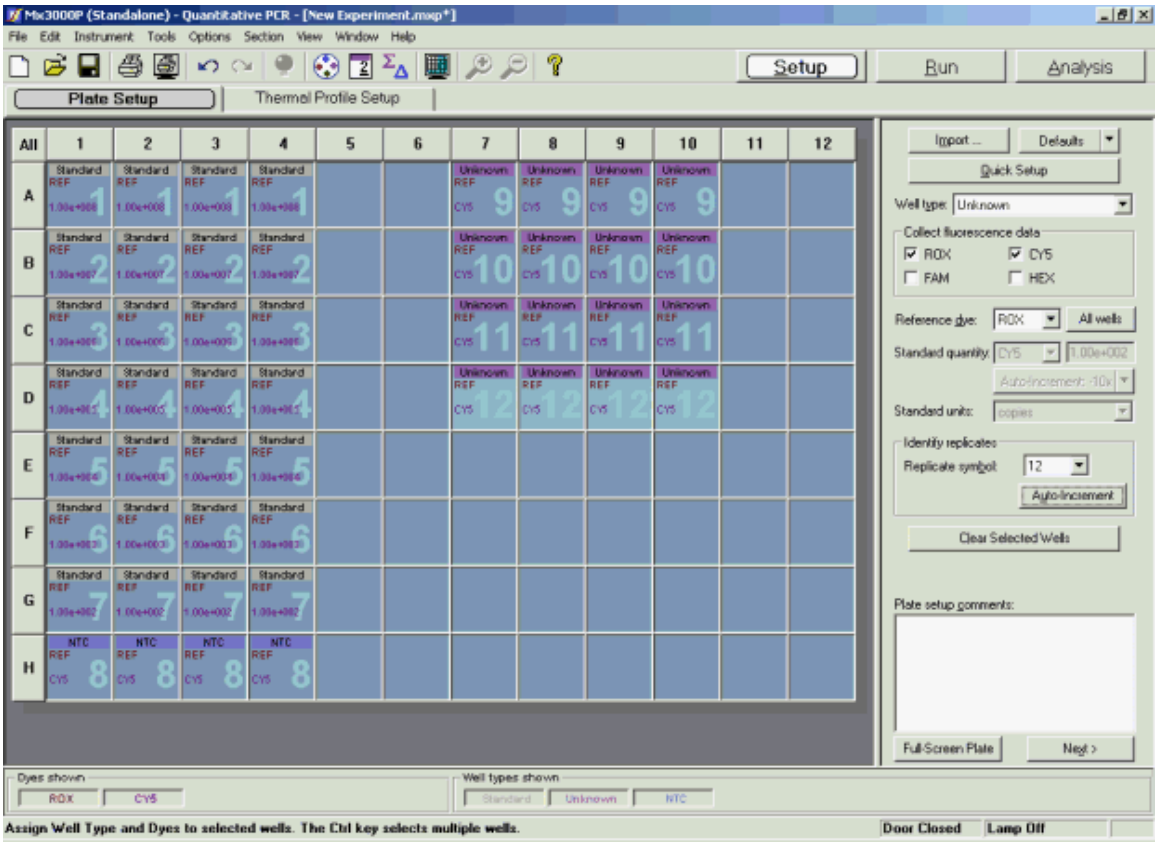
It is also possible to perform some forms of relative quantitation using **Quantitative PCR** experiments. In one common form, a relative standard curve is generated using a dilution series of a reference sample without knowledge of the absolute amount of the specific target in the **Standard** wells.

Multiplexing Quantitative PCR Experiments

Fluorescence readings may be taken for each sample using up to four different detection paths that are assigned to detect four specific dyes (in the **Optics Configuration** dialog box of the Mx3000P software). In this way, samples may be multiplexed for quantitation of multiple targets in the same well, by using spectrally-distinct dyes to detect each target. Each well/dye combination is reported separately on amplification plots and other results displays.

Quantitative PCR Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical plate setup for **Quantitative PCR**.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) by using the **Well type** menu on the command panel. The well types available in a Quantitative PCR experiment are described in the table below.

Well Type	Description
Unknown	Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target.
Buffer	Contains only buffer, used to monitor the background fluorescence attributable to the buffer.
NAC	No amplification control; contains all reaction components except DNA polymerase.
NPC	No probe control; contains all reaction components except the fluorescence-labeled probe.
NTC	No template control; contains all reaction components except the template nucleic acid.
Standard	Contains a complete reaction mixture including a known concentration of target nucleic acid. Used to generate a standard curve, which is then used to relate the threshold cycle (**Ct**) to initial template quantity in **Unknown** wells.
No RT	No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.
Dye-specific Negative Control	Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment is rarely used in Quantitative PCR experiments. (Most QPCR experiments will use the **NTC** well type assignment for reactions lacking template.)
Dye-specific Positive Control	Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment, which qualitatively designates a positive signal for the target without specifying the absolute amount, is rarely used in QPCR experiments. (Most QPCR experiments will include **Standard** wells with known amounts of target.)

After assigning the **Well type** to the selected well(s), select the dyes for which data should be collected in the wells using the **Collect fluorescence data** check boxes.

If you are using a passive reference dye in the selected well(s) select the dye from the **Reference dye** menu. If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.

If the selected well(s) is a **Standard**, for each dye for which standard curve data will be collected, enter the amount of template. To do this, first select the dye from the pulldown menu following **Standard quantity.** Next, enter the quantity of template added to the selected well(s) as a decimal number (for example, 400 or 1.234). Specify the units for the quantity in the **Standard units** menu. Instead of assigning quantities to wells on an individual basis, it is also possible to assign standard quantities to **Standard** wells sequentially using the **Auto-Increment** feature.

Assign the replicate set number to the selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

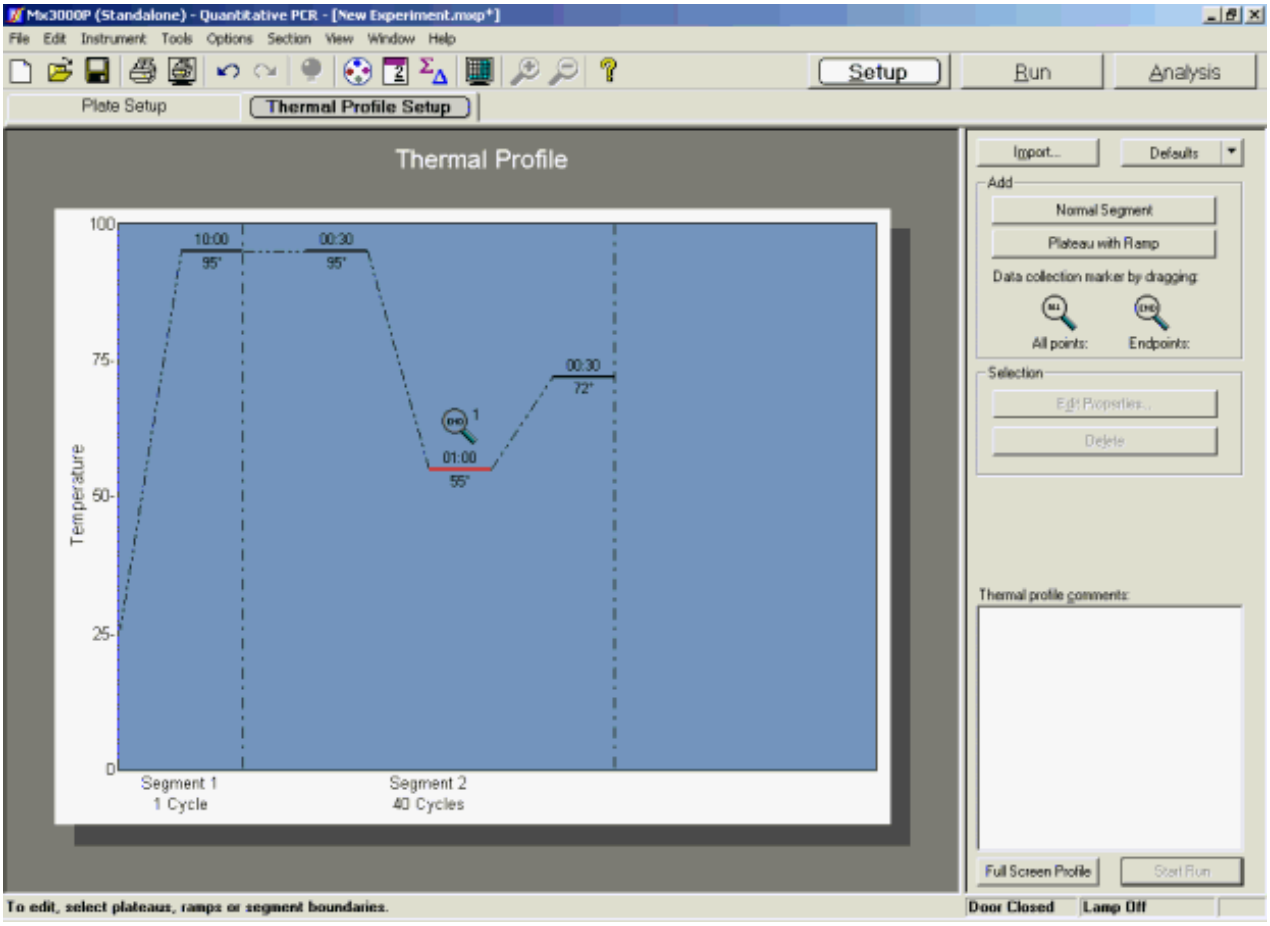
Once plate setup is complete, click **Next** to proceed to the **Thermal Profile Setup** screen.

Quantitative PCR Thermal Profile Setup

When the **Thermal Profile Setup** tab is selected in a new Quantitative PCR experiment, a default thermal profile opens. The default thermal profile should be modified to the specific requirements of the new experiment, as discussed below. It is also possible to **Import** the thermal profile from an existing experiment into the new experiment.

Shown in the figure below is the default thermal profile for a Quantitative PCR when using the software's **Original Settings**. (If you are using a user-defined default set, the default thermal profile stored in that default set will appear when the **Thermal Profile Setup** screen is opened. See *Preferences-Defaults* for more information about the use and management of **Defaults**.)

In the profile below, segment 1 is an activation step of 95°C for 10 minutes that is compatible with most hot start reactions. Segment 2 consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 55°C/1 minute for annealing, and a plateau of 72°C/30 seconds for extension.



Some applications use a two-step amplification protocol instead of the three-step protocol in the default thermal profile. A typical two-step amplification segment consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 60°C/1 minute for annealing and extension.

Data are collected at the end of each annealing plateau in the default thermal profile. These collection settings are suitable for a typical Quantitative PCR experiment. See *Data Collection* for more information on data collection options.

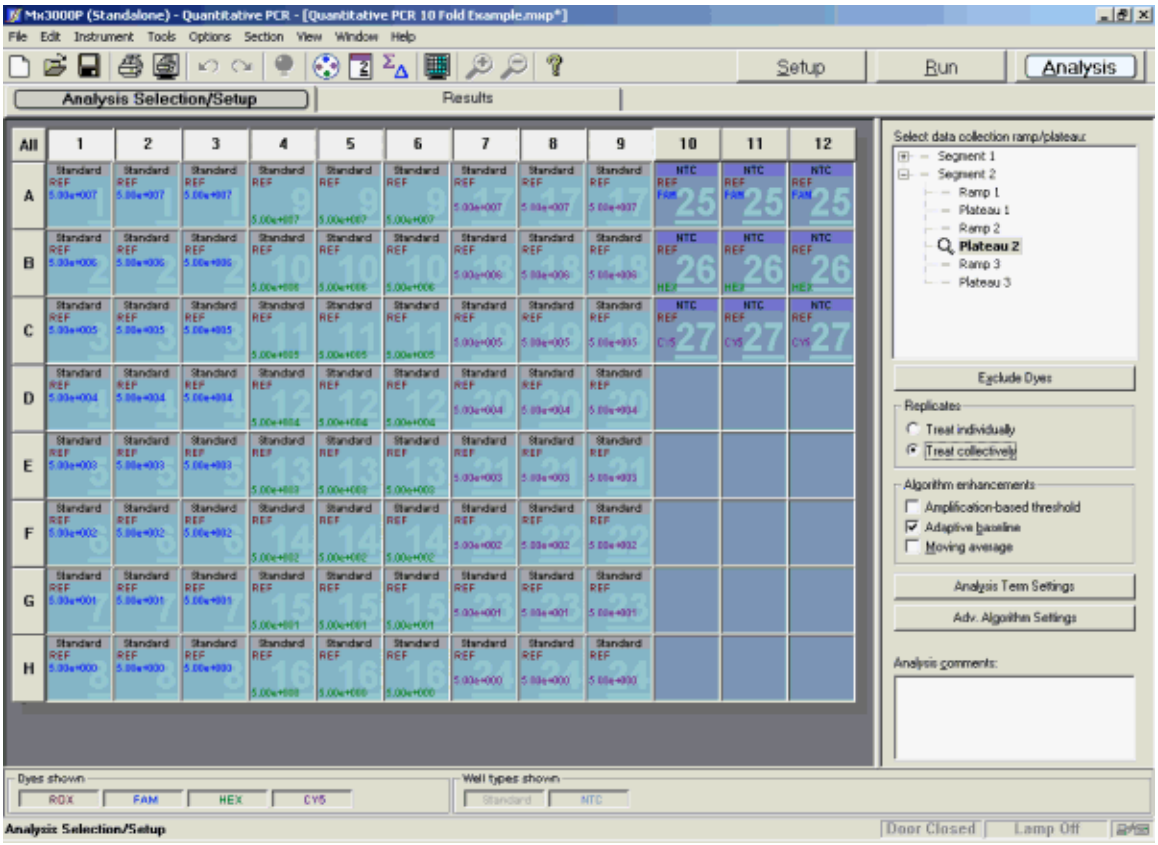
Modify the default thermal profile as necessary. At minimum, ensure that the temperature setting for the annealing plateau in Segment 2 is appropriate for the PCR primer pair used in the current experiment. The properties of all other plateaus, ramps, and segments may also be adjusted. See *Thermal Profile Setup* in the *How-To (Detailed Protocols)* section for more information about changing the default thermal profile.

Quantitative PCR Data Analysis

Analysis Setup

On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.
2. **Select** the **data collection ramp/plateau** from which data should be analyzed.
3. Specify the desired analysis settings on the command panel.



See Analysis Selection and Setup for more detailed instructions on using the features on this screen.

When all selections and settings are made, click the **Results** tab. One of several available results screens will appear. Use the radio buttons under **Area to analyze** to navigate to different analysis screens. The following screens are available for Quantitative PCR analysis:

Amplification Plots

Plate Sample Values

Standard Curve

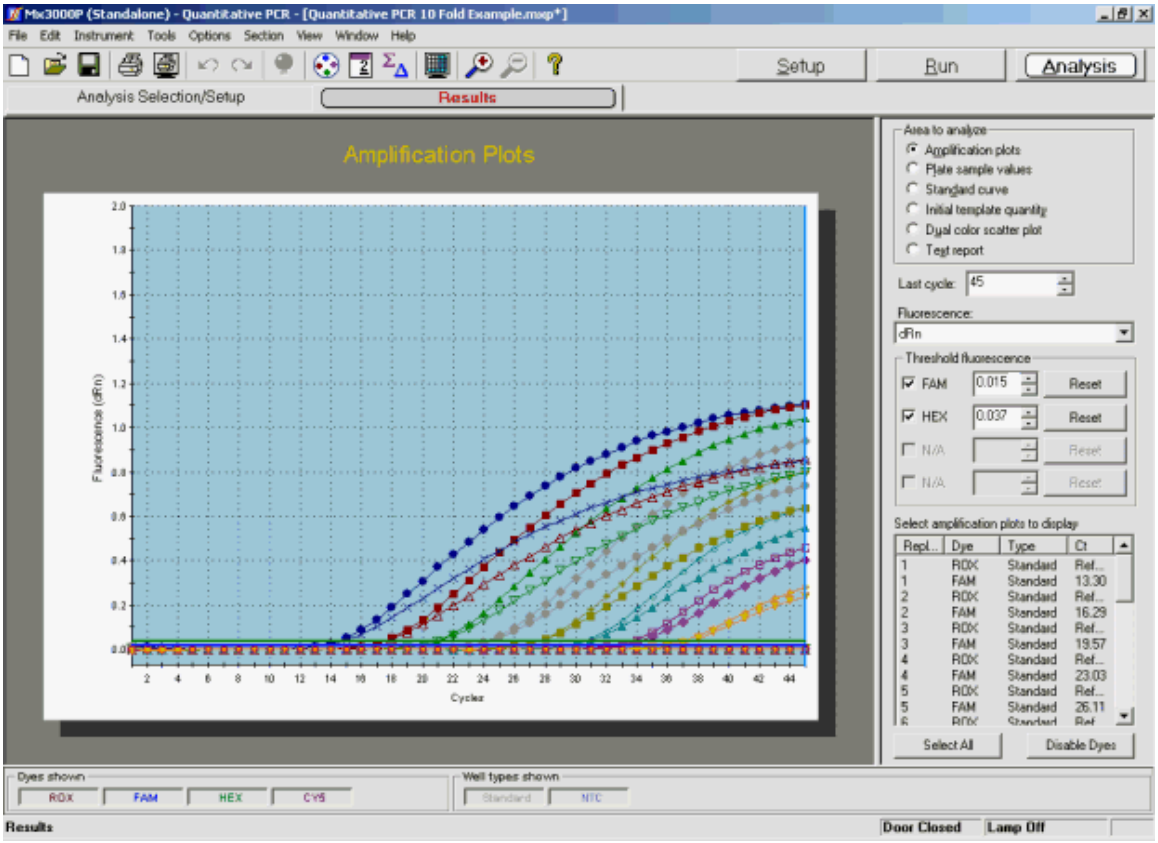
Initial Template Quantity

Dual Color Scatter Plot

Text Report

Quantitative PCR Amplification Plots

The **Amplification plots** screen, accessed through the **Results** tab, shows a plot of cycles versus fluorescence for a ramp or plateau on which data are gathered. Select the ramp or plateau for which data should be analyzed using the **Analysis Selection/Setup** screen.



Analysis Options

Fluorescence Data Type Plotted

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are: **R** (raw fluorescence), **dR** (baseline-corrected raw fluorescence), **Rn** (normalized fluorescence), and **dRn** (baseline-corrected normalized fluorescence).

Threshold Fluorescence

When basing plots on normalized fluorescence (**dR** or on **dRn**), the **Threshold fluorescence** value for each dye is listed on the command panel and is marked on the graph by a horizontal line. (Note that if a dye is hidden using the **Dyes shown** controls or if the check box next to the dye is cleared, the line corresponding to threshold fluorescence for that dye will not be shown on the graph.)

Threshold fluorescence is calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**. If desired, the threshold fluorescence may be adjusted manually. (To restore the software-calculated values, click **Reset**).

Baseline correction is required to establish a threshold fluorescence. Thus selections related to threshold fluorescence are unavailable for **R** or **Rn** data plots.

Last Cycle Setting

The **Last cycle** setting specifies the cycle from which the final (last) fluorescence values will be reported in the **Text Report** and **Plate Sample Values** screens. The position of the last cycle is marked on the graph with a solid vertical line.

This setting also affects the way Ct values are reported on other **Results** screens. While the **Last cycle** is not directly used to determine Ct, any wells with Ct values greater than the **Last cycle** value will be designated as **No Ct** in screens that display Ct.

Excluding Well/Dye Data from Analysis

To exclude the curve for one or more specific well/dye combinations from analysis, use either of the following two methods. (Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen.)

- From the list under **Select amplification plots to display**, select the curve(s) to be excluded and then click **Disable Dyes**.
- Double-click on any data point on the plot to be excluded. This action will exclude the entire data set for the well/dye corresponding to the plot.

To restore a plot that was disabled using either of these methods, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To restrict the plots shown to a subset of the well/dye combinations, use the **Select amplification plots to display** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

To display information about a specific data point on the plots, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

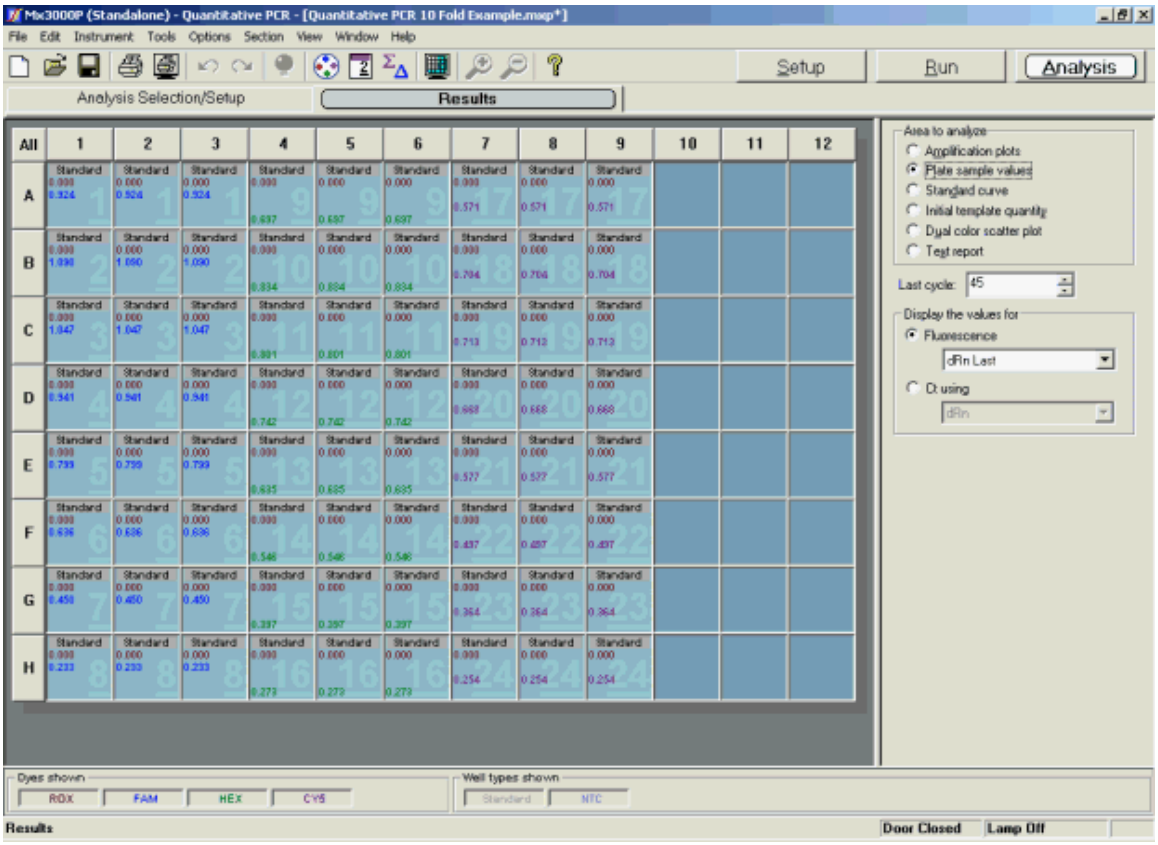
The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** and **Plot Markers** are displayed or hidden. See *Graphs and Graph Properties* for more information.

Quantitative PCR Plate Sample Values

The **Plate Sample Values** screen displays fluorescence values or Ct values for the sampled wells in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Either the fluorescence or the Ct value for each dye for which data was collected
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)



Analysis Options

Display the Values for Fluorescence or Ct

Under **Display the values for**, specify the form of the data to be displayed by selecting either the **Fluorescence** or the **Ct using** radio button. You will also need to specify the type of fluorescence data to be either displayed or used in calculations by selecting a data type from the menu below the selected radio button. Refer to the tables below for descriptions of the fluorescence data type abbreviations in the menus.

When displaying **Fluorescence** values, the following fluorescence data types are available:

Data Type	How Calculated
R Last	final fluorescence
dR Last	final fluorescence minus the initial fluorescence
Rn Last	normalized final fluorescence
dRn Last	normalized final fluorescence minus the normalized initial fluorescence
R Last/R First	final fluorescence divided by the initial fluorescence

When displaying **Ct** values, Ct calculations may be based on the following fluorescence data types:

Data Type	How Calculated
dR	baseline-corrected fluorescence
dRn	baseline-corrected normalized fluorescence

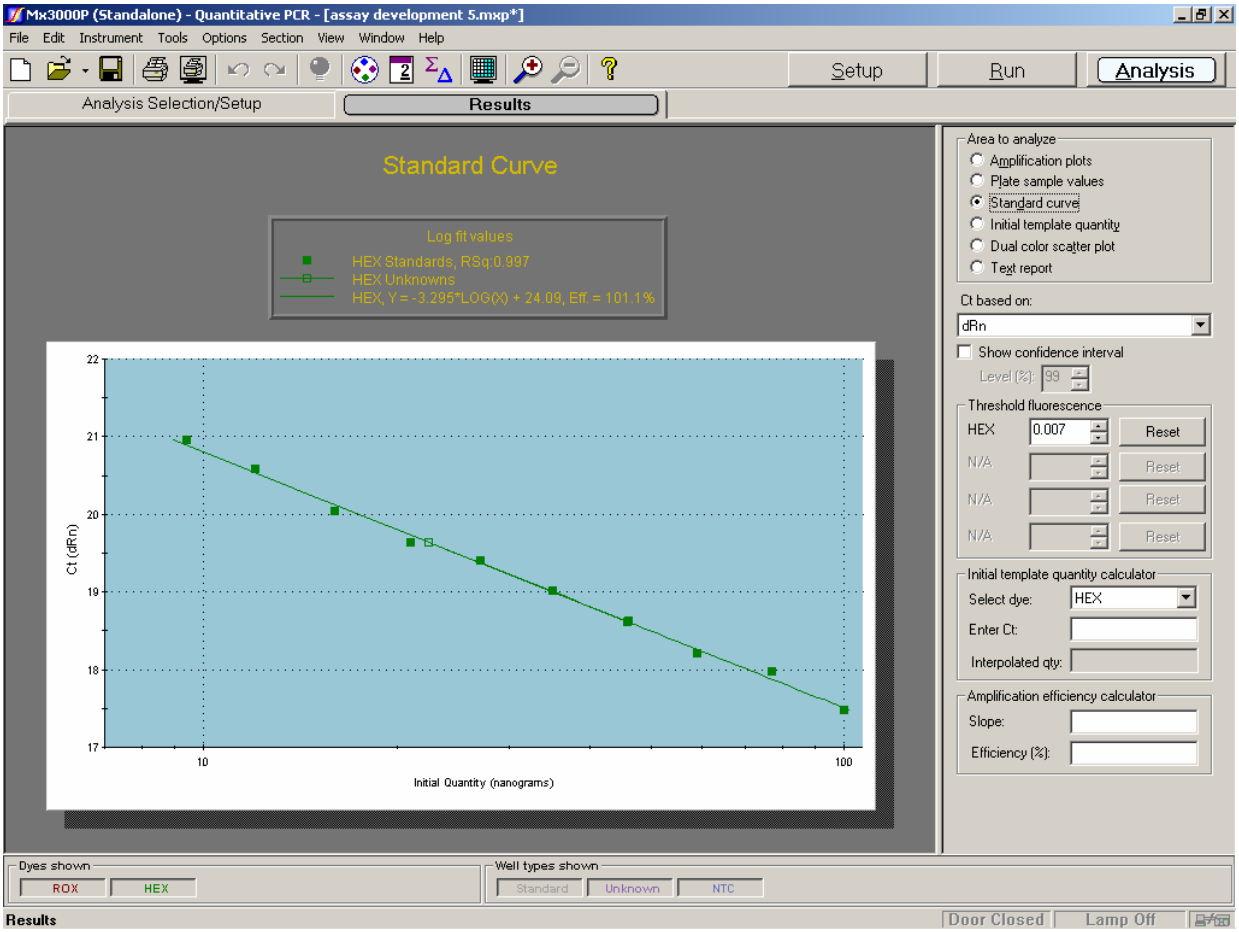
Last Cycle

The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

The **Last cycle** setting also affects the reporting of Ct. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct**.

Quantitative PCR Standard Curve

The **Standard Curve** is a plot of the initial template quantity in the **Standard** wells (X-axis), versus the **Ct** (threshold cycle). A least mean squares curve fitting algorithm is used to generate the standard curves displayed. Curves are displayed for each dye for which data was collected in **Standard** wells. **Unknown** wells selected during **Analysis Selection/Setup** are also plotted on the standard curve (using a different data point marker) based on their measured **Ct** values.

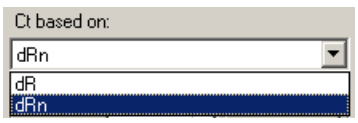


If less than two **Standard** wells were selected in **Analysis Selection/Setup**, no data will be plotted on this chart.

Analysis Options

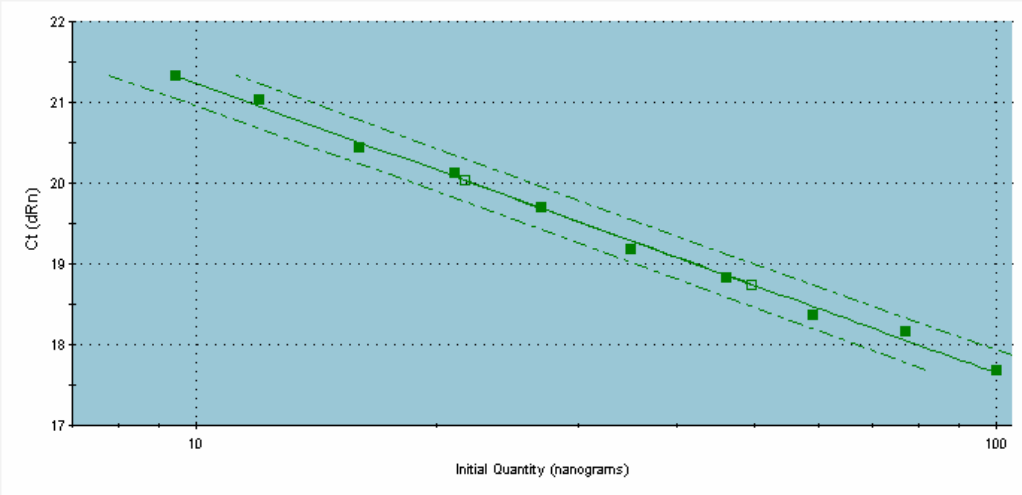
Fluorescence Data Type for Ct Determinations

Select the type of fluorescence data to be used for Ct determinations from the **Ct based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected normalized fluorescence).



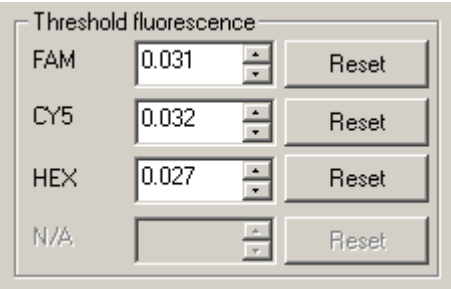
Confidence Interval

The confidence interval for each standard curve may be displayed by selecting the **Show confidence interval** checkbox. The resulting graph shows the confidence limits as hashed lines. The lines show the range of Initial Quantity values at a particular Ct that cannot be statistically distinguished from the fit line with more certainty than the confidence level (**Level %**) selected. The width of the confidence interval is an indicator of the quality of the fit of the data to the standard curve. Confidence interval calculations require a **Replicates** setting of **Treat individually**. Confidence level values may be exported to an Excel file, a text file or an XML file by using the **Export Chart Data** commands on the **File** menu.



Threshold Fluorescence

The **Threshold fluorescence** value for each dye, as calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**, is listed on the command panel. If desired, the threshold fluorescence for a given dye may be adjusted manually by entering a new value in the spin control box for the dye. (To restore the software-calculated values, click **Reset**).

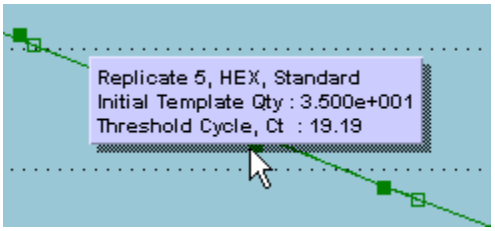


Excluding Well/Dye Data from Analysis

To exclude a specific well/dye data point from analysis, double click on the data point on the chart. Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen. To restore an excluded data point, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To display information about a specific data point on the plots, place the cursor over the point of interest to display the following information: well location or replicate number, dye name, well type, the initial template quantity and the Ct.



If the well is a **Standard**, the initial template quantity is the amount assigned to the well during plate setup. If the well is an **Unknown**, the initial template quantity is the interpolated amount based on the standard curve.

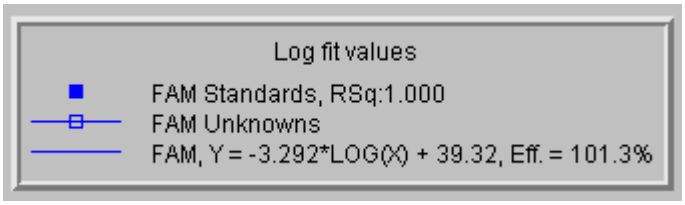
Note *If* **Display tooltips** *is cleared (deselected) on the* **Display** *tab of the* **Preferences** *dialog box, this well information will not be displayed.*

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** is displayed or hidden. See *Graphs and Graph Properties* for more information.

Interpretation and Use of Curve Attributes

RSq Value, Slope, and Amplification Efficiency

The graph legend displays information about each curve plotted. (If no legend is displayed on the screen, open the **Graph Properties** dialog box by double-clicking on the graph, and then select the **On** radio button under **Legend**.) Besides correlating plot markers with dyes and well types, the legend displays the R Squared (**RSq**) value and the equation for the line [y = m*log(x) + b, where m is the slope of the line]. The amplification efficiency (**Eff.**), calculated from the slope, is also displayed.



The **RSq** value is an indicator of the quality of the fit of the standard curve to the **Standard** data points plotted. The value will always be between 0 and 1. The closer the value is to 1, the better the fit of the line.

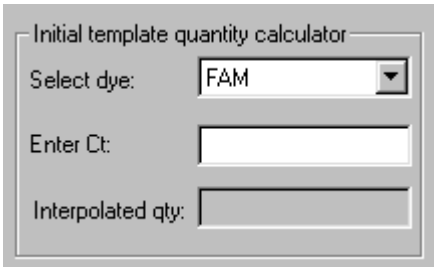
The slope of the curve is directly related to the average amplification efficiency throughout the cycling reaction. The equation that relates the slope to amplification efficiency is:

PCR Efficiency = $10^{(-1/slope)} - 1$

where PCR Efficiency corresponds to the proportion of template molecules that are doubled every cycle. From this equation it follows that a reaction with 100% efficiency will result in a slope of –3.322.

Initial Template Quantity Calculator

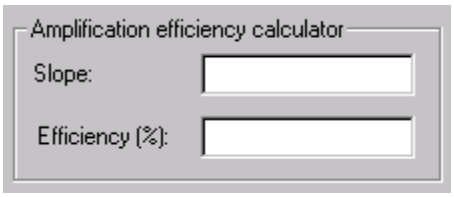
To use the displayed standard curve to calculate an interpolated quantity of template in another sample from the run based on the Ct determined for the sample, choose the dye from the **Select dye** menu, then enter a **Ct** value. The software will calculate and display the interpolated initial template quantity (**Interpolated qty**). Note that initial template quantities are automatically determined, based on the standard curves, for all **Unknowns** in the same experiment, and these results may be viewed on the Initial Template Quantity screen.



Amplification Efficiency Calculator

Enter either a **Slope** value or an amplification **Efficiency** value into the **Amplification efficiency calculator** to convert between values, based on the following equation:

PCR Efficiency = $10^{(-1/slope)} - 1$



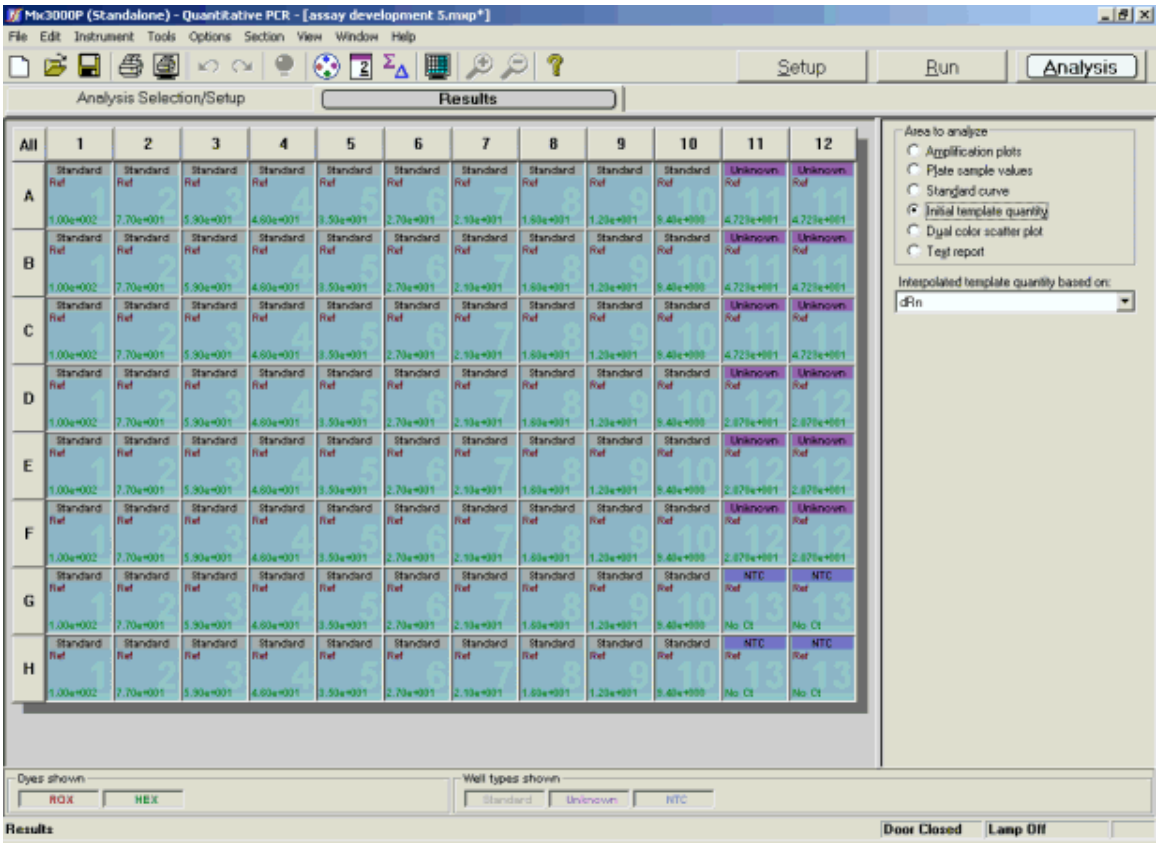
Quantitative PCR Initial Template Quantity

The **Initial Template Quantity** screen displays interpolated quantities of template added to **Unknown** wells before thermal cycling in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Initial template quantity (for **Unknowns**, the value interpolated from the standard curve based on the Ct calculated for the **U**nknown; for **Standards**, the amounts entered during plate setup)
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)

If less than two **Standard** wells were selected in **Analysis Selection/Setup**, a standard curve cannot be plotted and an error message will appear in place of results in the **Unknown** wells.



Analysis Options

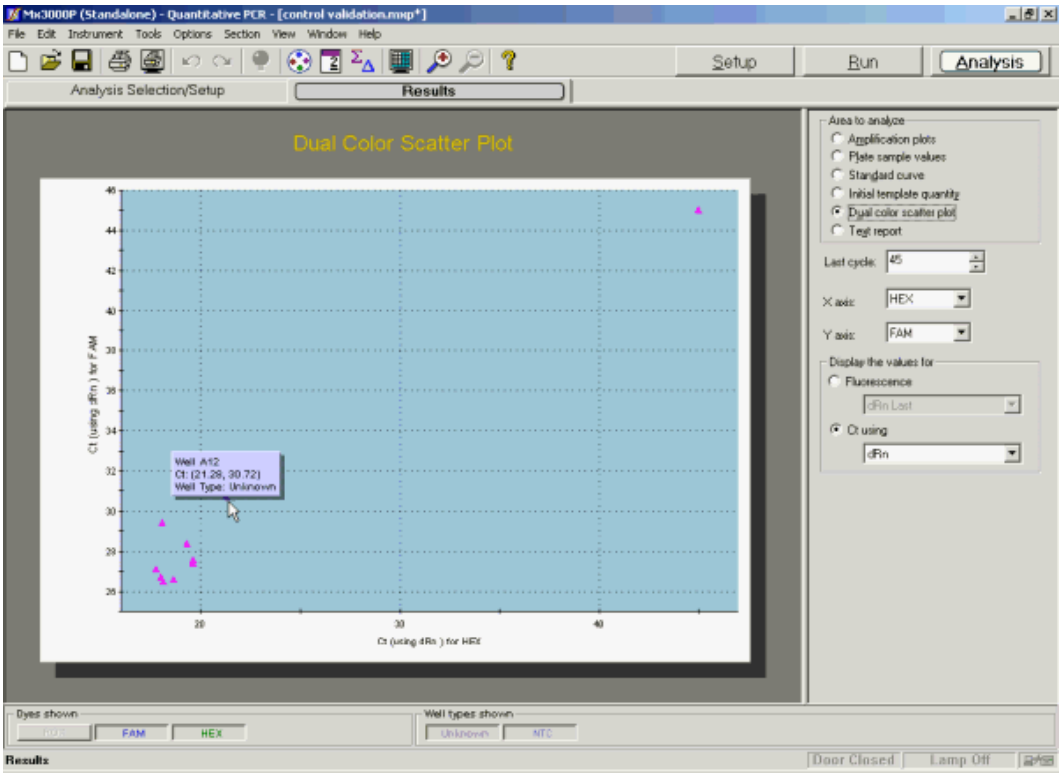
Fluorescence Data Type for Ct Determinations

Select the type of fluorescence data to be used to calculate the Ct values to be used for interpolation from the **Interpolated template quantity based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected normalized fluorescence).

Quantitative PCR Dual Color Scatter Plot

The **Dual Color Scatter Plot** screen is useful to compare the amplification properties of two different targets (represented by two different dyes) in the same well. In **Quantitative PCR** experiments, this plot is typically used to assess the suitability of a specific control target by displaying any correlation between the amplification of a gene of interest and the amplification of the control target.

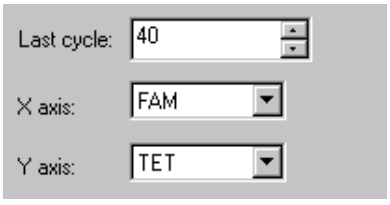
Each plotted point represents the coordinates of either the fluorescence values or threshold cycle (Ct) values for two dyes in a single well. For example, the X-axis may correspond to HEX Ct while the Y-axis corresponds to FAM Ct and the plotted point (x,y) corresponds to the coordinates describing the two Ct values determined for a given well. The positions of the plotted points provide a convenient indicator of whether the control target quantity is uniform or variable across samples.



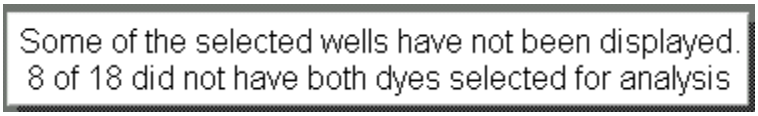
Analysis Options

Dye Selection

Specify the dye for which data should be plotted on each axis by selecting the dyes of interest from the **X axis** and **Y axis** menus on the command panel. Any two dyes assigned during **Plate Setup** may be specified.



A well will not be included in the scatter plot if the two dyes selected for the plot are not assigned to that well. An error message similar to the example shown below will appear if wells selected during **Analysis Selection/Setup** cannot be displayed.



Plot the Values for Fluorescence or Ct

Under **Display the values for**, specify the form of the data to be plotted by selecting either the **Fluorescence** or the **Ct using** radio button. For either of these options, you will need to specify the type of fluorescence data to be used in the analysis by selecting a data type from the menu below the selected radio button. Refer to the tables below for descriptions of the fluorescence data type abbreviations in the menus.

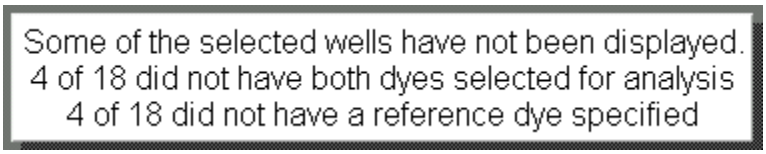
When plotting **Fluorescence** values, the following fluorescence data types are available:

Data Type	How Calculated
R Last	final fluorescence
dR Last	final fluorescence minus the initial fluorescence
Rn Last	normalized final fluorescence
dRn Last	normalized final fluorescence minus the normalized initial fluorescence
R Last/R First	final fluorescence divided by the initial fluorescence

When plotting **Ct** values, Ct calculations may be based on the following fluorescence data types:

Data Type	How Calculated
dR	baseline-corrected fluorescence
dRn	baseline-corrected normalized fluorescence

Note that if no reference dye has been assigned to the wells and **dRn**, **Rn Last** or **dRn Last** is selected, a message like the following will appear below the graph:



Last Cycle Setting

The **Last cycle** setting specifies the cycle from which the fluorescence values will be plotted if **Fluorescence** is selected under **Display the values for**. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

This setting also affects the way Ct values are reported on this and other **Results** screens. While the **Last cycle** is not directly used to determine Ct, any wells with Ct values greater than the **Last cycle** value will be plotted with a Ct value corresponding to the last cycle for which data was collected. The same wells will be designated as **No Ct** in screens that display Ct values.

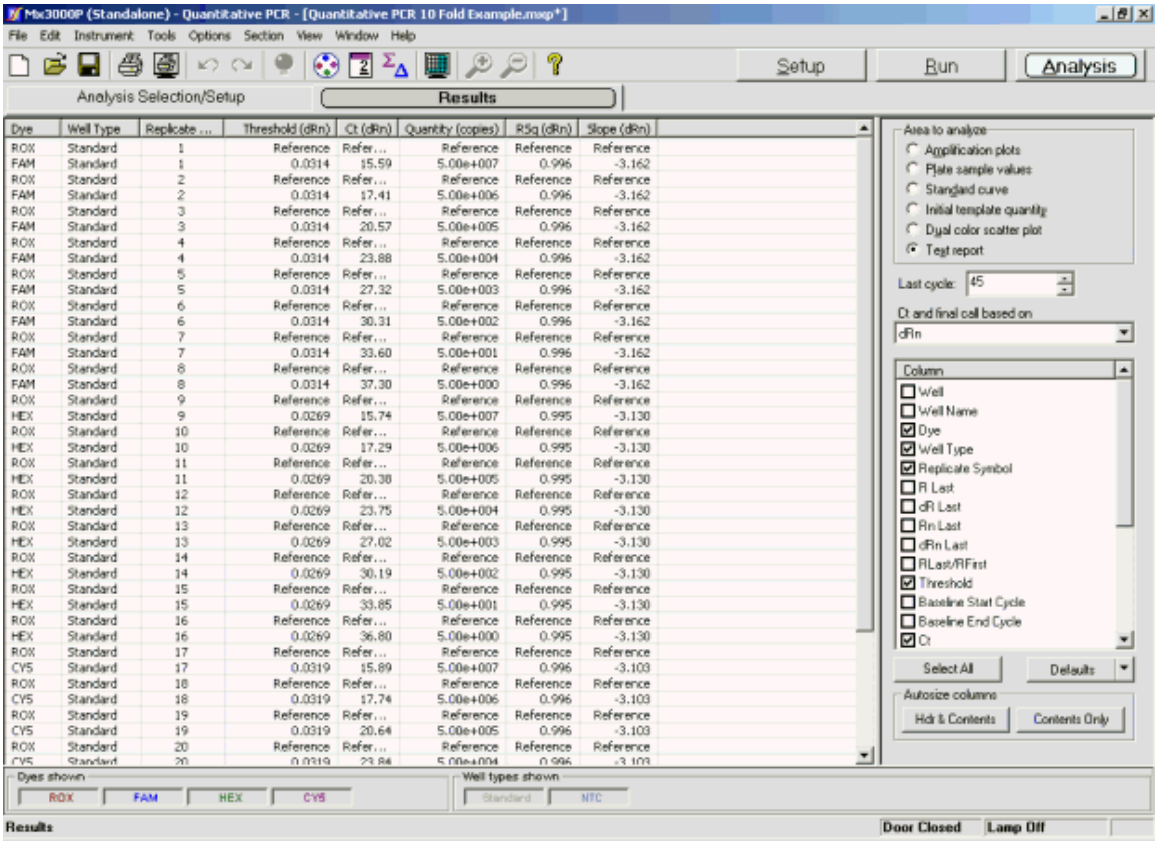
Display Options

To display information about a specific data point on the scatter plot, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including the attributes of the axes. See *Graphs and Graph Properties* for more information.

Quantitative PCR Text Report

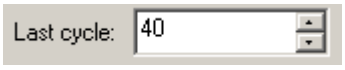
The **Text Report** screen shows the data from a run in text format.



Analysis Options

Last Cycle

The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported when fluorescence value columns (such as **dRn Last**) are selected for display. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.



The **Last cycle** setting also affects the reporting of **Ct** and the **Final Call**. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct** and will be called as negative (–).

Ct and Final Call Based On

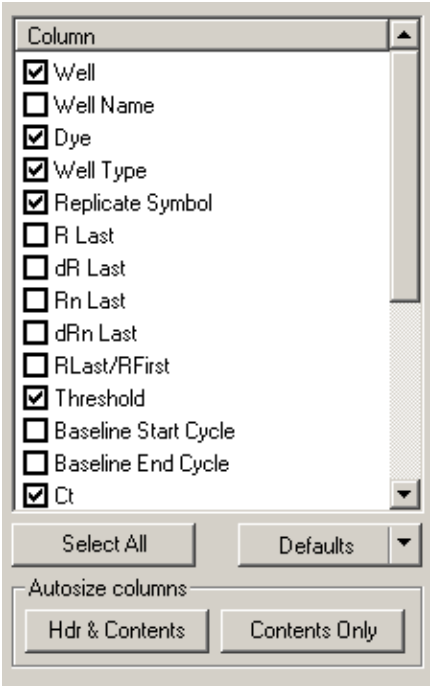
Select the type of fluorescence data that the reported **Ct** and **Final Call** should be based on from the **Ct and final call based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected fluorescence) and **dRn** (baseline-corrected normalized fluorescence).

Display Options

Columns Included in the Report

Select the **Columns** to be included in the text report by checking the boxes next to the column names. To exclude columns that are selected by default, click on the check box to deselect the column name. To select all columns for display, click **Select All**. The default settings for column selection can be restored by expanding the **Defaults** menu and then clicking **Get**.

If the included columns will not fit in the screen, a horizontal scroll bar appears at the bottom of the window to allow horizontal scrolling.



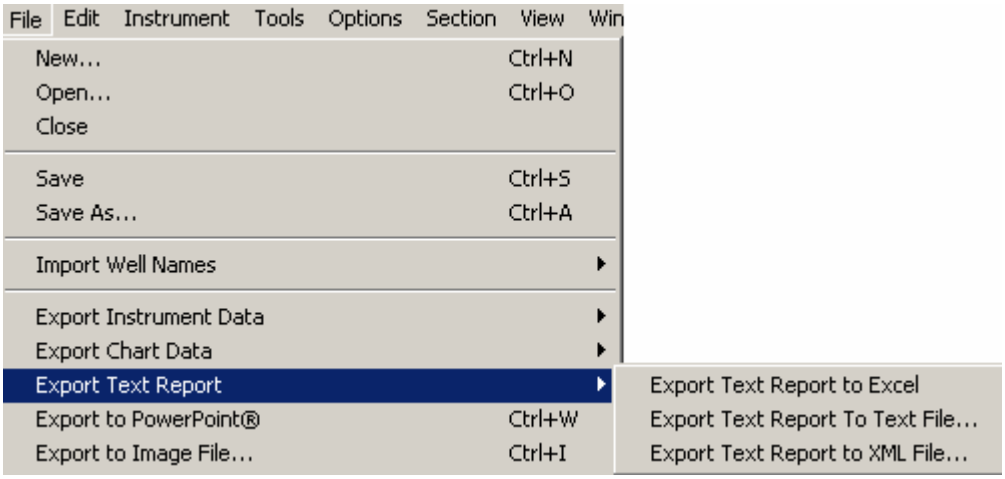
Column Width

To facilitate viewing data, columns can be automatically sized. Clicking the **Contents Only** button will cause each column to be automatically resized to the width of the widest data cell in that column (some column headers may be only partially displayed using this setting). Clicking the **Hdr & Contents** button will cause each column to be resized to the widest cell in that column, including the header cell.

Columns can also be resized manually by dragging the column dividers with the mouse.

Exporting Text Report Data

Text report data can be exported from the application in text, Microsoft Excel spreadsheet or XML format for analysis. Text report exporting options are available from the **File** menu. See *Exporting Data to Other Applications* in the *File Menu* section for more information.



Mx3000P System Experiments: Comparative Quantitation

The Comparative Quantitation Experiment Type

The **Comparative Quantitation** experiment provides an efficient method for comparing levels of RNA or DNA across samples when information about the absolute amounts of target in any sample is not required. Most common is the comparison of amounts of mRNA in treated versus untreated, or normal versus diseased cells or tissues.

For many gene expression studies it is not necessary to determine the absolute amount of a target in a particular sample; evidence of a relative increase or decrease in expression, compared to a sample of reference, is sufficient. The sample of reference is referred to as the **Calibrator.** For example, in a study in which a large number of compounds are screened for the ability to induce the expression of a certain set of genes in HeLa cells, the **Calibrator** might be a nucleic acid sample isolated from an untreated HeLa cell culture. In a study involving the expression of a cancer marker gene, the **Calibrator** might be a nucleic acid sample isolated from the normal, non-diseased part of the organ, whereas the test samples (referred to in the Mx3000P software as **Unknowns**) are nucleic acids isolated from the diseased tissue of the same patient. The expression level of the gene of interest (GOI) in the **Calibrator** is defined as 1× (or 1.0). Expression levels for the GOI in all **Unknowns** are reported relative to this **Calibrator** benchmark.

The amount of the GOI present in any sample is subject to many variables such as sample-to-sample differences in total amount of input nucleic acid and differences in the efficiency of RNA extraction or reverse transcription. A **Normalizer** target is included in the assay to reduce the effect of these spurious variations. Any gene with little to no variance in expression due to the treatment can serve as a **Normalizer**. (Commonly-used examples include GAPDH, cyclophilin, GUS, TFIID, or 18S, or 28S ribosomal RNA.) The abundance of the **Normalizer** and the GOI should be similar.

The **Normalizer** is necessary to correct for differences in total cDNA input between samples, whether the differences be due to efficiency of RNA purification, reverse transcription, or pipetting error. In order to use **Normalizer** levels to correct for spurious differences in GOI across samples, the relative amplification efficiencies of the **Normalizer** and the GOI must be taken into account. After either establishing that the **Normalizer** and GOI have equal amplification efficiencies or compensating for any differences in the amplification efficiencies, **Normalizer** target levels may be used to correct the relative quantities of the GOI target, allowing for a direct comparison of the GOI levels in **Unknowns** versus **Calibrator** wells.

Another level of correction can be introduced by the use of a passive reference dye such as ROX. The **Reference** dye is not reactive during QPCR and therefore it can be used to normalize slight differences in the volume of the QPCR reaction, transparency of the plastic caps and other sources of well-to-well differences. Note that references to normalized fluorescence values (abbreviated as **Rn** or **dRn** in the analysis screens) are normalized using **Reference** dye fluorescence values, not using any measurements of the **Normalizer** target.

For **Comparative Quantitation** analysis, it is assumed that the amplification efficiency for a given target is uniform between **Unknown** and **Calibrator** samples. If the experimental treatment affects PCR efficiency, **Comparative Quantitation** is not a valid experiment type. In this situation, **Quantitative PCR** experiments that include relative standard curves for each sample in each experiment may be used to compare initial quantities in different samples.

Comparative Quantitation Analysis using the Mx3000P Software

In a typical validated **Comparative Quantitation** assay, a replicate set of **Calibrator** wells containing the reference sample are run alongside a variety of **Unknown** replicate sets to test the effect of some variable on the expression level of one or more genes. The **Normalizer** target may either be measured in the same well as the gene of interest (using multiplexing) or be measured in different wells by **Identifying associations** of wells with the same sample source during plate setup.

During analysis, the Mx3000P software automatically adjusts the levels of the gene of interest in both **Unknown** and **Calibrator** wells to compensate for differences in the levels of the **Normalizer**. The normalized value for each **Unknown** sample is then compared to the normalized **Calibrator** value, and the **Relative Quantity** is reported for each **Unknown**. (The expression level of the gene of interest in the **Calibrator** is set to 1.0.)

The validity of using a **Normalizer** target to compensate for spurious differences in amounts of the gene of interest across samples is dependent on first establishing the efficiency of amplification for the gene of interest and **Normalizer** by generating standard curves for both targets.

Use of Standard Curves in Comparative Quantitation Experiments

In developing a comparative quantitation assay, it is important to show that the amplification/detection efficiencies of the GOI and of the **Normalizer** are reproducible and, ideally, very similar. The **Amplification efficiency** for each target is usually measured by generating a standard curve. The Mx3000P software analyzes the standard curve data and calculates the **Amplification efficiency** from the standard curve **Slope**.

In **Comparative Quantitation**, a **Standard Curve** is based on relative amounts of template, not on copy numbers or absolute amounts. The relative amounts are entered in the Mx3000P software using arbitrary units. A typical **Standard Curve** used for validation employs 2× dilutions of a reference sample.

In general, for the **Comparative Quantitation** experiment type, standard curves for the GOI and the **Normalizer** are prepared only during the initial development and validation of the assay. Once the amplification/detection efficiencies of both targets are established, there is no need to generate standard curves in subsequent assays, unless assay components or conditions are changed.

Correcting for Differences in Amplification Efficiencies for the GOI and Normalizer

In an idealized **Comparative Quantitation** experiment, the amplification efficiencies for the GOI and **Normalizer** targets must be identical in order to allow a direct correction of GOI levels across samples. The Mx3000P software allows you to compensate for differences in the amplification efficiencies for the GOI and **Normalizer** when reporting relative quantities by using the **Analysis Term Settings - Efficiency Settings** dialog box. Entering either the standard curve **Slopes** or the **Amplification efficiencies** for both the GOI and the **Normalizer** in this dialog box causes the software to compensate for the difference in efficiencies when correcting for **Normalizer** levels.

Using a Single Dye for Detection of Gene of Interest and Normalizer

The following considerations and procedures facilitate **Comparative Quantitation** experiments using a single dye (e.g. SYBR Green I dye) to detect both gene of interest and **Normalizer** targets.

Plate Setup

When setting up a single-dye experiment, for each template, assign separate wells for detection of the gene of interest and the **Normalizer**. Indicate the association between the two wells containing the same template by assigning the same **Association symbol** to both wells. Associations between gene of interest and **Normalizer** targets in separate wells are designated during **Plate Setup** by using the **Identify associations** menu.

Standard Curves

It is possible to analyze standard curves for two targets, such as the gene of interest and **Normalizer**, using a single dye on a single plate. In this situation, it is beneficial to distinguish the two sets of **Standard** wells corresponding to the two targets during **Plate Setup** using particular replicate symbol assignments or plate configurations. During analysis, either perform separate rounds of analysis by selecting the wells corresponding to only one target for each round, or reassign the dye detecting the **Normalizer** target with a unique name, as illustrated in the example below.

Efficiency Settings

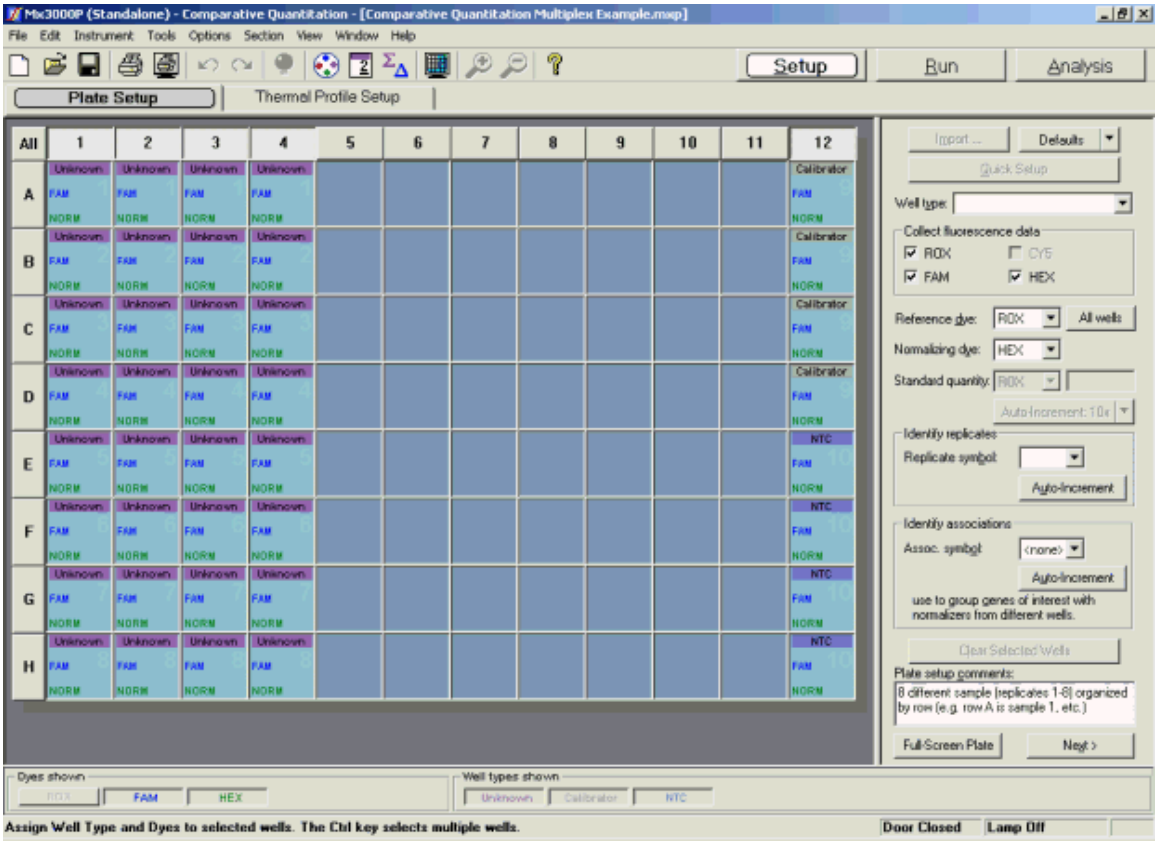
In order to use the **Analysis Term Settings - Efficiency Settings** dialog box to record amplification efficiencies for the two targets detected using the same dye, it is necessary to first reassign the wells detecting the **Normalizer** to a uniquely-named dye. For example, the steps outlined below may be used in an experiment using SYBR Green dye for detection of both targets.

Example: Distinguishing the Normalizer from the Gene of Interest when using SYBR Green Detection

1. Complete **Plate Setup** with SYBR Green data collection in all sample wells.
2. After running the experiment, on the **Plate Setup** screen, select the wells corresponding to the **Normalizer**.
3. In the **Optics Configuration - Dyes & Filters** dialog box, define a mock custom dye with a descriptive name, such as NRML, and associate this dye with the FAM filter set.
4. In the **Optics Configuration - Dye Assignment** dialog box, assign the NRML dye to the FAM filter set.
5. On the **Plate Setup** screen, double click on the SYBR checkbox under **Collect fluorescence data**. This will change the dye assignment for the selected wells, detecting the **Normalizer**, to NRML. Unselected wells, detecting the gene of interest, will retain the SYBR assignment.
6. With the wells corresponding to the **Normalizer** still selected, expand the **Normalizing dye** menu and then select NRML.
7. If the experiment is being used to generate standard curves for both targets, navigate to the **Analysis Setup/Selection** screen and select the **Standard** wells for both targets. On the **Standard Curve** screen, separate curves for each target will be displayed with distinctive plot markers for SYBR and NRML data.
8. If the experiment is being used to analyze relative quantities, open the **Analysis Term Settings - Efficiency Settings** dialog box and enter the **Efficiency** value for the gene of interest in the SYBR field and for the **Normalizer** in the NRML field.

Comparative Quantitation Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical plate setup for **Comparative Quantitation** using a **Normalizer** multiplexed with the gene of interest (both targets measured in the same wells). Click here to see a plate setup in which data are collected for the gene of interest and the **Normalizer** in separate wells.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) using the **Well type** menu on the command panel. The well types available in a Comparative Quantitation experiment are described in the table below.

Well Type	Description
Unknown	Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target. Quantities of a gene of interest in the **Unknown** wells is reported relative to the quantity of the same gene in the **Calibrator** wells, after correction for the relative quantities of a **Normalizer** target in the **Unknown** and **Calibrator** wells.
Buffer	Contains only buffer, used to monitor the background fluorescence attributable to the buffer.
NAC	No amplification control; contains all reaction components except DNA polymerase.
NPC	No probe control; contains all reaction components except the fluorescence-labeled probe.
NTC	No template control; contains all reaction components except the template nucleic acid. If the gene of interest and the **Normalizer** are detected in separate wells, a **NTC** well is required for each primer/probe set.
Standard	Contains a complete reaction mixture including a known relative amount of target nucleic acid. Typically a series of serial dilutions of a reference template are included in **Standards** for the purpose of initial validation of a Comparative Quantitation assay.
Calibrator	Contains a complete reaction mixture including a characterized target. The level of a gene of interest in the **Calibrator** wells is set to 1.0 for comparison to the relative quantities in **Unknown** samples.
No RT	No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.
Dye-specific Negative Control	Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment is rarely used in Comparative Quantitation experiments (most will use the **NTC** well type assignment for reactions lacking template).
Dye-specific Positive Control	Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment is rarely used in Comparative Quantitation experiments.

After assigning the **Well type** to the selected well(s), select the dyes for which data should be collected in these wells using the **Collect fluorescence data** check boxes.

If you are using a passive reference dye in the selected well(s) select the dye from the **Reference dye** menu. If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.

If **Normalizer** data will be collected in the selected well, identify the dye corresponding to the **Normalizer** target from the **Normalizing dye** menu.

If the selected well(s) is a **Standard**, for each dye for which standard curve data will be collected, enter the amount of template. To do this, first select the dye name from the pull-down menu following **Standard quantity.** Next, enter the relative amount of template added to the selected well(s) as a decimal number with arbitrary units. For example, the sample containing the lowest amount in the dilution series may be assigned a value of 1, with the quantity assigned to each increasingly-concentrated **Standard** well in the dilution series multiplied by the dilution factor of 2. Instead of assigning quantities to wells on an individual basis, it is also possible to assign quantities to **Standard** wells sequentially using the **Auto-Increment** feature.

More than one **Standard Curve** may be generated on a single plate using a single dye. See Using a Single Dye for Detection of Gene of Interest and Normalizer.

Assign the replicate set number to the selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

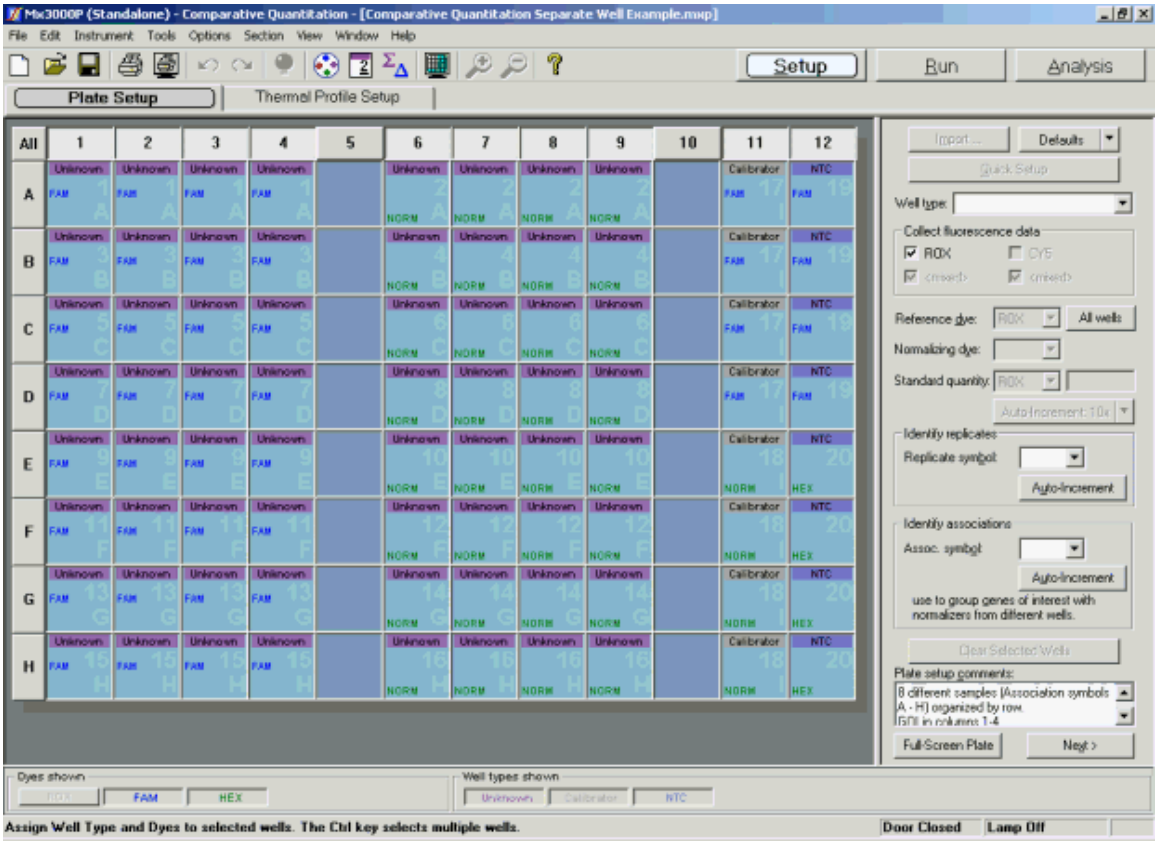
If the levels of the gene of interest and the **Normalizer** present in a given sample are to be measured in two separate wells, it is necessary to **Identify associations** between wells containing the same template. Select the wells to be associated on the plate, then select the appropriate symbol from the **Assoc. symbol** menu. If the gene of interest and **Normalizer** are multiplexed in all wells, it is not necessary to make any selections in the **Identify associations** section.

Once plate setup is complete, click **Next** to proceed to the **Thermal Profile Setup** screen.

Sample Plate Setup for a Non-multiplexed Comparative Quantitation Experiment

Shown below is a typical plate setup for **Comparative Quantitation** in which data are collected for a **Normalizer** and a gene of interest in separate wells. In this example, the gene of interest and the **Normalizer** are detected using different dyes. A similar plate setup would be appropriate for experiments using a single dye for detection, with the single dye (only) marked for data collection.

The association between wells set up to detect gene of interest or Normalizer levels from the same template are indicated by a capital letter **Association symbol** in the wells.

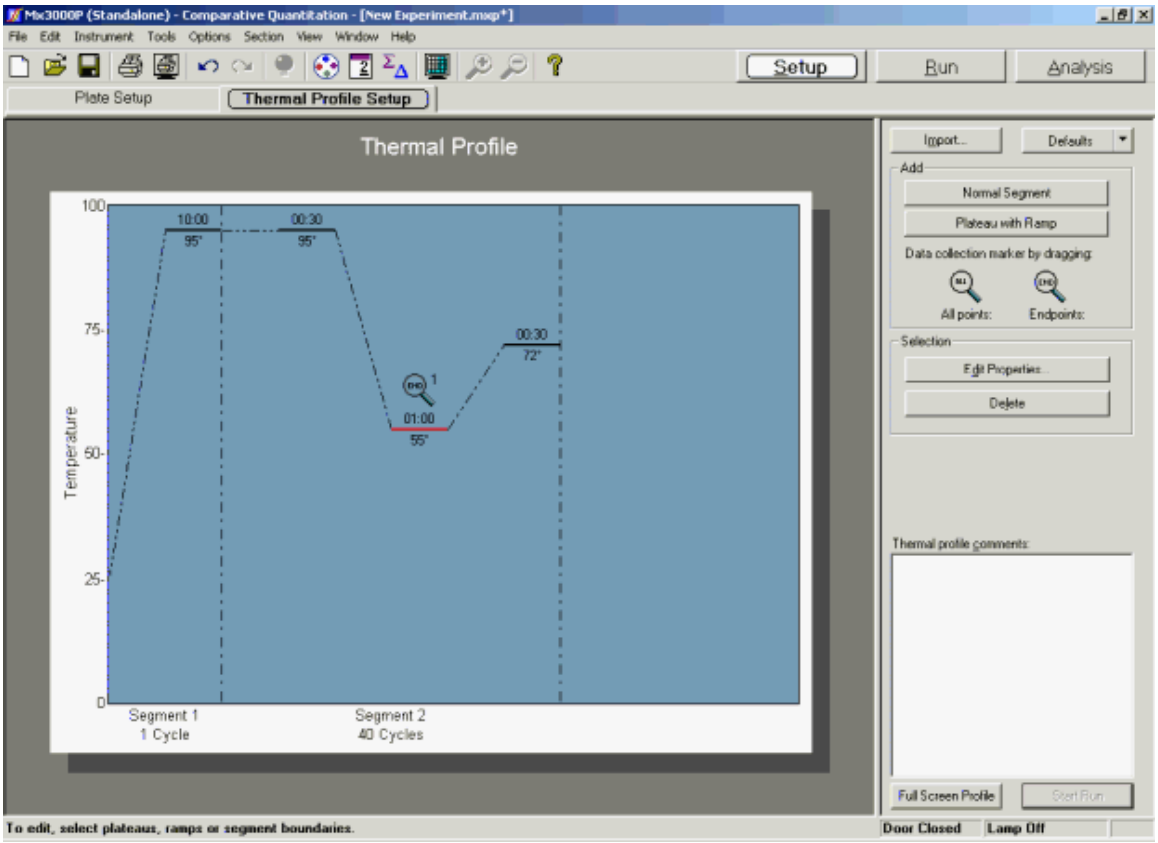


Comparative Quantitation Thermal Profile Setup

When the **Thermal Profile Setup** tab is selected in a new Comparative Quantitation experiment, a default thermal profile opens. The default thermal profile should be modified to the specific requirements of the new experiment, as discussed below. It is also possible to **Import** the thermal profile from an existing experiment into the new experiment.

Shown in the figure below is the default thermal profile for a Comparative Quantitation experiment when using the software's **Original Settings**. (If you are using a user-defined default set, the default thermal profile stored in that default set will appear when the **Thermal Profile Setup** screen is opened. See *Preferences-Defaults* for more information about the use and management of **Defaults**.)

In the profile below, segment 1 is an activation step of 95°C for 10 minutes that is compatible with a hot start reaction. Segment 2 consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 55°C/1 minute for annealing, and a plateau of 72°C/30 seconds for extension.



Some applications use a two-step amplification protocol instead of the three-step protocol in the default thermal profile. A typical two-step amplification segment consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 60°C/1 minute for annealing and extension.

Data are collected at the end of each annealing plateau in the default thermal profile. These collection settings are suitable for a typical Comparative Quantitation experiment.

Modify the default thermal profile as necessary. At minimum, ensure that the temperature setting for the annealing plateau in Segment 2 is appropriate for the PCR primer pair used in the current experiment. The properties of all other plateaus, ramps, and segments may also be adjusted. See *Thermal Profile Setup* in the *How-To (Detailed Protocols)* section for more information about changing the default thermal profile and data collection options.

Comparative Quantitation Data Analysis

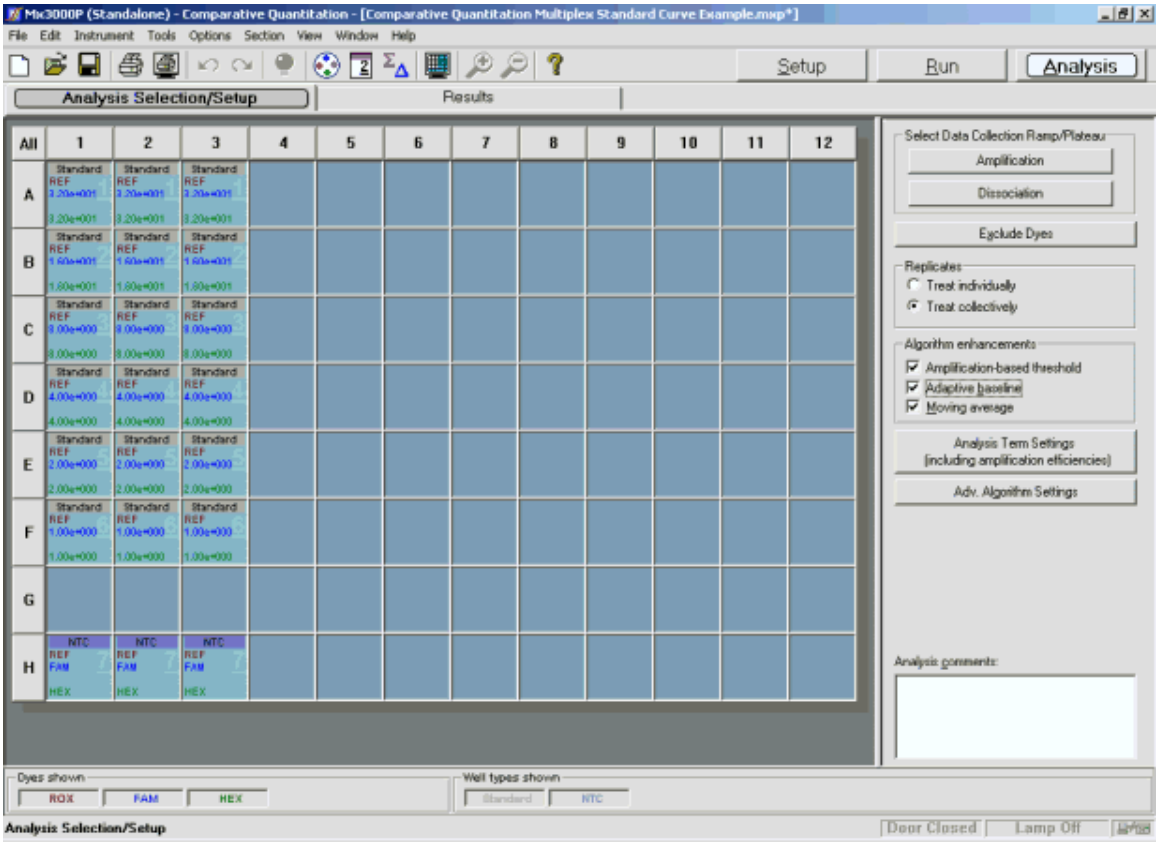
Analysis Setup

On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.

2. Select the segment (**Amplification** or **Dissociation**), and then the **position** (either the **ramp** or the **plateau**) from which data should be analyzed. The **Dissociation** segment should be selected only for viewing the **Dissociation Curve**. All other **Results** screens require selection of a data set collected during **Amplification**.

3. Specify the desired analysis settings on the command panel.



See Analysis Selection and Setup for more detailed instructions on using the features on this screen.

When all selections and settings are made, click the **Results** tab. One of several available results screens will appear. Use the radio buttons under **Area to analyze** to navigate to different analysis screens. The following screens are available for Comparative Quantitation analysis:

Amplification Plots

Dissociation Curve

Plate Sample Values

Standard Curve (for validation)

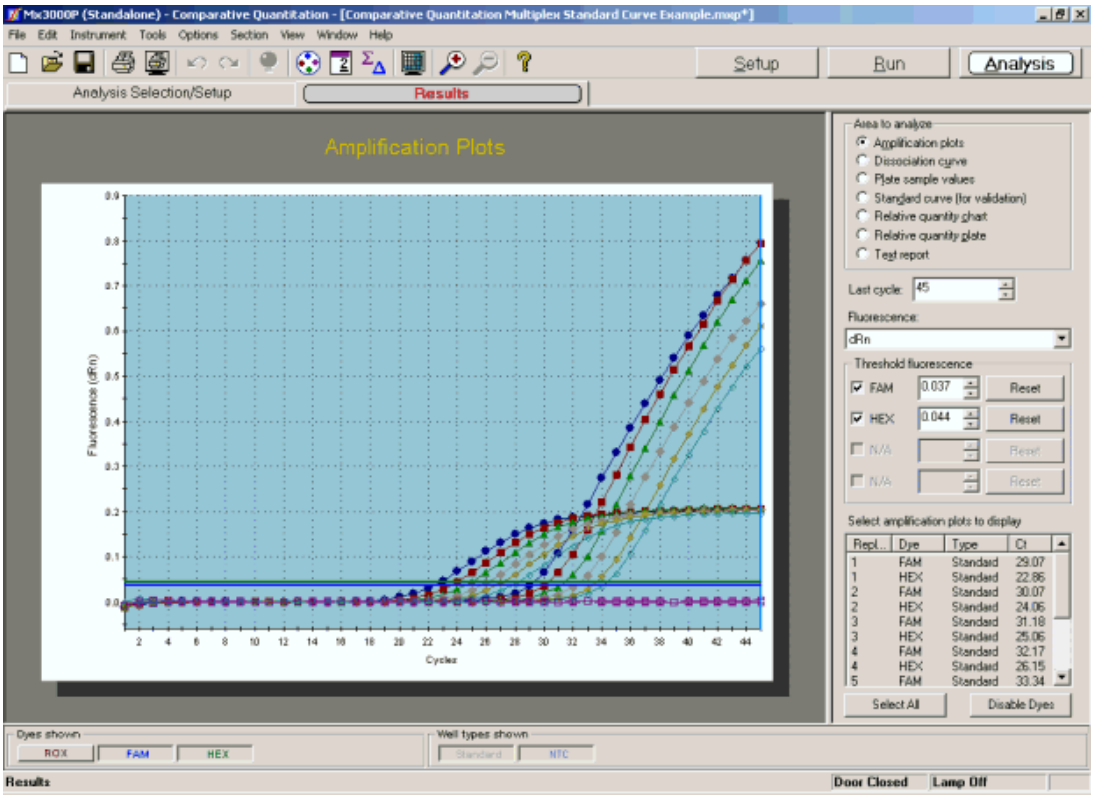
Relative Quantity Chart

Relative Quantity Plate

Text Report

Comparative Quantitation Amplification Plots

The **Amplification Plots** screen, accessed through the **Results** tab, shows a plot of cycles versus fluorescence for a ramp or plateau on which data are gathered. Select the ramp or plateau for which data should be analyzed using the **Analysis Selection/Setup** screen.



Analysis Options

Fluorescence Data Type Plotted

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are: **R** (raw fluorescence), **dR** (baseline-corrected raw fluorescence), **Rn** (normalized fluorescence), and **dRn** (baseline-corrected, normalized fluorescence).

Note that the fluorescence data designated as **Rn** and **dRn** refers to data normalized according to the passive reference dye (if used). Measurements from the dye defined as the **Normalizer** do not enter into normalization at the level of fluorescence reporting.

Threshold Fluorescence

When basing plots on normalized fluorescence (**dR** or on **dRn**), the **Threshold fluorescence** value for each dye is listed on the command panel and is marked on the graph by a horizontal line. (Note that if a dye is hidden using the **Dyes shown** controls or if the check box next to the dye is cleared, the line corresponding to threshold fluorescence for that dye will not be shown on the graph.)

Threshold fluorescence is calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**. If desired, the threshold fluorescence may be adjusted manually. (To restore the software-calculated values, click **Reset**).

Baseline correction is required to establish a threshold fluorescence. Thus selections related to threshold fluorescence are unavailable for **R** or **Rn** data plots.

Last Cycle Setting

The **Last cycle** setting specifies the cycle from which the final (last) fluorescence values will be reported in the **Text Report** and **Plate Sample Values** screens. The position of the last cycle is marked on the graph with a solid vertical line.

This setting also affects the way Ct values are reported on other **Results** screens. While the **Last cycle** is not directly used to determine Ct, any wells with Ct values greater than the **Last cycle** value will be designated as **No Ct** in screens that display Ct.

Excluding Well/Dye Data from Analysis

To exclude the curve for one or more specific well/dye combinations from analysis, use either of the following two methods. (Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen.)

- From the list under **Select amplification plots to display**, select the curve(s) to be excluded and then click **Disable Dyes**.
- Double-click on any data point on the plot to be excluded. This action will exclude the entire data set for the well/dye corresponding to the plot.

To restore a plot that was disabled using either of these methods, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To restrict the plots shown to a subset of the selected well/dye combinations, use the **Select amplification plots to display** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

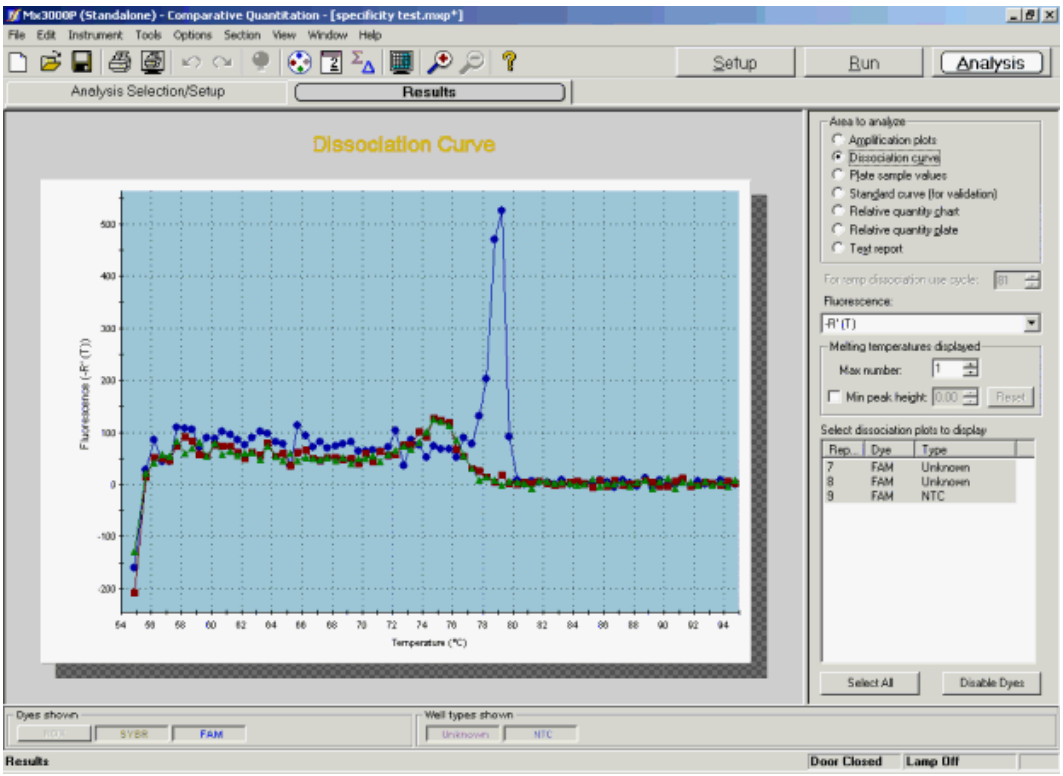
To display information about a specific data point on the plots, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** and **Plot Markers** are displayed or hidden. See *Graphs and Graph Properties* for more information.

Comparative Quantitation Dissociation Curve

For experiments that use SYBR Green detection and that include a **Dissociation** segment, use the **Dissociation Curve** screen, accessed through the **Results** tab, to view the dissociation profile. This screen shows a plot of the fluorescence (or its first derivative) as a function of temperature. Ensure that the data set collected during the **Dissociation** segment of the experiment is selected for analysis using the **Analysis Selection/Setup** screen.

The plot shown below is based on the first derivative of the fluorescence reading multiplied by –1 [designated **–R´(T)**]. Plotting results in this way is generally appropriate for complex nucleic acid mixtures such as those generated during PCR amplification. In samples with amplicon, this type of plot will typically display at least two populations with different transition temperatures. Populations with a Tm of 80°C or higher correspond to the larger PCR products, and can usually be assigned as specific DNA product. DNA products displaying melting temperatures of < 75°C correspond to non-specific DNA products. It is important to note that these populations are not necessarily homogeneous, and may contain multiple PCR product species.



Analysis Options

Fluorescence Data Type Plotted

Dissociation curves may be based on four different types of fluorescence: **R** (raw fluorescence), **Rn** (reference dye-normalized fluorescence), **–R´(T)**, (the first derivative of the raw fluorescence reading multiplied by –1), and **–Rn´(T)** (the first derivative of the reference dye-normalized fluorescence reading multiplied by –1). Select the desired type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel.

Product Melting Temperature Listings Settings

The Mx3000P software will identify and report **Product melting temperatures** for up to six product populations from each dissociation curve. Set the maximum number of products for which a melting temperature should be calculated using the **Max number** spin controls. This setting controls only the maximum number; the software may detect and report a smaller number of product populations for a given curve.

When basing plots on the negative derivative of fluorescence [**–R´(T)** or **–Rn´(T)**], you may specify the minimum peak height to be used for designating product populations. This peak height minimum may be entered in the **Min peak height** box as a decimal number or may be set manually by dragging the horizontal line indicator on the graph to the desired position.

Display Options

To restrict the plots shown to a subset of the well/dye combinations, use the **Select dissociation plots to display** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

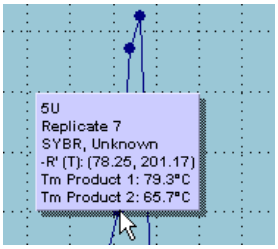
To exclude the curve for one or more specific well/dye combinations from view, select the curve(s) to be excluded and then click **Disable Dyes**. Disabling a particular well/dye combination requires replicates to be treated **individually** (this may be specified on the **Analysis Selection/Setup** screen). To restore a disabled plot, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

To display information about a specific data point on the plots, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** and **Plot markers** are displayed or hidden. See *Graphs and Graph Properties* for more information.

Reporting of Melting Temperatures (Tm)

Melting temperatures for each of the product populations are displayed when the cursor is placed over any point on the curve of interest.



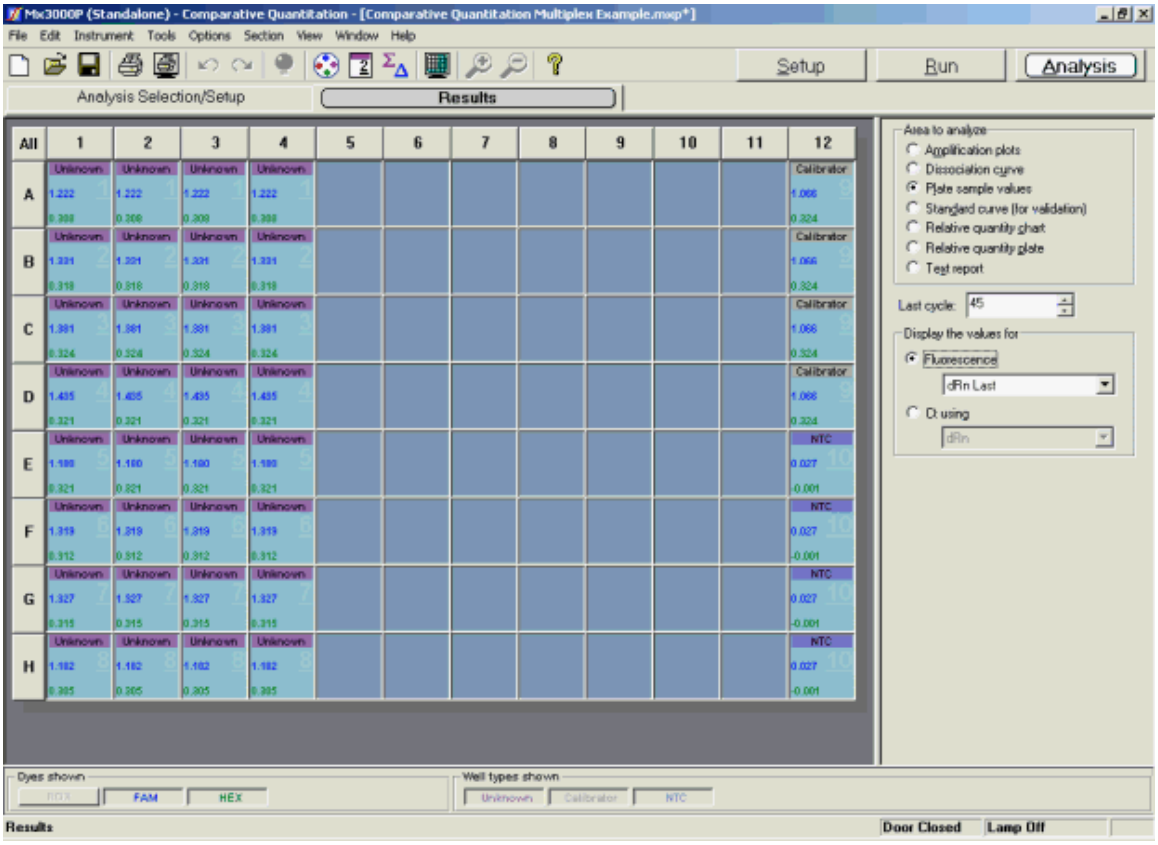
Melting temperatures will also be reported on the **Text Report** screen when the appropriate **Tm Product** boxes are selected in the **Columns** selection on that screen.

Comparative Quantitation Plate Sample Values

The **Plate Sample Values** screen displays **Fluorescence** values or **Ct** values for the sampled wells in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Either the fluorescence or the Ct value for each dye for which data was collected
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)
- Association symbol, if used



Analysis Options

Display the Values for Fluorescence or Ct

Under **Display the values for**, specify the form of the data to be displayed by selecting either the **Fluorescence** or the **Ct using** radio button. You will also need to specify the type of fluorescence data to be either displayed or used in calculations by selecting a data type from the menu below the selected radio button. Refer to the tables below for descriptions of the fluorescence data type abbreviations in the menus.

When displaying **Fluorescence** values, the following fluorescence data types are available:

Data Type	How Calculated
R Last	final fluorescence
dR Last	final fluorescence minus the initial fluorescence
Rn Last	normalized final fluorescence (with normalization based on the reference dye)
dRn Last	normalized final fluorescence minus the normalized initial fluorescence (with normalization based on the reference dye)
R Last/R First	final fluorescence divided by the initial fluorescence

When displaying **Ct** values, Ct calculations may be based on the following fluorescence data types:

Data Type	How Calculated
dR	baseline-corrected fluorescence
dRn	baseline-corrected normalized fluorescence (with normalization based on the reference dye)

Last Cycle

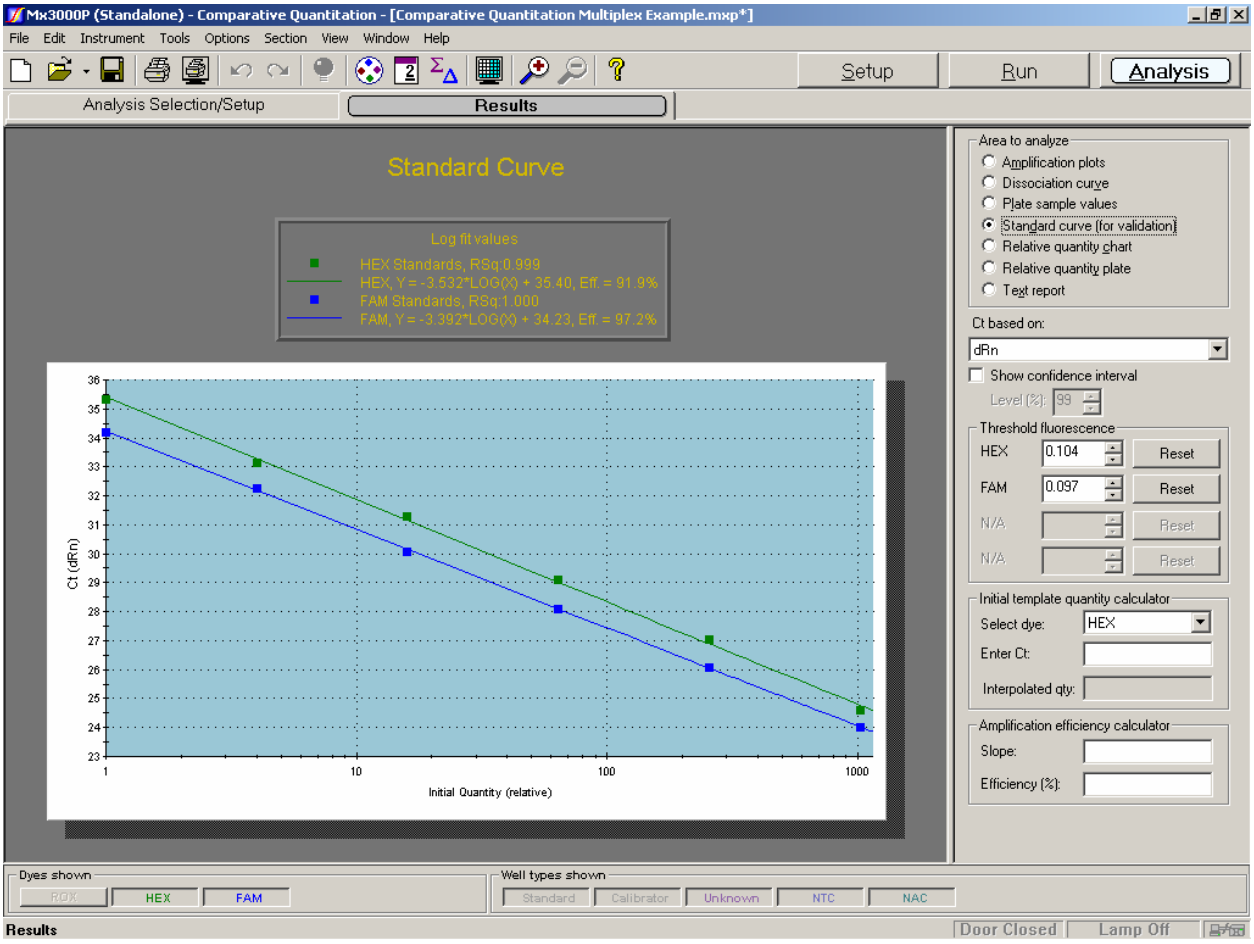
The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

The **Last cycle** setting also affects the reporting of Ct. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct**.

Comparative Quantitation Standard Curve

The **Standard Curve** is a plot of the initial template quantity in the **Standard** wells (X-axis), versus the **Ct** (threshold cycle). A least mean squares curve fitting algorithm is used to generate the standard curves displayed. Curves are displayed for each dye for which data was collected in **Standard** wells. **Unknown** wells selected during **Analysis Selection/Setup** are also plotted on the standard curve (using a different data point marker) based on their measured **Ct** values.

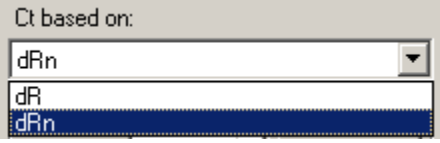
If less than two **Standard** wells were selected in **Analysis Selection/Setup**, no data will be plotted on this chart. (Note that after validation, many Comparative Quantitation experiments will not include a standard curve.)



Analysis Options

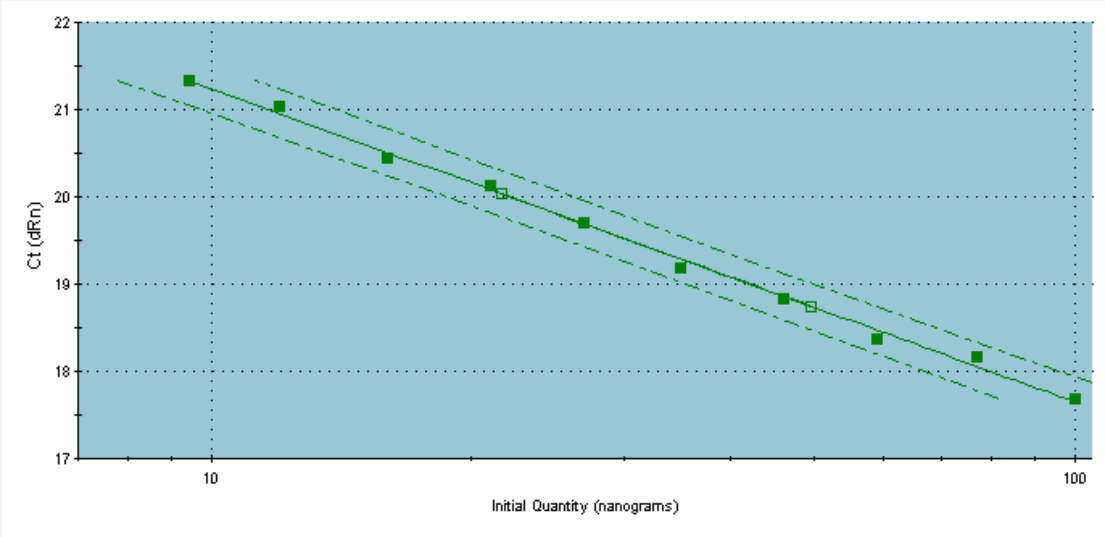
Fluorescence Data Type for Ct Determinations

Select the type of fluorescence data to be used for Ct determinations from the **Ct based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected, reference dye-normalized fluorescence).



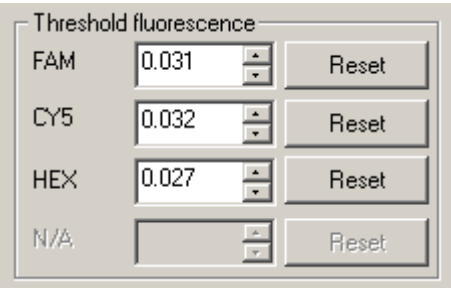
Confidence Interval

The confidence interval for each standard curve may be displayed by selecting the **Show confidence interval** checkbox. The resulting graph shows the confidence limits as hashed lines. The lines show the range of Initial Quantity values at a particular Ct that cannot be statistically distinguished from the fit line with more certainty than the confidence level (**Level %**) selected. The width of the confidence interval is an indicator of the quality of the fit of the data to the standard curve. Confidence interval calculations require a **Replicates** setting of **Treat individually**. Confidence level values may be exported to an Excel file, a text file or an XML file by using the **Export Chart Data** commands on the **File** menu.



Threshold Fluorescence

The **Threshold fluorescence** value for each dye, as calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**, is listed on the command panel. If desired, the threshold fluorescence for a given dye may be adjusted manually by entering a new value in the spin control box for the dye. (To restore the software-calculated values, click **Reset**).

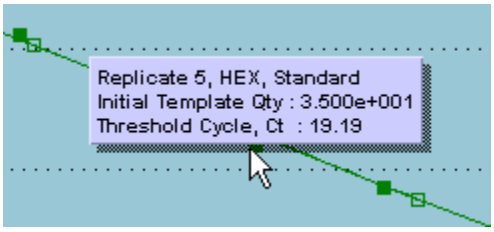


Excluding Well/Dye Data from Analysis

To exclude a specific well/dye data point from analysis, double click on the data point on the chart. Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen. To restore an excluded data point, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To display information about a specific data point on the plots, place the cursor over the point of interest to display the following information: well location or replicate number, dye name, well type, the initial template quantity and the Ct.



If the well is a **Standard**, the initial template quantity is the amount assigned to the well during plate setup. If the well is an **Unknown**, the initial template quantity is the interpolated amount based on the standard curve.

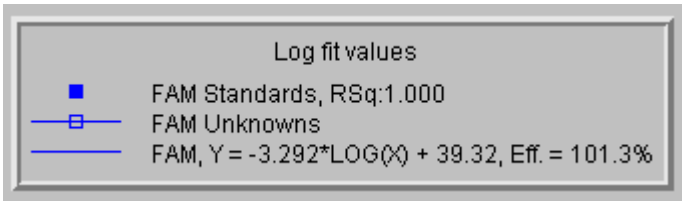
Note *If* ***Display tooltips*** *is cleared (deselected) on the* **Display** *tab of the* **Preferences** *dialog box, this well information will not be displayed.*

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls important display features including whether the **Legend** is displayed or hidden. See *Graphs and Graph Properties* for more information.

Interpretation and Use of Curve Attributes

RSq Value, Slope, and Amplification Efficiency

The graph legend displays information about each curve plotted. (If no legend is displayed on the screen, open the **Graph Properties** dialog box by double-clicking on the graph, and then select the **On** radio button under **Legend**.) Besides correlating plot markers with dyes and well types, the legend displays the R Squared (**RSq**) value and the equation for the line [y = m*log(x) + b, where m is the slope of the line]. The amplification efficiency (**Eff.**), calculated from the slope, is also displayed.



The **RSq** value is an indicator of the quality of the fit of the standard curve to the **Standard** data points plotted. The value will always be between 0 and 1. The closer the value is to 1, the better the fit of the line.

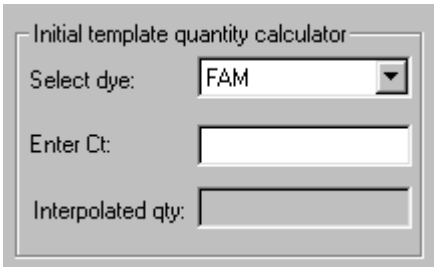
The slope of the curve is directly related to the average amplification efficiency throughout the cycling reaction. The equation that relates the slope to amplification efficiency is:

PCR Efficiency = $10^{(-1/\text{slope})} - 1$

where **PCR Efficiency** corresponds to the proportion of template molecules that are doubled every cycle. From this equation it follows that a reaction with 100% efficiency will result in a slope of –3.322.

Initial Template Quantity Calculator

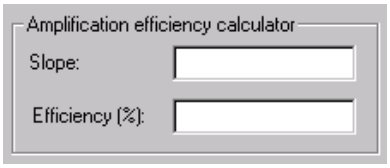
To use the displayed standard curve to calculate an interpolated relative quantity of template in another sample from the run based on the Ct, choose the dye from the **Select dye** menu, then enter the **Ct** value. The software will calculate and display the interpolated relative initial template quantity (**Interpolated qty**).



Amplification Efficiency Calculator

Enter either a **Slope** value or an amplification **Efficiency** value into the **Amplification efficiency calculator** to convert between values, based on the following equation:

PCR Efficiency = $10^{(-1/\text{slope})} - 1$



Implementing Standard Curve Results in Comparative Quantitation Experiments

The **Standard Curve (for validation)** functionality is included in this experiment type to facilitate the determination of the efficiencies of amplification for the gene of interest and the **Normalizer**, a critical step in the initial optimization of **Comparative Quantitation** experiments.

After a particular assay using a particular **Normalizer** has been validated, standard curves may be omitted from later experiments. Some researchers also include standard curves in optimized assays for validation of results.

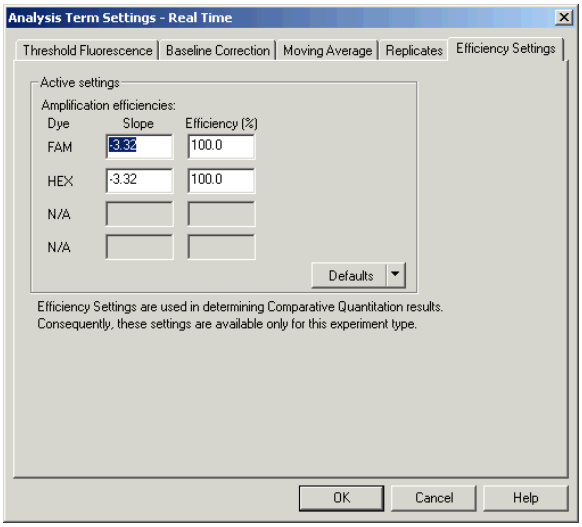
Determination of Amplification Efficiencies

Define a serial dilution set of wells for two different targets as **Standards** with their corresponding initial quantities, and then analyze the results using the **Standard Curve (for validation)** plot. To determine the amplification efficiencies, enter the reported slope of each curve into the **Amplification efficiency calculator**.

Recording Efficiencies for Use in Relative Quantity Calculations

Enter the slope or efficiency values for the two targets in the **Efficiency Settings** tabbed page of the **Analysis Term Settings** dialog box. Changing these values from the default values (corresponding to 100% efficiency) will cause the Mx3000P software to automatically compensate for differences in efficiencies for the two targets when reporting relative quantities on other **Results** screens. Note that using this feature to compensate for differences in amplification efficiency requires that the GOI and **Normalizer** be detected with different dyes.

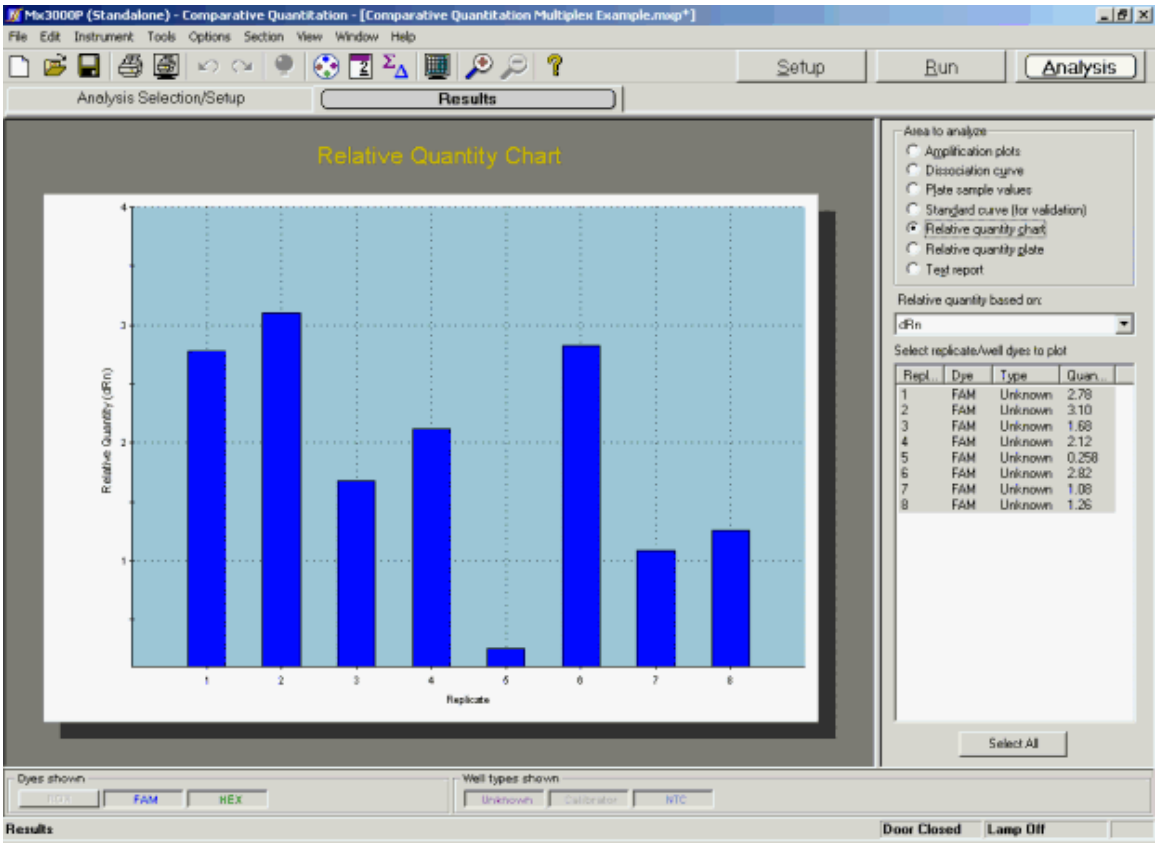
Access the **Efficiency Settings** page by selecting **Analysis Term Settings** from the **Options** menu.



Comparative Quantitation Relative Quantity Chart

The **Relative Quantity Chart** screen, accessed through the **Results** tab, shows a graph depicting the relative quantity values for each well relative to the **Calibrator.** The relative quantity of the **Calibrator** is automatically defined as 1.0 and is not displayed on the graph. Relative quantities may be displayed in either bar graph (shown) or line plot format. See *Display Options*, below, for more information.

When replicates are treated individually (in **Analysis Selection/Setup**), the X-axis displays **Well Ids** (in A1 through H12 format). When replicates are treated collectively, relative quantity values are plotted against the **Replicate** symbol.



Analysis Options

Relative Quantity Based On

Under **Relative quantity based on,** specify the form of the fluorescence data to be used in relative quantity calculations. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected, reference dye-normalized fluorescence).

Display Options

To change the style of the relative quantity chart from bar graph to line plot, or the reverse, double-click on the graph to open the **Graph Properties** dialog box. In the **Style** section of the dialog, select **Plot** to display the data as a line plot or **Bar** to display the data as a bar graph.

To restrict the results shown to a subset of the replicate set or well/dye combinations, use the **Select replicate/well dyes to plot** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

To display information about a specific well or replicate set, place the cursor over the bar or point corresponding to the well/replicate set of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

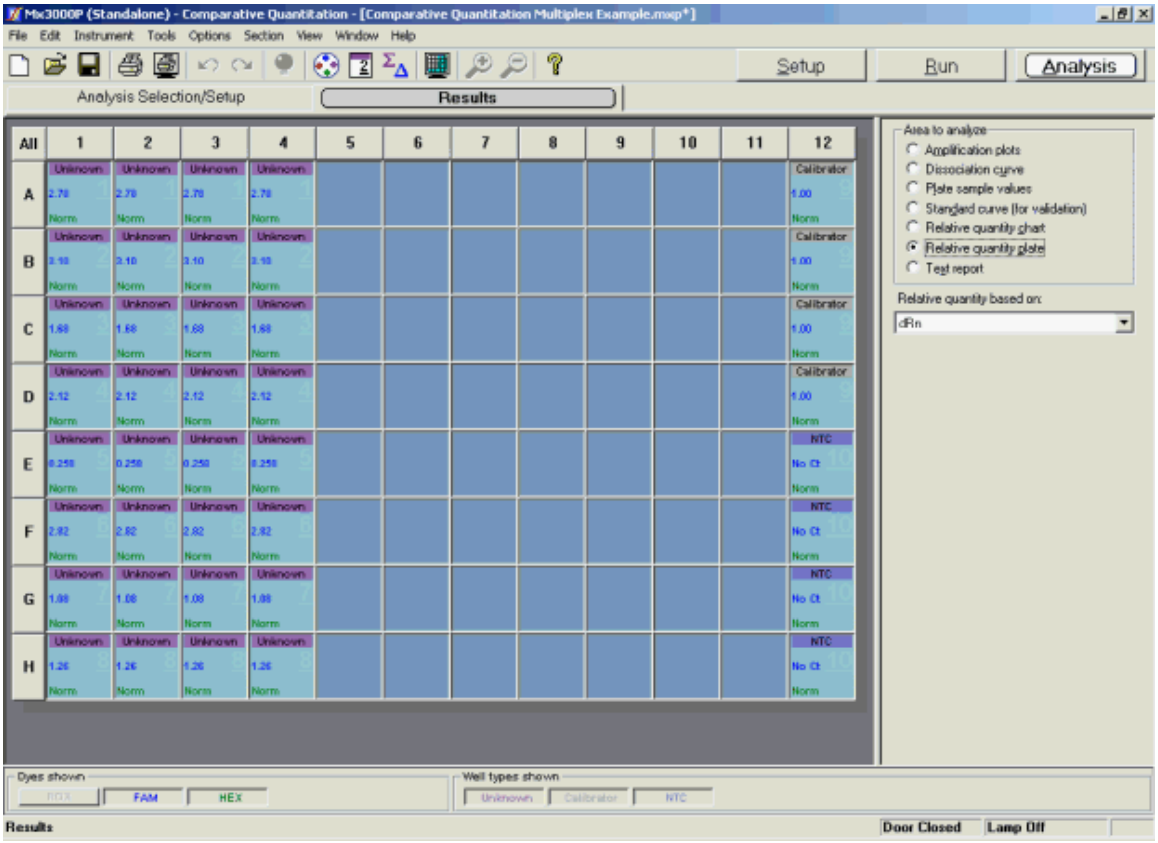
The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including the attributes of the axes. See *Graphs and Graph Properties* for more information.

Comparative Quantitation Relative Quantity Plate

The **Relative Quantity Plate** screen displays the calculated relative quantities of template present in the wells before thermal cycling in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Relative initial template quantity for each dye associated with a gene of interest. For **Unknowns**, the value relative to the **Calibrator** after correction for **Normalizer** quantities is reported. For all well types, the reference dye (**Ref**) and the normalizer dye (**Norm**) are not quantified
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)
- Association symbol, if used



Analysis Options

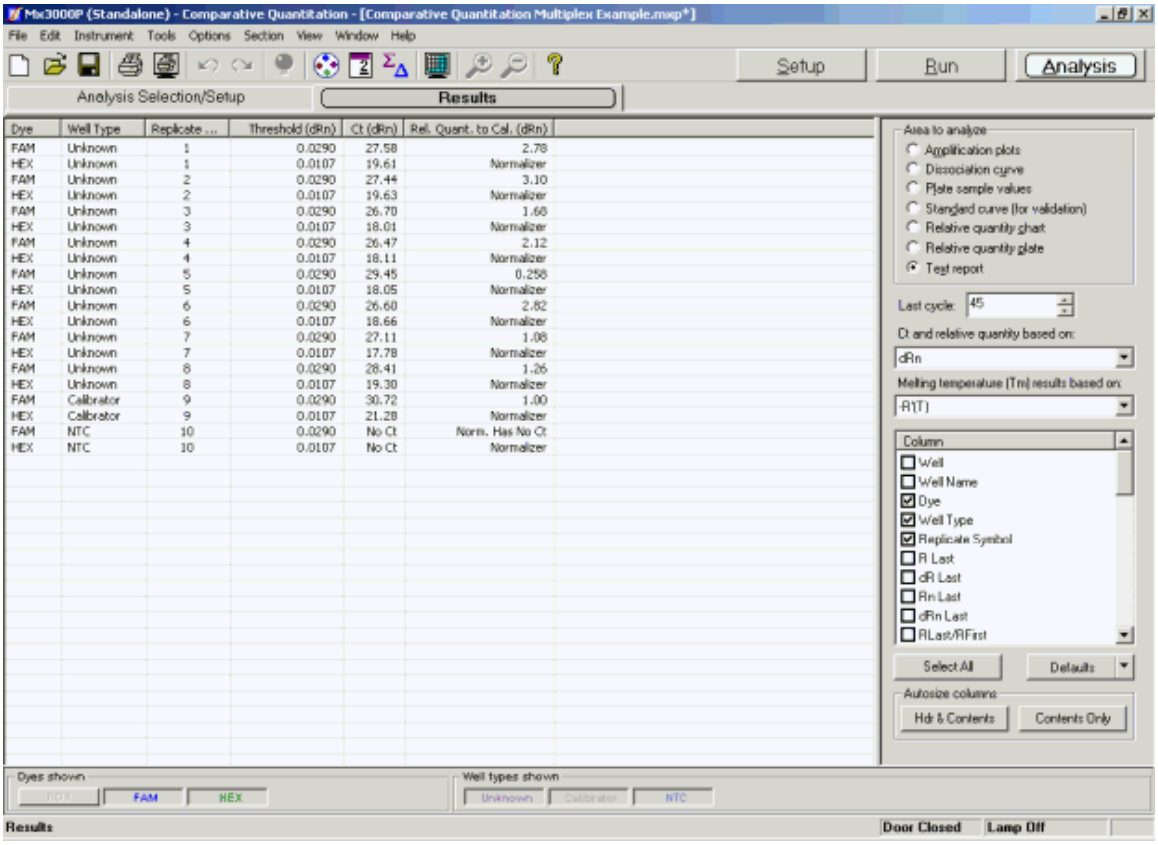
Relative Quantity Based On

Under **Relative quantity based on,** specify the form of the fluorescence data to be used to in relative quantity calculations. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected, reference-dye normalized fluorescence).

Relative quantities are calculated from the **Ct** values for the gene of interest and the **Normalizer** in both the **Unknown** wells and in the **Calibrator** wells. Therefore, normalizing the data for the reference dye indirectly affects the relative quantity values through changing the **Ct** values.

Comparative Quantitation Text Report

The **Text Report** screen shows the data from a run in text format.



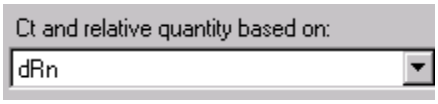
Analysis Options

Last Cycle

The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported when fluorescence value columns (such as **dRn Last**) are selected for display. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

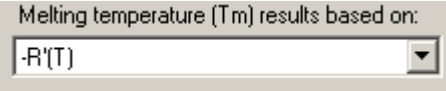
The **Last Cycle** setting also affects the reporting of **Ct** and **Relative Quantity**. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct**.

Ct and Relative Quantity Based On



Select the type of fluorescence data that the reported **Ct** and **Relative Quantity** should be based on from the **Ct and relative quantity based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected fluorescence) and **dRn** (baseline-corrected, reference dye-normalized fluorescence).

Tm Results Based On



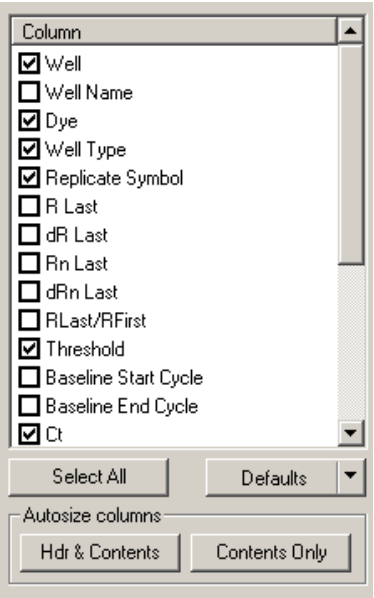
Select the type of fluorescence data plot that the reported melting temperature (**Tm**) should be based on from the **Melting temperature (Tm) based on** menu on the command panel. Available designations are: **-R'(T),** for plots based on raw fluorescence (the negative of the first derivative) of and **-Rn'(T),** for plots based on the normalized fluorescence (the negative of the first derivative).

Display Options

Columns Included in the Report

Select the **Columns** to be included in the text report by checking the boxes next to the column names. To exclude columns that are selected by default, click on the check box to deselect the column name. To select all columns for display, click **Select All**. The default settings for column selection can be restored by expanding the **Defaults** menu and then clicking **Get**.

If the included columns will not fit in the screen, a horizontal scroll bar appears at the bottom of the window to allow horizontal scrolling.



Column Width

To facilitate viewing data, columns can be automatically sized. Clicking the **Contents Only** button will cause each column to be automatically resized to the width of the widest data cell in that column (some column headers may be only partially displayed using this setting). Clicking the **Hdr & Contents** button will cause each column to be resized to the widest cell in that column, including the header cell.

Columns can also be resized manually by dragging the column dividers with the mouse.

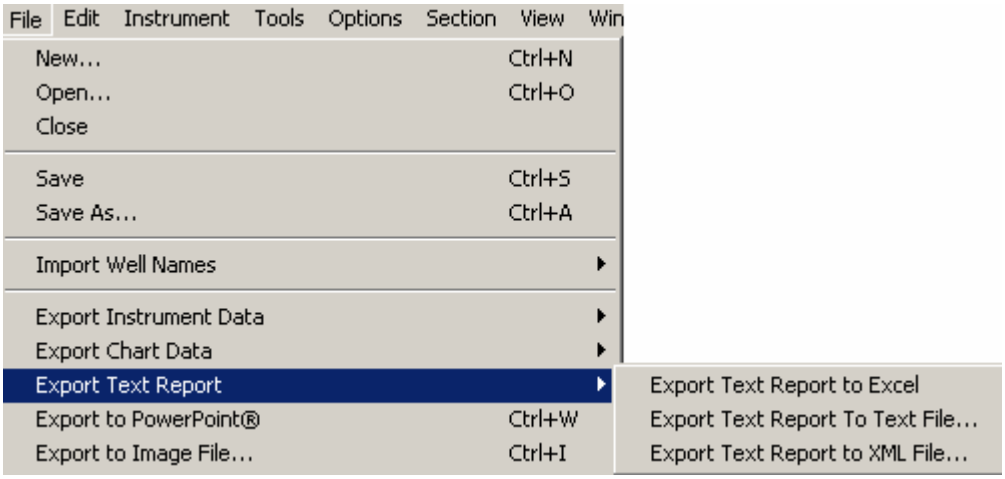
Specialized Comparative Quantitation Text Report Columns

Rel. Quant. to Norm.	The estimated quantities of gene of interest relative to the **Normalizer** target within that well or associated to it during **Plate Setup**, regardless of the **Calibrator.** This value is simply an approximation and is not intended as a quantitative result. The calculations are done for each individual well (or set of replicates), regardless of well type.
Rel. Quant. to Std. (for validation)	The quantitative determination of the (relative) initial quantity based on the standard curve for the same dye and target. Note that for experiments that include two standard curves using the same dye (e.g. one for the gene of interest and one for the **Normalizer**), wells for only one standard curve should be selected for each round of analysis. Alternatively, detection of two targets with the same dye may be distinguished by renaming the dye used for one target as discussed in Using a Single Dye for Detection of Gene of Interest and Normalizer.
Rel. Quant. to Cal.	The relative quantity of the gene of interest corrected for the quantity of the **Normalizer** target, and relative to the **Calibrator** sample. (For example, a **Rel. Quant. to Cal** of 2 means that there is twice as much target in the **Unknown** sample as in the **Calibrator**, when the numbers are corrected for the **Normalizer** quantity in each well.)

A note on standard deviations: Each reported quantity is associated with a standard deviation and a coefficient of variation (cv). When replicates are treated collectively, these values are calculated as zero, since replicate variability is eliminated in the collective treatment. When replicates are treated individually, quantities are calculated for each well based on their individual Ct values and on the average Ct values for the respective **Normalizer** and **Calibrator**. The standard deviation of the calculated relative quantities for a group of replicate wells is calculated for each replicate set. The coefficient of variation is calculated as the standard deviation divided by the average.

Exporting Text Report Data

Text report data can be exported from the application in text, Microsoft Excel spreadsheet or XML format for analysis. Text report exporting options are available from the **File** menu. See *Exporting Data to Other Applications* in the *File Menu* section for more information.



Mx3000P System Experiments: SYBR Green (with Dissociation Curve)

The SYBR Green Experiment Type

SYBR Green experiments typically use a **Standard Curve** to quantitate the amount of target present in **Unknown** samples using SYBR Green I dye for detection of double-stranded DNA (dsDNA). The **Thermal Profile** includes a dissociation curve, used to verify that the majority of fluorescence detected can be attributed to the labeling of specific PCR products, and to verify the absence of primer-dimers and sample contamination. See *Detection using SYBR Green Dye* for more information on the general properties of SYBR Green I dye.

The accumulation of dsDNA can be assessed by the incorporation of SYBR Green I dye into a PCR reaction. A major advantage of this approach is that no fluorescence-labeled probes are required. A limitation is that dye binding to both specific and non-specific PCR products generates the same fluorescence signal. Including a dissociation curve in the experiment facilitates the process of optimizing the assay for conditions that minimize the formation of non-specific products, and provides a means for verifying the predominance of specific products in each run. Using this approach, SYBR Green dye-based assays have been successfully used for a wide variety of QPCR and QRT-PCR applications.

In **SYBR Green** experiments, the Mx3000P instrument detects the fluorescence of the SYBR Green fluorophore during each cycle of the thermal cycling process and a **Fluorescence** value is reported at each cycle. Fluorescence values are generally acquired in the annealing and extension stages of thermal cycling. **Fluorescence** results are typically displayed in an **Amplification plot**, which reflects the change in fluorescence during cycling.

The initial copy numbers of RNA or DNA targets can be quantified using real-time PCR analysis based on threshold cycle (**Ct**) determinations. The Ct is defined as the cycle at which a statistically-significant increase in fluorescence (above background signal measured in the early rounds of amplification) is detected. The threshold cycle is inversely proportional to the log of the initial copy number. In other words, the more template that is present initially, the fewer the number of cycles required for the fluorescence signal to be detectable above background.

Typical **SYBR Green** experiments use a standard curve to quantitate the amount of target present in an **Unknown** sample. In this method, a series of **Standards**, containing a dilution series of a known amount of target, are amplified to generate a curve that relates the initial quantity of the specific target in the **Standard** sample to the **Ct**. The standard curve is then used to interpolate the initial template quantity in **Unknown** wells based on the **Ct** values determined for the **Unknown** wells.

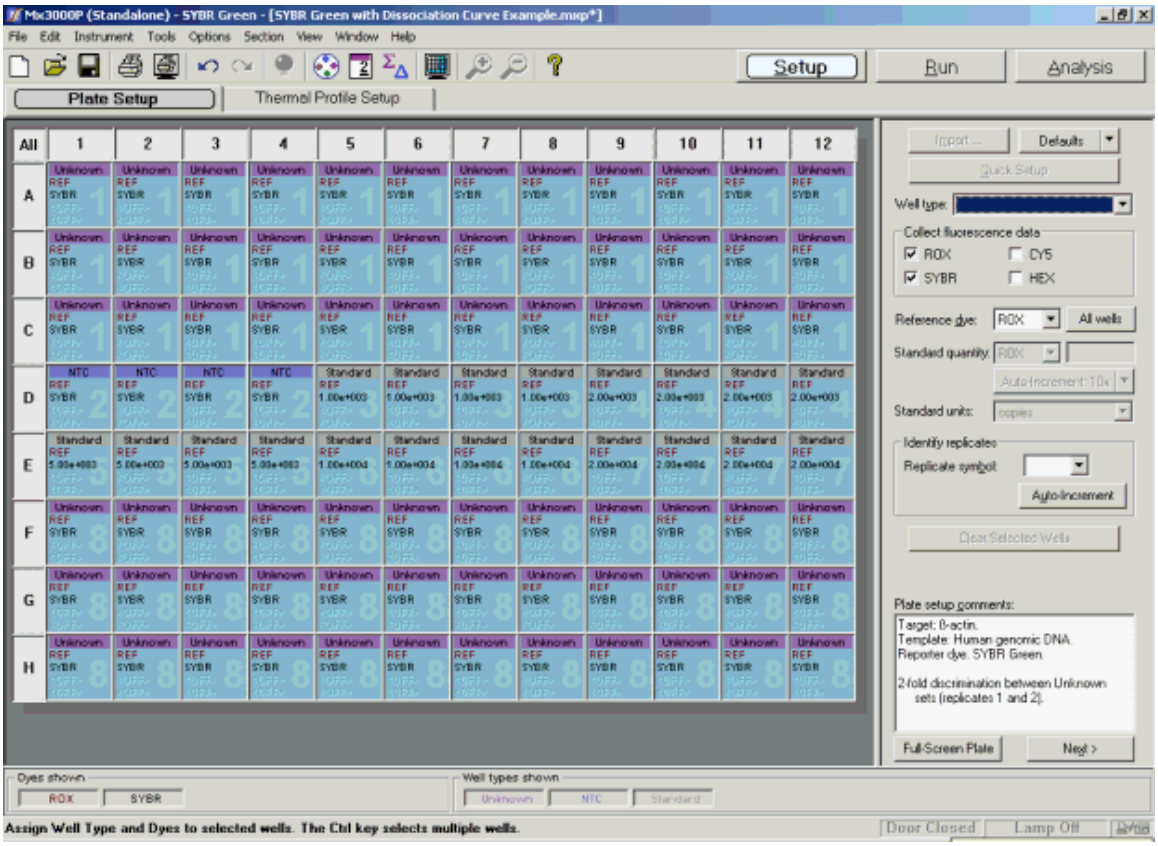
Use of the Dissociation Curve

The **SYBR Green** experiment has two major phases: **Amplification** and **Dissociation**. The **Amplification** phase corresponds to the PCR portion of the experiment, and results in the generation of dsDNA. In the **Dissociation** phase, the dsDNA product is melted into ssDNA by a stepwise increase in temperature, with fluorescence data being collected at each temperature step. The magnitude of the reduction in fluorescence intensity of the SYBR Green dye due to its release from dsDNA provides an indicator of the amount of dsDNA dissociated at each step in the dissociation curve. For data analysis, **Fluorescence** (or its first derivative) is plotted as a function of **Temperature**. In thermal denaturation profiles for complex nucleic acid mixtures such as those generated during PCR amplification, two semi-discrete populations with different transition temperatures can typically be identified in the first derivative plots. Populations with a Tm of 80°C or higher correspond to the larger PCR products, and can usually be assigned to the specific DNA product. DNA products displaying melting temperatures of < 75°C correspond to non-specific DNA products. It is important to note that these populations are not necessarily homogeneous, and may contain multiple PCR product species.

Analysis often includes evaluation of the dissociation curve for no-template-control (**NTC**) reactions in order to monitor the presence of primer-dimers and of contaminating template that might be contributing to the fluorescence signal.

SYBR Green Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical plate setup for a **SYBR Green** experiment.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) by using the **Well type** menu on the command panel. The well types available in a **SYBR Green** experiment are described in the table below.

Well Type	Description
Unknown	Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target.
Buffer	Contains only buffer, used to monitor the background fluorescence attributable to the buffer.
NAC	No amplification control; contains all reaction components except DNA polymerase.
NPC	No probe control; contains all reaction components except the fluorescence-labeled probe.
NTC	No template control; contains all reaction components except the template nucleic acid.
Standard	Contains a complete reaction mixture including a known concentration of target nucleic acid. Used to generate a standard curve, which is then used to relate the threshold cycle (**Ct**) to initial template quantity in **Unknown** wells.
No RT	No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.
Dye-specific Negative Control	Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment is rarely used in SYBR Green experiments (most will use the **NTC** well type assignment for reactions lacking template).
Dye-specific Positive Control	Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment, which qualitatively designates a positive signal for the target without specifying the absolute amount, is rarely used in SYBR Green experiments (most will include **Standard** wells with known amounts of target).

After assigning the **Well type** to the selected well(s), select the dyes for which data should be collected in these wells using the **Collect fluorescence data** check boxes. SYBR Green I dye data are collected using the FAM filter path. If **FAM**, rather than **SYBR** appears on the setup screen, SYBR Green dye may be assigned to this path in the **Optics Configuration** dialog box.

If you are using a passive reference dye in the selected well(s) select the dye from the **Reference dye** menu. If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.

If the selected well(s) is a **Standard**, enter the amount of template. To do this, first select the dye from the pulldown menu following **Standard quantity.** Next, enter the quantity of template added to the selected well(s) as a decimal number (for example, 400 or 1.234). Specify the units for the quantity in the **Standard Units** dropdown box. Instead of assigning quantities to wells on an individual basis, it is also possible to assign quantities to **Standard** wells sequentially using the **Auto-Increment** feature.

Assign the replicate set number to the selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

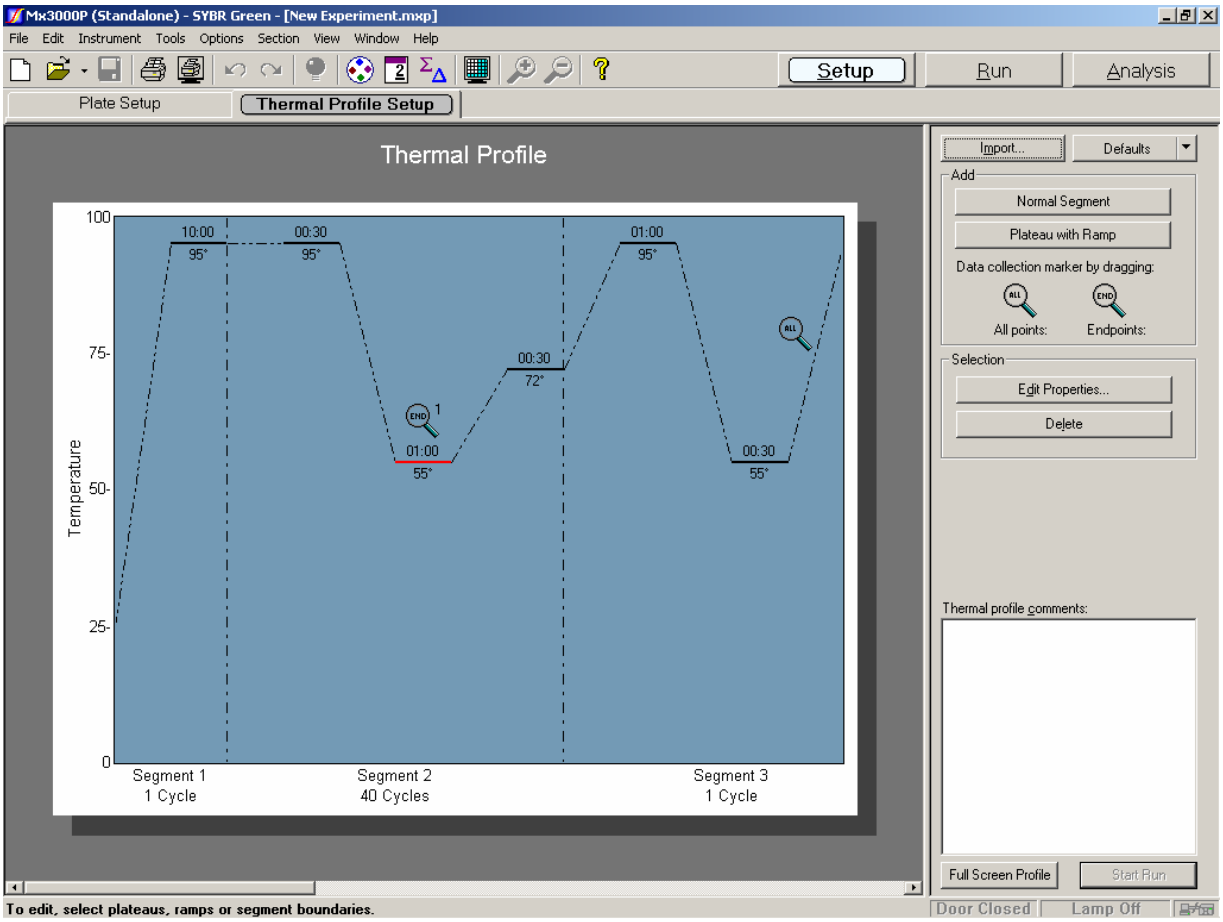
Once plate setup is complete, click **Next** to proceed to the **Thermal Profile Setup** screen.

SYBR Green Thermal Profile Setup

When the **Thermal Profile Setup** tab is selected in a new SYBR Green experiment, a default thermal profile opens. The default thermal profile should be modified to the specific requirements of the new experiment, as discussed below. It is also possible to **Import** the thermal profile from an existing experiment into the new experiment.

Shown in the figure below is the default thermal profile for a SYBR Green experiment when using the software's **Original Settings**. (If you are using a user-defined default set, the default thermal profile stored in that default set will appear when the **Thermal Profile Setup** screen is opened. See *Preferences-Defaults* for more information about the use and management of **Defaults**.)

In the profile below, segment 1 is an activation step of 95°C for 10 minutes that is compatible with a hot start reaction. Segment 2 is the PCR **Amplification** phase of the experiment and consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 55°C/1 minute for annealing, and a plateau of 72°C/30 seconds for extension. Amplification data are collected at the end of each annealing plateau. Segment 3 contains the **Dissociation curve**. The default profile dissociation curve begins with a 1-minute incubation at 95°C to melt the DNA and then a 30-second incubation at 55°C. This is followed by a ramp up to 95°C with **Allpoints** data collection performed during the ramp. Data collection during the ramp slows the ramp rate to 0.01°C/sec to allow for a slow melt of the PCR product.



Modify the default thermal profile as necessary. At minimum, ensure that the **Amplification** (Segment 2) annealing temperature setting is appropriate for the PCR primer pair used in the current experiment. The properties of all other plateaus, ramps, and segments may also be adjusted. See *Thermal Profile Setup* in the *How-To (Detailed Protocols)* section for more information about changing the default thermal profile.

SYBR Green Data Analysis

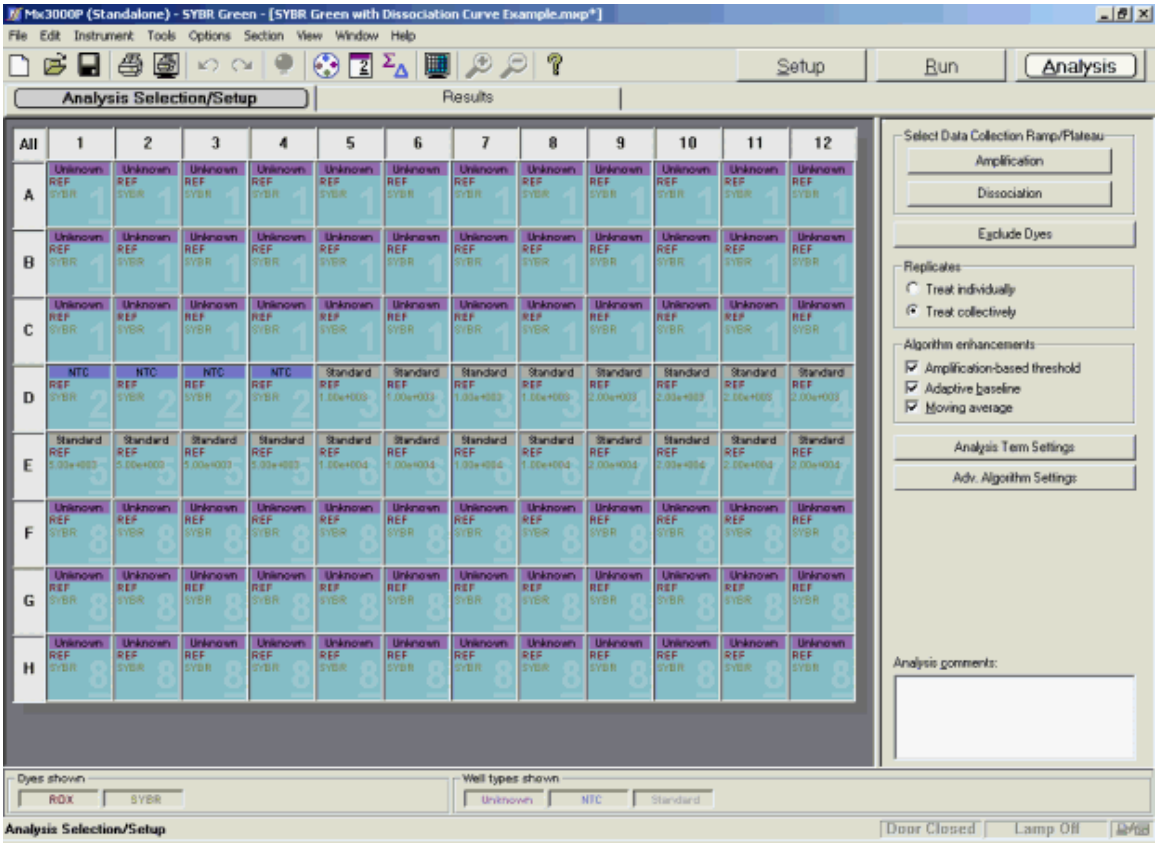
Analysis Setup

On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.

2. Select the segment (**Amplification** or **Dissociation**), and then the **position** (either the **ramp** or the **plateau**) from which data should be analyzed. The **Dissociation** segment should be selected only for viewing the **Dissociation curve**. All other **Results** screens require selection of a data set collected during amplification.

3. Specify the desired analysis settings on the command panel.



See Analysis Selection and Setup for more detailed instructions on using the features on this screen.

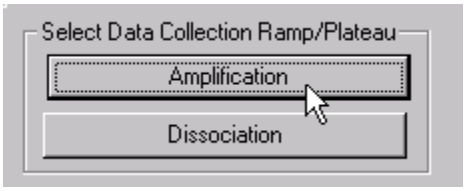
When all selections and settings are made, click the **Results** tab. The first of several available results screens will appear. Use the radio buttons under **Area to analyze** to navigate to different analysis screens. The following screens are available for SYBR Green analysis:

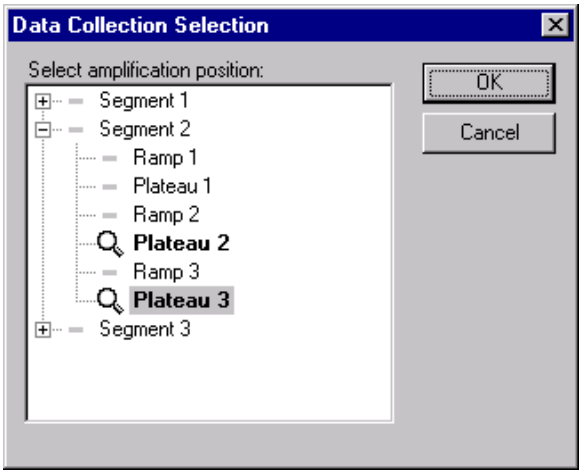
Amplification Plots
Dissociation Curve
Plate Sample Values
Standard Curve
Initial Template Quantity
Text Report

Selecting the Data Collection Ramp/Plateau

The **SYBR Green Dissociation Curve** experiment typically has two phases: **Amplification** and **Dissociation** (or melting curve). Data can be collected for each of these parts. Prior to data analysis, it is necessary to select which data set will be used in the **Dissociation Curve** analysis (**Dissociation** data set) and which data set will be used for quantitative analyses (**Amplification** data set).

To select the data set to be used in the **Amplification** analysis (**Amplification plots**, **Plate sample values**, **Standard curve**, **Initial template quantity**, and **Text report**), click **Amplification** from the **Select Data Collection Ramp/Plateau** box in **Analysis Selection/Setup**. Then select the appropriate ramp/plateau.

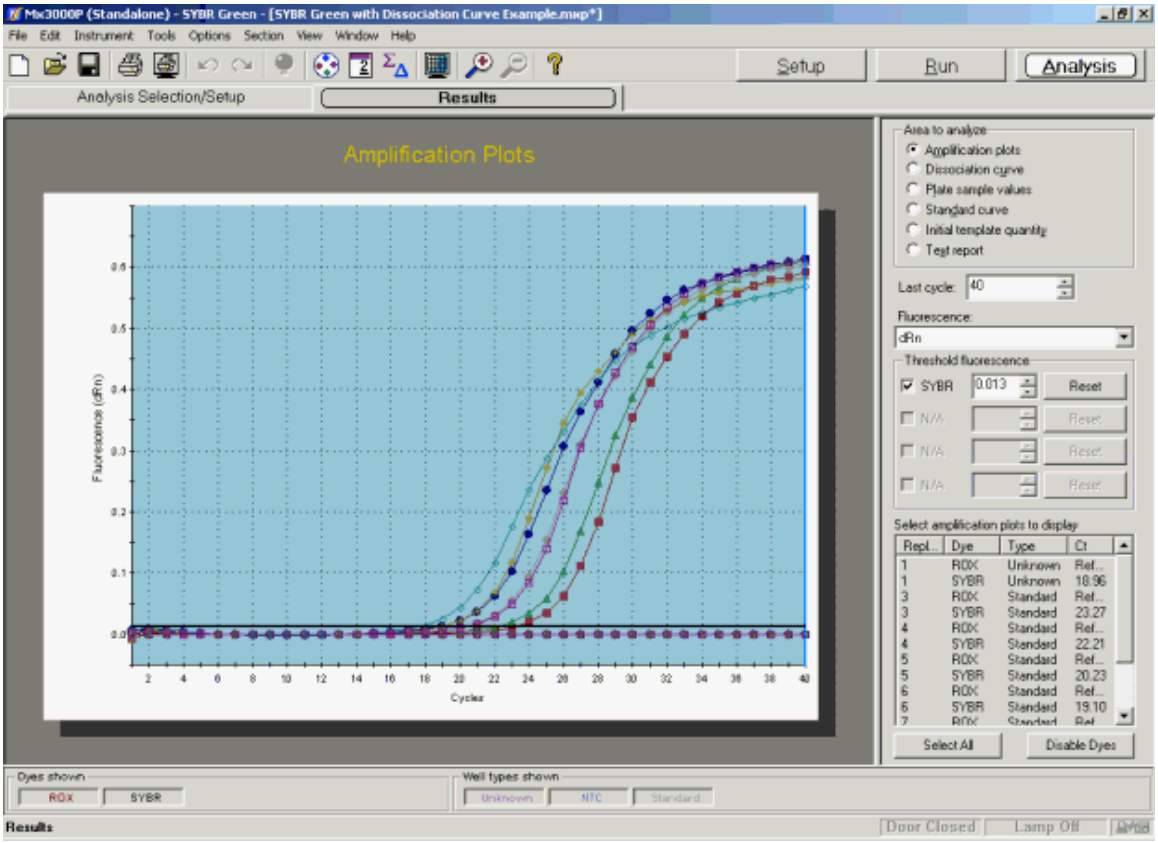




To assign a data set to use in the **Dissociation Curve** analysis, click **Dissociation** from the **Select Data Collection Ramp/Plateau** box and choose the appropriate data set.

SYBR Green Amplification Plots

The **Amplification Plots** screen, accessed through the **Results** tab, shows a plot of cycles versus fluorescence for a ramp or plateau on which data are gathered. Select the ramp or plateau for which data should be analyzed using the **Analysis Selection/Setup** screen.



Analysis Options

Fluorescence Data Type Plotted

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are: **R** (raw fluorescence), **dR** (baseline-corrected raw fluorescence), **Rn** (normalized fluorescence), and **dRn** (baseline-corrected normalized fluorescence).

Threshold Fluorescence

When basing plots on normalized fluorescence (**dR** or on **dRn**), the **Threshold fluorescence** value for each dye is listed on the command panel and is marked on the graph by a horizontal line. (Note that if a dye is hidden using the **Dyes shown** controls or if the check box next to the dye is cleared, the line corresponding to threshold fluorescence for that dye will not be shown on the graph.)

Threshold fluorescence is calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**. If desired, the threshold fluorescence may be adjusted manually. (To restore the software-calculated values, click **Reset**).

Baseline correction is required to establish a threshold fluorescence. Thus selections related to threshold fluorescence are unavailable for **R** or **Rn** data plots.

Last Cycle Setting

The **Last cycle** setting specifies the cycle from which the final (last) fluorescence values will be reported in the **Text Report** and **Plate Sample Values** screens. The position of the last cycle is marked on the graph with a solid vertical line.

This setting also affects the way Ct values are reported on other **Results** screens. While the **Last cycle** is not directly used to determine Ct, any wells with Ct values greater than the **Last cycle** value will be designated as **No Ct** in screens that display Ct.

Excluding Well/Dye Data from Analysis

To exclude the curve for one or more specific well/dye combinations from analysis, use either of the following two methods. (Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen.)

- From the list under **Select amplification plots to display**, select the curve(s) to be excluded and then click **Disable Dyes**.
- Double-click on any data point on the plot to be excluded. This action will exclude the entire data set for the well/dye corresponding to the plot.

To restore a plot that was disabled using either of these methods, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To restrict the plots shown to a subset of the selected well/dye combinations, use the **Select amplification plots to display** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

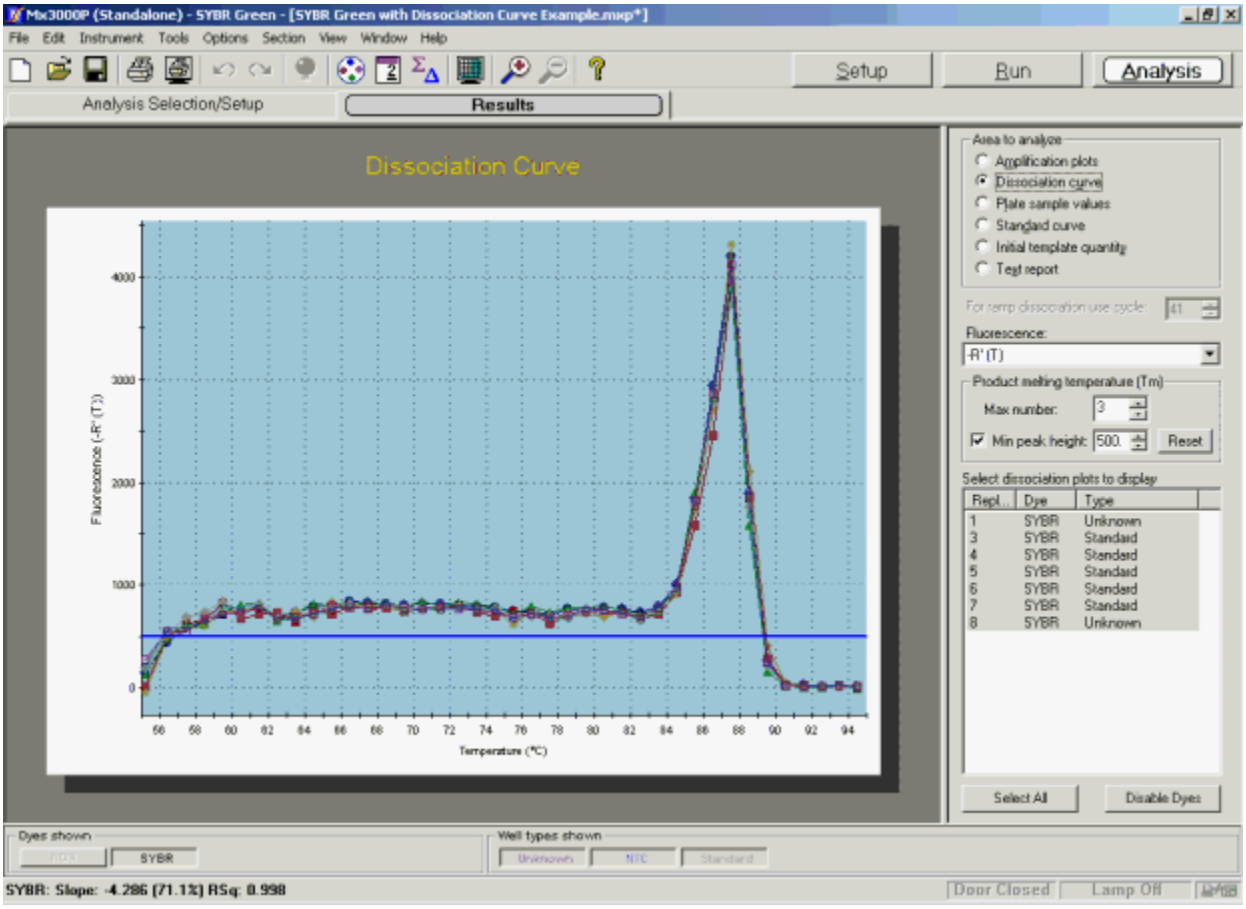
To display information about a specific data point on the plots, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** and **Plot Markers** are displayed or hidden. See *Graphs and Graph Properties* for more information.

SYBR Green Dissociation Curve

The **Dissociation Curve** screen, accessed through the **Results** tab, shows a plot of fluorescence (or its first derivative) as a function of temperature for the **Dissociation** segment of the experiment. Ensure that the data set collected during the **Dissociation** segment of the experiment is selected for analysis using the **Analysis Selection/Setup** screen.

The plot shown below is based on the first derivative of the normalized fluorescence reading multiplied by –1 [designated **–Rn´(T)**]. Plotting results in this way is generally appropriate for complex nucleic acid mixtures such as those generated during PCR amplification. In samples with amplicon, this type of plot will typically display at least two populations with different transition temperatures. Populations with a Tm of 80°C or higher correspond to the larger PCR products, and can usually be assigned as specific DNA product. DNA products displaying melting temperatures of < 75°C correspond to non-specific DNA products. It is important to note that these populations are not necessarily homogeneous, and may contain multiple PCR product species.



Analysis Options

Fluorescence Data Type Plotted

Dissociation curves may be based on four different types of fluorescence: **R** (raw fluorescence), **Rn** (normalized fluorescence), **–R´(T)**, (the first derivative of the raw fluorescence reading multiplied by –1), and **–Rn´(T)** (the first derivative of the normalized fluorescence reading multiplied by –1). Select the desired type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel.

Product Melting Temperature Listings Settings

The Mx3000P software will identify and report **Product melting temperatures** for up to six product populations from each dissociation curve. Set the maximum number of products for which a melting temperature should be calculated using the **Max number** spin control box. This setting controls only the maximum number; the software may detect and report a smaller number of product populations for a given curve.

When basing plots on the negative derivative of fluorescence [**–R´(T)** or **–Rn´(T)**], you may specify the minimum peak height to be used for designating product populations. This peak height minimum may be entered in the **Min peak height** box as a decimal number or may be set manually by dragging the horizontal line indicator on the graph to the desired position.

Display Options

To restrict the plots shown to a subset of the selected well/dye combinations, use the **Select dissociation plots to display** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

To exclude the curve for one or more specific well/dye combinations from view, select the curve(s) to be excluded and then click **Disable Dyes**. Disabling a particular well/dye combination requires replicates to be treated **individually** (this may be specified on the **Analysis Selection/Setup** screen). To restore a disabled plot, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

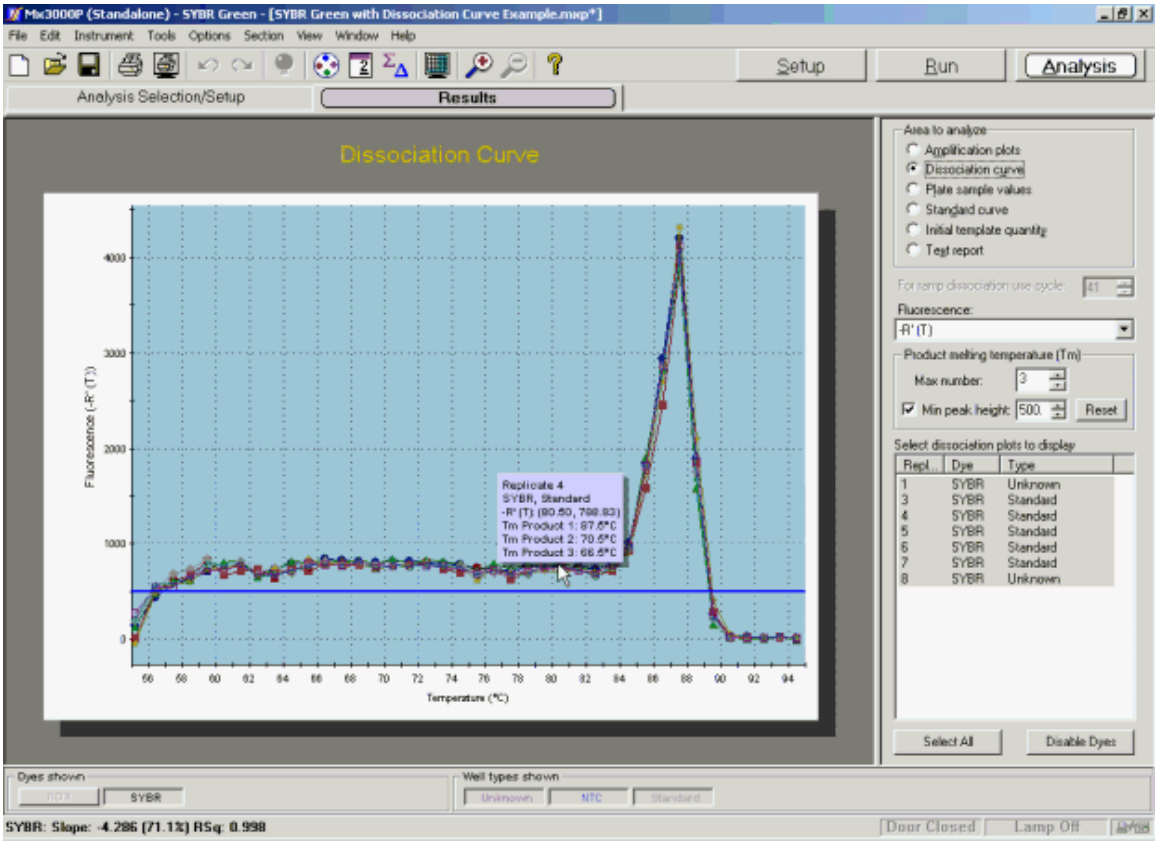
To display information about a specific data point on the plots, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** and **Plot markers** are displayed or hidden. See *Graphs and Graph Properties* for more information.

Reporting of Melting Temperatures (Tm)

Melting temperatures for each of the identified product populations are displayed when the cursor is placed over any point on the curve of interest.

Melting temperatures will also be reported on the **Text Report** screen when the appropriate **Tm Product** boxes are selected in the **Columns** selection on that screen.

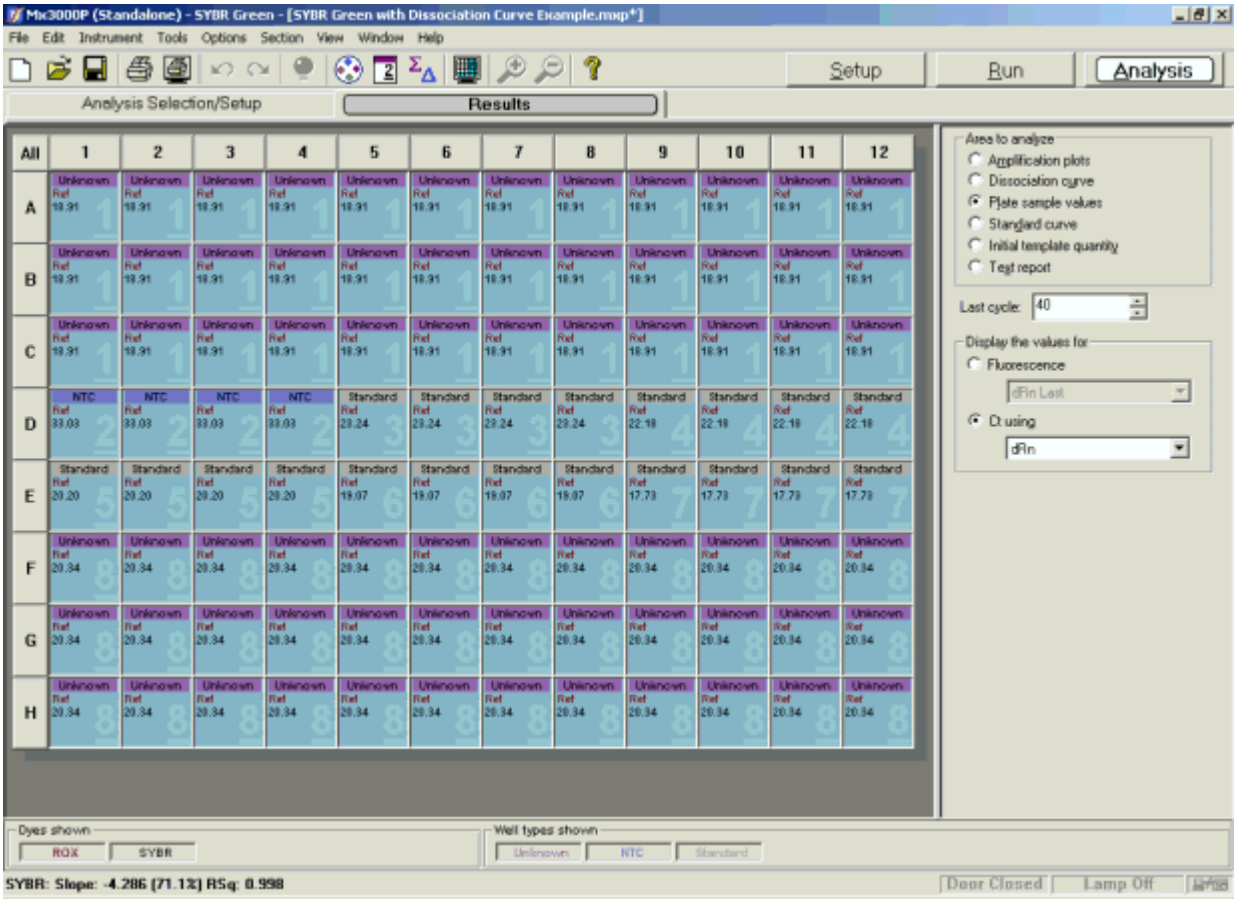


SYBR Green Plate Sample Values

The **Plate Sample Values** screen displays **Fluorescence** values or **Ct** values for the sampled wells in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Either the fluorescence or the Ct value for each dye for which data was collected
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)



Analysis Options

Display the Values for Fluorescence or Ct

Under **Display the values for**, specify the form of the data to be displayed by selecting either the **Fluorescence** or the **Ct using** radio button. You will also need to specify the type of fluorescence data to be either displayed or used in calculations by selecting a data type from the menu below the selected radio button. Refer to the tables below for descriptions of the fluorescence data type abbreviations in the menus.

When displaying **Fluorescence** values, the following fluorescence data types are available:

Data Type	How Calculated
R Last	final fluorescence
dR Last	final fluorescence minus the initial fluorescence
Rn Last	normalized final fluorescence
dRn Last	normalized final fluorescence minus the normalized initial fluorescence
R Last/R First	final fluorescence divided by the initial fluorescence

When displaying **Ct** values, Ct calculations may be based on the following fluorescence data types:

Data Type	How Calculated
dR	baseline-corrected fluorescence
dRn	baseline-corrected normalized fluorescence

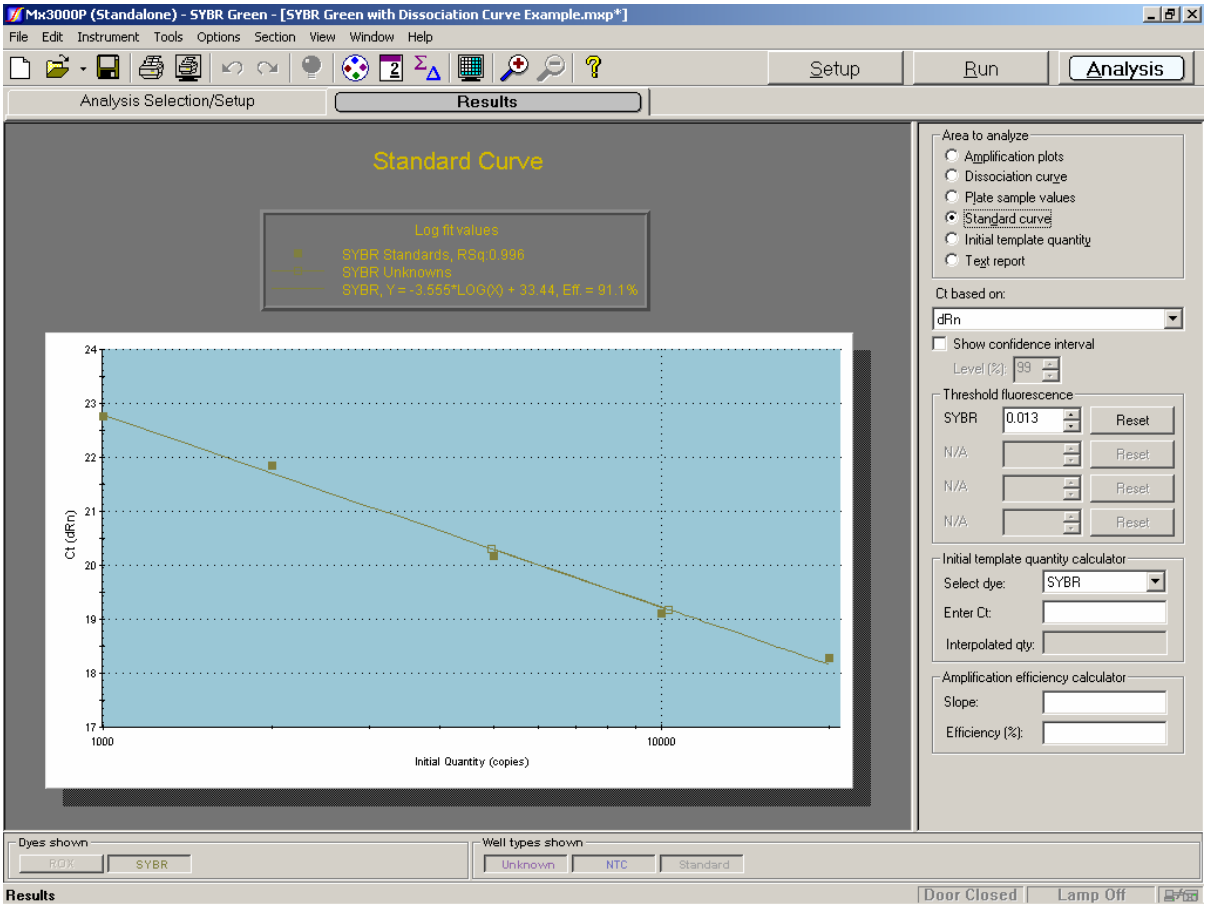
Last Cycle

The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

The **Last cycle** setting also affects the reporting of Ct. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct**.

SYBR Green Standard Curve

The **Standard Curve** is a plot of the initial template quantity in the **Standard** wells (X-axis), versus the **Ct** (threshold cycle). A least mean squares curve fitting algorithm is used to generate the standard curves displayed. Curves are displayed for each dye for which data was collected in **Standard** wells. **Unknown** wells selected during **Analysis Selection/Setup** are also plotted on the standard curve (using a different data point marker) based on their measured **Ct** values.

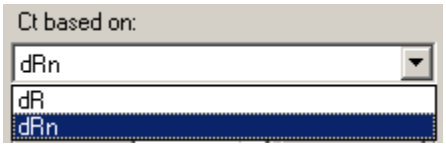


If less than two **Standard** wells were selected in **Analysis Selection/Setup**, no data will be plotted on this chart.

Analysis Options

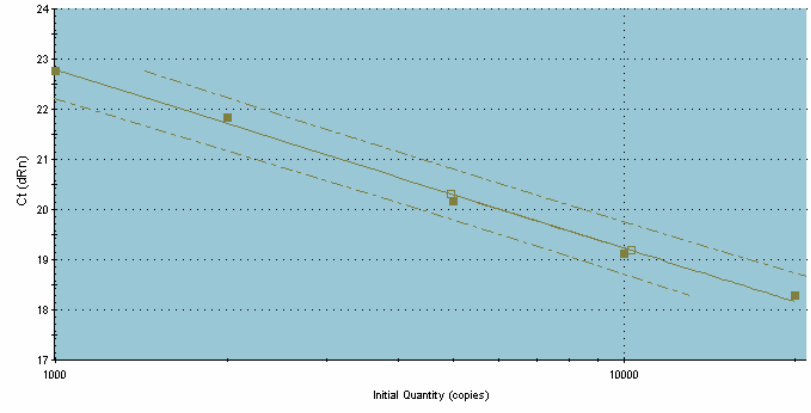
Fluorescence Data Type for Ct Determinations

Select the type of fluorescence data to be used for Ct determinations from the **Ct based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected normalized fluorescence).



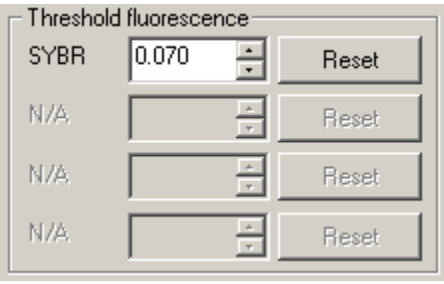
Confidence Interval

The confidence interval for each standard curve may be displayed by selecting the **Show confidence interval** checkbox. The resulting graph shows the confidence limits as hashed lines. The lines show the range of Initial Quantity values at a particular Ct that cannot be statistically distinguished from the fit line with more certainty than the confidence level (**Level %**) selected. The width of the confidence interval is an indicator of the quality of the fit of the data to the standard curve. Confidence interval calculations require a **Replicates** setting of **Treat individually**. Confidence level values may be exported to an Excel file, a text file or an XML file by using the **Export Chart Data** commands on the **File** menu.



Threshold Fluorescence

The **Threshold fluorescence** value for each dye, as calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**, is listed on the command panel. If desired, the threshold fluorescence for a given dye may be adjusted manually by entering a new value in the spin control box for the dye. (To restore the software-calculated values, click **Reset**).

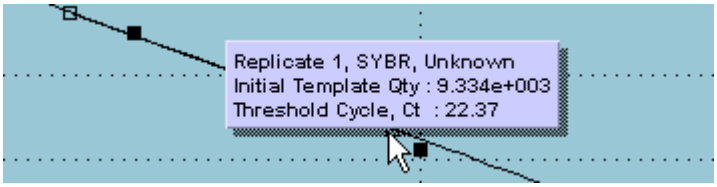


Excluding Well/Dye Data from Analysis

To exclude a specific well/dye data point from analysis, double click on the data point on the chart. Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen. To restore an excluded data point, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To display information about a specific data point on the plot, place the cursor over the point of interest to display the following information: well location or replicate number, dye name, well type, the initial template quantity and the Ct.



If the well is a **Standard**, the initial template quantity is the amount assigned to the well during plate setup. If the well is an **Unknown**, the initial template quantity is the interpolated amount based on the standard curve.

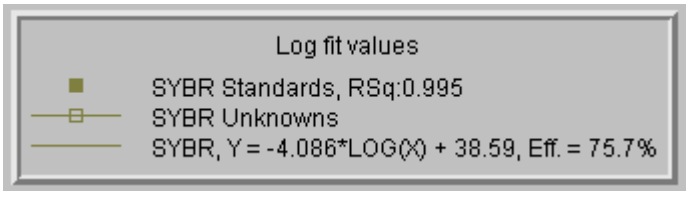
Note *If* ***Display tooltips*** *is cleared (deselected) on the* **Display** *tab of the* ***Preferences*** *dialog box, this well information will not be displayed.*

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** is displayed or hidden. See *Graphs and Graph Properties* for more information.

Interpretation and Use of Curve Attributes

RSq Value, Slope, and Amplification Efficiency

The graph legend displays information about each curve plotted. (If no legend is displayed on the screen, open the **Graph Properties** dialog box by double-clicking on the graph, and then select the **On** radio button under **Legend**.) Besides correlating plot markers with dyes and well types, the legend displays the R Squared (**RSq**) value and the equation for the line [y = m*log(x) + b, where m is the slope of the line]. The amplification efficiency (**Eff.**), calculated from the slope, is also displayed.



The **RSq** value is an indicator of the quality of the fit of the standard curve to the **Standard** data points plotted. The value will always be between 0 and 1. The closer the value is to 1, the better the fit of the line.

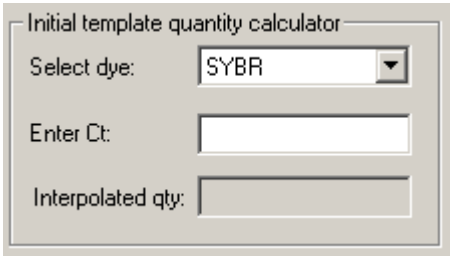
The slope of the curve is directly related to the average amplification efficiency throughout the cycling reaction. The equation that relates the slope to amplification efficiency is:

PCR Efficiency = $10^{(-1/slope)} - 1$

where PCR Efficiency corresponds to the proportion of template molecules that are doubled every cycle. From this equation it follows that a reaction with 100% efficiency will result in a slope of –3.322.

Initial Template Quantity Calculator

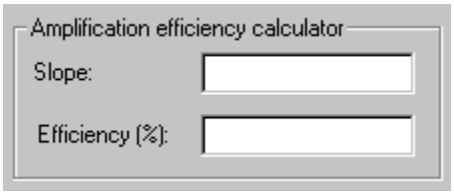
To use the displayed standard curve to calculate an interpolated quantity of template in another sample from the run based on the Ct determined for the sample, choose the dye from the **Select dye** menu, then enter a **Ct** value. The software will calculate and display the interpolated initial template quantity (**Interpolated qty**). Note that initial template quantities are automatically determined, based on the standard curves, for all **Unknowns** in the same experiment, and these results may be viewed on the Initial Template Quantity results screen.



Amplification Efficiency Calculator

Enter either a **Slope** value or an amplification **Efficiency** value into the **Amplification efficiency calculator** to convert between values, based on the following equation:

PCR Efficiency = $10^{(-1/slope)} - 1$



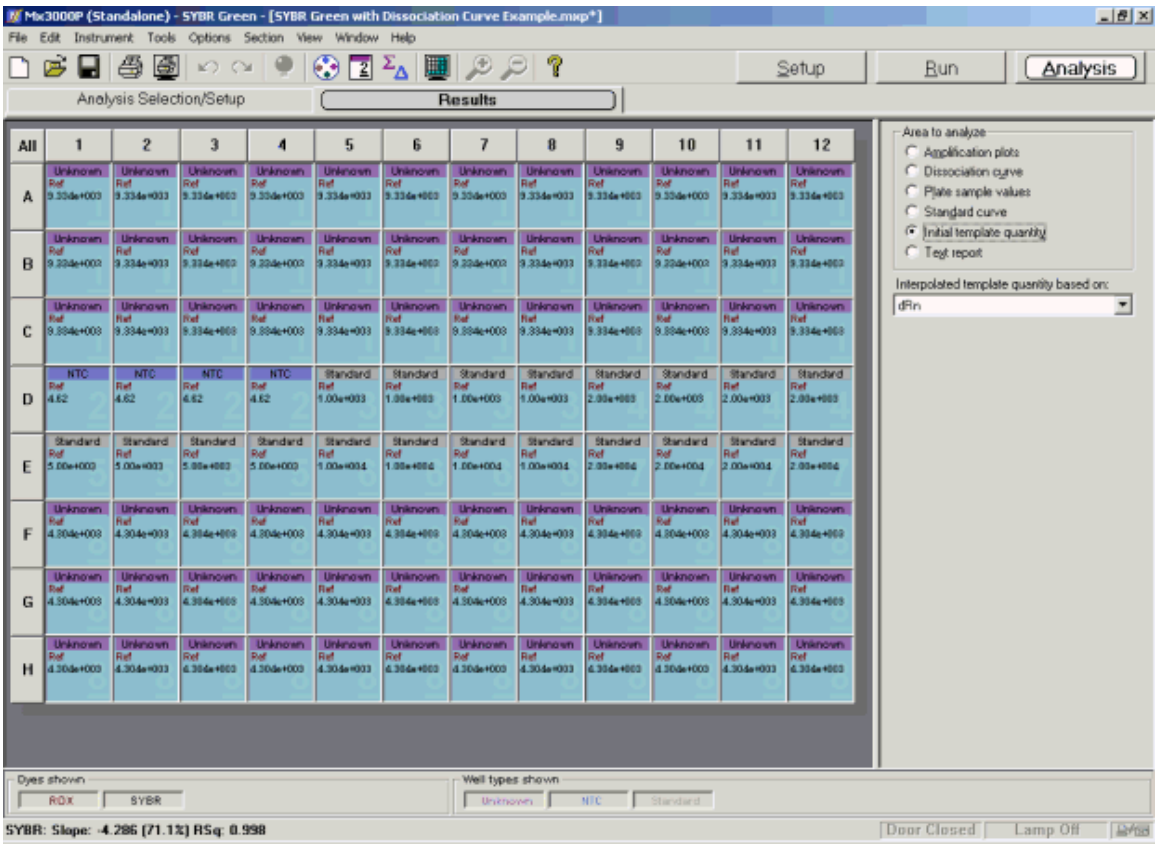
SYBR Green Initial Template Quantity

The **Initial Template Quantity** screen displays interpolated quantities of template added to **Unknown** wells before thermal cycling in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Initial template quantity (for **Unknowns**, the value interpolated from the standard curve based on the Ct calculated for the **U**nknown; for **Standards**, the amounts entered during plate setup)
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)

If less than two **Standard** wells were selected in **Analysis Selection/Setup**, a standard curve cannot be plotted and an error message will appear in place of results in the **Unknown** wells.



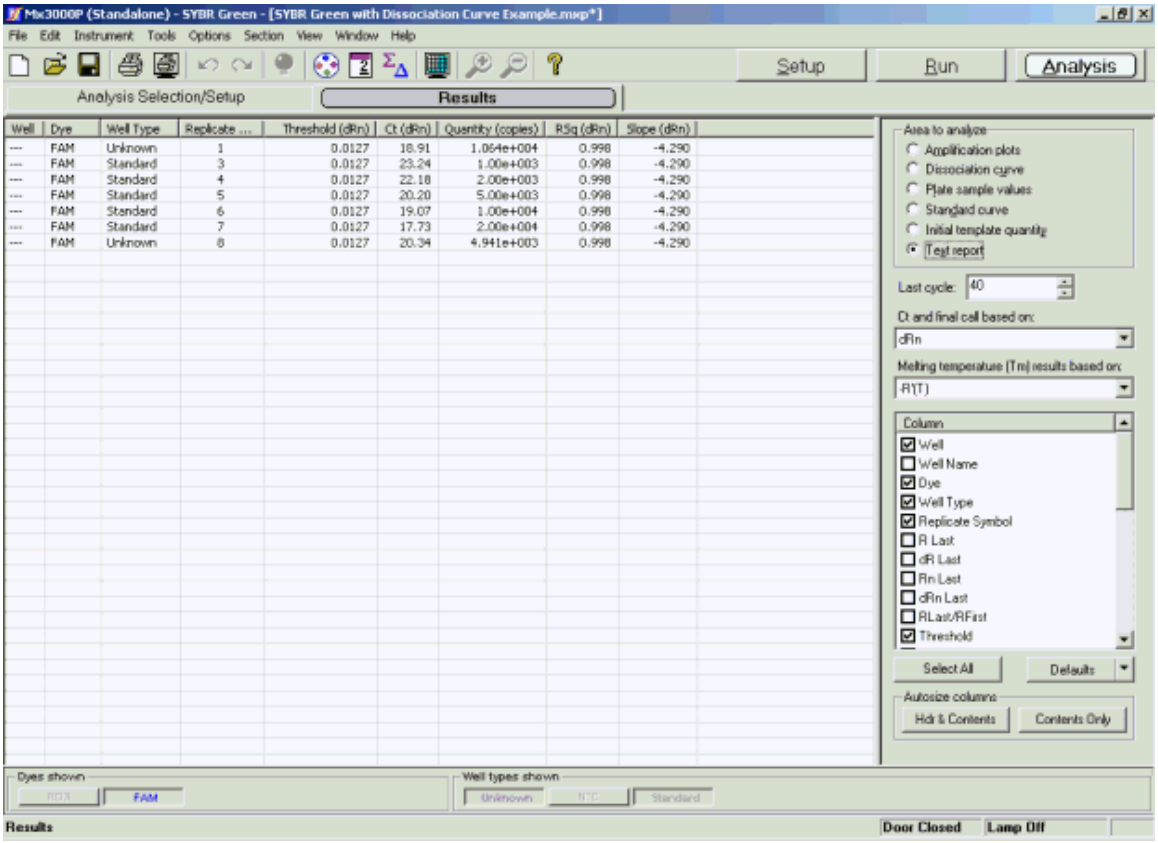
Analysis Options

Fluorescence Data Type for Ct Determinations

Select the type of fluorescence data to be used to calculate the Ct values to be used for interpolation from the **Interpolated template quantity based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected normalized fluorescence).

SYBR Green Text Report

The **Text Report** screen shows the data from a run in text format.



Analysis Options

Last Cycle

The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported when fluorescence value columns (such as **dRn Last**) are selected for display. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

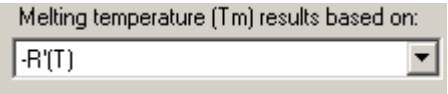
The **Last cycle** setting also affects the reporting of **Ct** and the **Final Call**. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct** and will be called as negative (–).

Ct and Final Call Based On

Select the type of fluorescence data that the reported **Ct** and **Final Call** should be based on from the **Ct and final call based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected fluorescence) and **dRn** (baseline-corrected normalized fluorescence).

Tm Results Based On

Select the type of fluorescence data plot that the reported melting temperature (**Tm**) should be based on from the **Melting temperature (Tm) results based on** menu on the command panel. Available designations are: **-R'(T),** for plots based on raw fluorescence (the negative of the first derivative) and **-Rn'(T),** for plots based on normalized fluorescence (the negative of the first derivative).

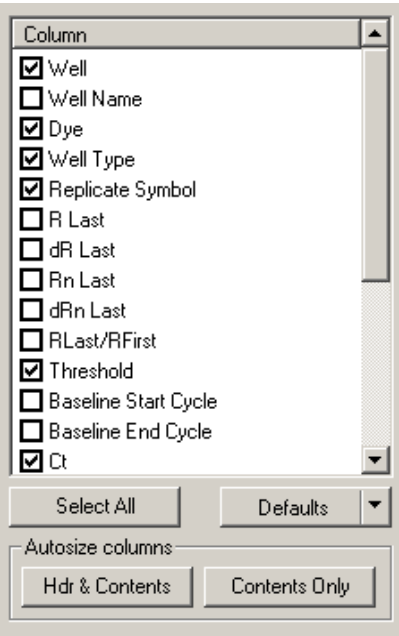


Display Options

Columns Included in the Report

Select the **Columns** to be included in the text report by checking the boxes next to the column names. To exclude columns that are selected by default, click on the check box to deselect the column name. To select all columns to display, click **Select All**. The default settings for column selection can be restored by expanding the **Defaults** menu and then clicking **Get**.

If the included columns will not fit in the screen, a horizontal scroll bar appears at the bottom of the window to allow horizontal scrolling.



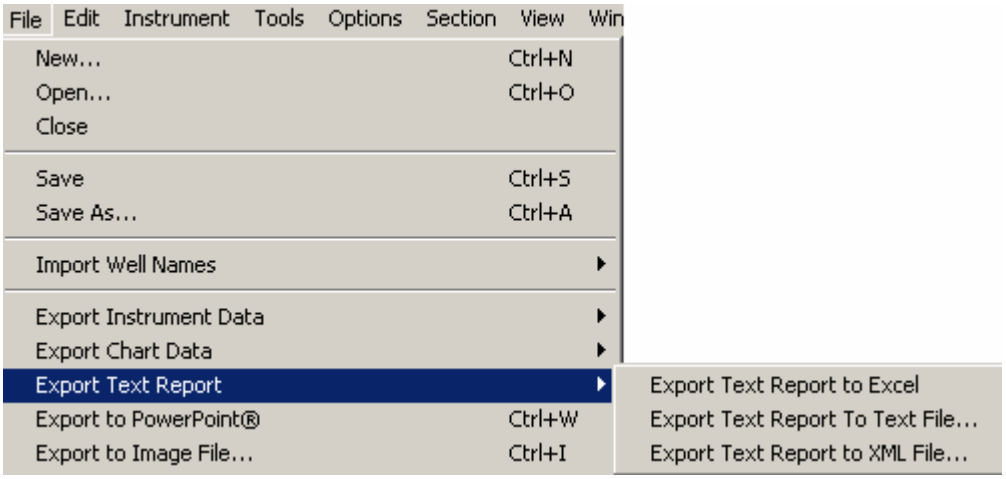
Column Width

To facilitate viewing data, columns can be automatically sized. Clicking the **Contents Only** button will cause each column to be automatically resized to the width of the widest data cell in that column (some column headers may be only partially displayed using this setting). Clicking the **Hdr & Contents** button will cause each column to be resized to the widest cell in that column, including the header cell.

Columns can also be resized manually by dragging the column dividers with the mouse.

Exporting Text Report Data

Text report data can be exported from the application in text, Microsoft Excel spreadsheet or XML format for analysis. Text report exporting options are available from the **File** menu. See *Exporting Data to Other Applications* in the *File Menu* section for more information.



Mx3000P System Experiments: Allele Discrimination/SNP's Real-Time

The Allele Discrimination/SNP's Real-Time Experiment Type

The **Allele Discrimination/SNP's Real-Time** experiment type uses fluorogenic probes to determine the allelic composition of DNA samples. Two fluorogenic probes, labeled with two spectrally distinct dyes, are used to discriminate between two alleles. For example, if amplification in an unknown DNA sample is detected for the dye identifying the wild-type allele but not for the dye identifying a mutant allele, the sample can be designated as wild-type homozygous. If amplification in an unknown DNA sample is detected for the dye identifying the mutant allele but not for the dye identifying the wild-type allele, the sample can be designated as mutant homozygous. If amplification is detected for both dyes, the unknown sample is designated as heterozygous for the two alleles. With properly designed probes, this assay is sensitive enough to detect a single-base difference (single-nucleotide-polymorphism or SNP) between two alleles.

Molecular beacons can be used in a variety of PCR applications, but are commonly used in Allele Discrimination assays due to the high specificity of the molecular beacons in recognizing nucleotide sequence mismatches in DNA and RNA. The hairpin shape of the molecular beacon causes mismatched probe/target hybrids to easily dissociate at significantly lower temperatures than exactly complementary hybrids. This thermal instability of mismatched hybrids increases the specificity of molecular beacons. See *Molecular Beacons Probes* in the *Getting Started* section for more information on the mechanism of action of molecular beacons in QPCR assays.

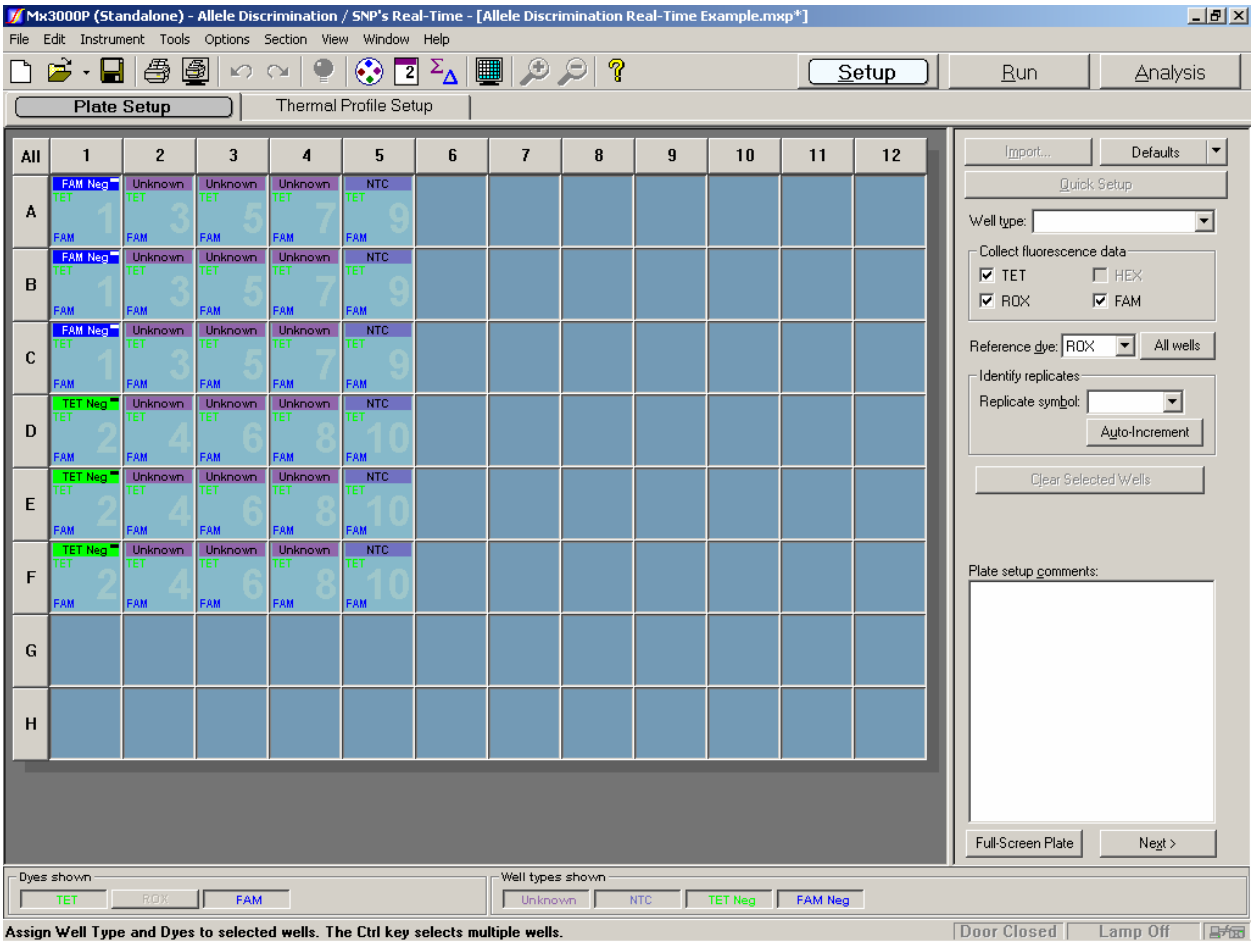
Monitoring PCR reactions in real-time produces a fluorescence signal during each cycle. Results are displayed as an **Amplification plot** that reflects the change in fluorescence during cycling. For Allele Discrimination/SNP's Real-Time experiments, fluorescence is typically monitored and reported during each annealing step when the molecular beacon is bound to its complementary target.

In the analysis of **Allele Discrimination/SNP's Real-Time** experiments, the Mx3000P software uses the threshold cycle (Ct) value for each dye in each sample to determine the genotypes of the samples. The threshold cycle is the cycle at which the fluorescence signal is first detectable above background. A Ct value of 24–32 is expected for a sample that contains the specific allele recognized by the probe. A Ct value equal to the final cycle of the PCR reaction (typically 40) indicates the absence of a specific allele. Results are conveniently displayed in the **Dual Color Scatter Plot**, which shows the Ct for the dye specific to one allele plotted against the Ct for the dye specific to the other allele. Plotted points are grouped according to their positions on the scatter plot, providing a convenient visualization of samples which share the same genotype (allelic composition).

For applications in which a highly quantitative measurement of copy number is not required, the Mx3000P system also offers the Plate Read/Allele Discrimination experiment type, in which fluorescence is monitored when cycling is complete.

Allele Discrimination/SNP's Real-Time Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical plate setup for **Allele Discrimination/SNP's Real-Time**.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) by using the **Well type** menu on the command panel. The well types available in an Allele Discrimination/SNP's Real-Time experiment are described in the table below.

Well Type	Description
Unknown	Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target.
Buffer	Contains only buffer, used to monitor the background fluorescence attributable to the buffer.
NAC	No amplification control; contains all reaction components except DNA polymerase.
NPC	No probe control; contains all reaction components except the fluorescence-labeled probe.
NTC	No template control; contains all reaction components except the template nucleic acid.
No RT	No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.
Dye-specific Negative Control	Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**. Used in combination with **NTC** wells to calculate positive and negative calls.
Dye-specific Positive Control	Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**. Used to validate the reaction mixture.

After assigning the **Well type** to the selected well(s), select the dyes for which data should be collected in these wells using the **Collect fluorescence data** check boxes.

If you are using a passive reference dye in the selected well(s) select the dye from the **Reference dye** menu. If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.

Assign the replicate set number to the selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

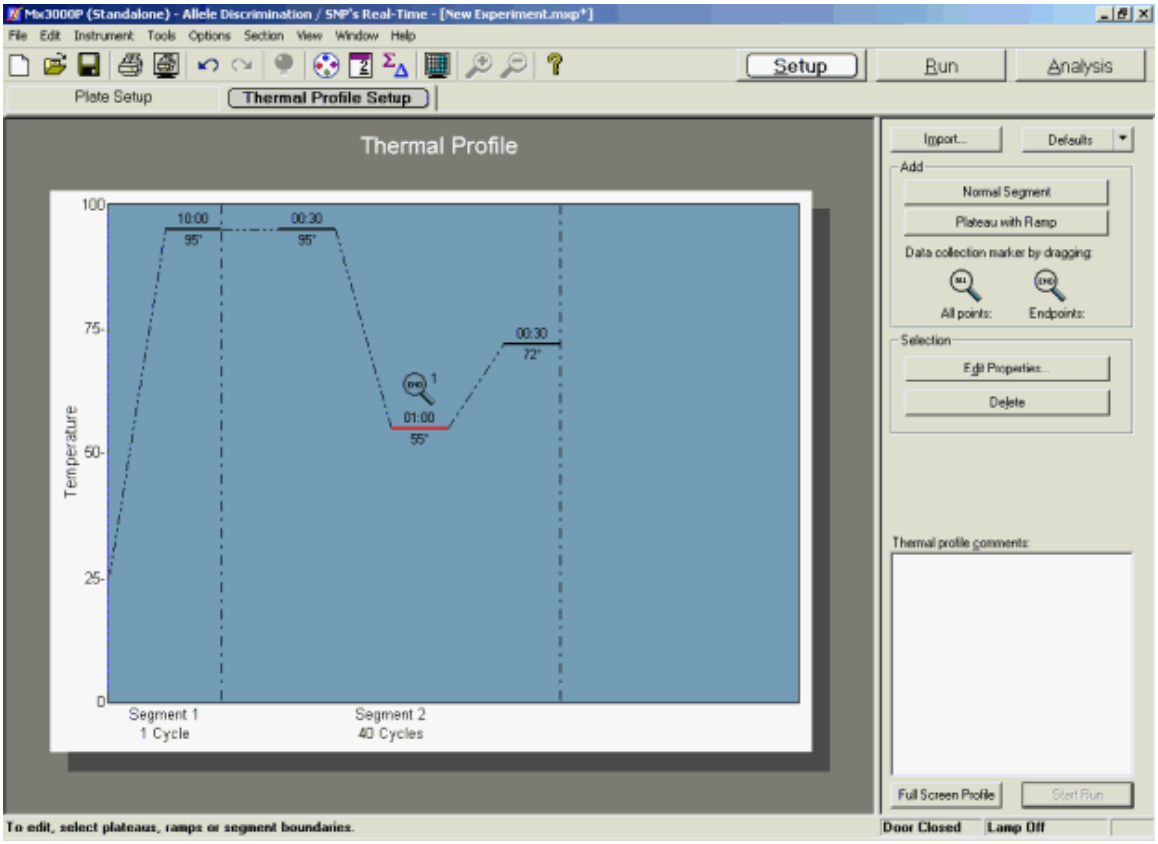
Once plate setup is complete, click **Next** to proceed to the **Thermal Profile Setup** screen.

Allele Discrimination/SNP's Real-Time Thermal Profile Setup

When the **Thermal Profile Setup** tab is selected in a new Allele Discrimination/SNP's Real-Time experiment, a default thermal profile opens. The default thermal profile should be modified to the specific requirements of the new experiment, as discussed below. It is also possible to **Import** the thermal profile from an existing experiment into the new experiment.

Shown in the figure below is the default thermal profile for an Allele Discrimination/SNP's Real-Time experiment when using the software's **Original Settings**. (If you are using a user-defined default set, the default thermal profile stored in that default set will appear when the **Thermal Profile Setup** screen is opened. See *Preferences-Defaults* for more information about the use and management of **Defaults**.)

In the profile below, segment 1 is an activation step of 95°C for 10 minutes that is compatible with a hot start reaction. Segment 2 consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 55°C/1 minute for annealing, and a plateau of 72°C/30 seconds for extension.



Some applications use a two-step amplification protocol instead of the three-step protocol in the default thermal profile. A typical two-step amplification segment consists of 40 cycles of amplification using a plateau of 95°C/30 seconds for denaturation, followed by a plateau of 60°C/1 minute for annealing and extension.

Data are collected at the end of each annealing plateau in the default thermal profile. These collection settings are suitable for a typical Allele Discrimination/SNP's Real-Time experiment.

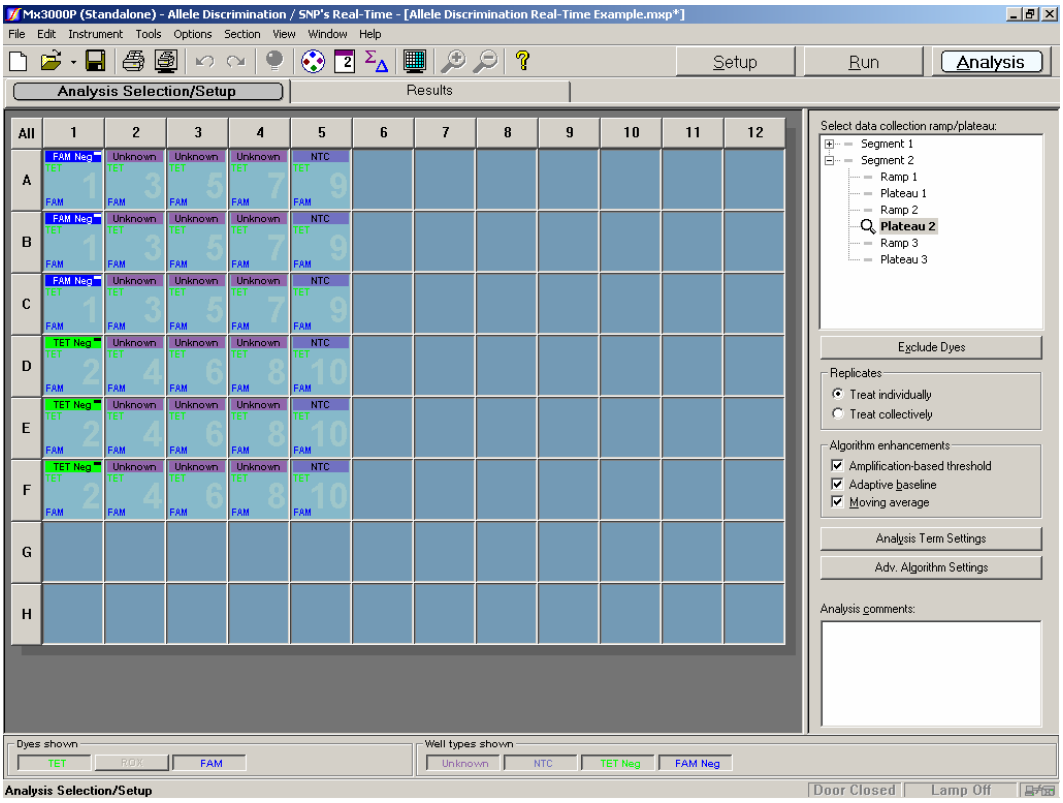
Modify the default thermal profile as necessary. At minimum, ensure that the temperature setting for the annealing plateau in Segment 2 is appropriate for the PCR primer pair used in the current experiment. The properties of all other plateaus, ramps, and segments may also be adjusted. See *Thermal Profile Setup* in the *How-To (Detailed Protocols)* section for more information about changing the default thermal profile and data collection options.

Allele Discrimination/SNP's Real-Time Data Analysis

Analysis Setup

On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.
2. **Select** the **data collection ramp/plateau** from which data should be analyzed.
3. Specify the desired analysis settings on the command panel.



See Analysis Selection and Setup for more detailed instructions on using the features on this screen.

When all selections and settings are made, click the **Results** tab. One of several available results screens will appear. Use the radio buttons under **Area to analyze** to navigate to different analysis screens. The following screens are available for Allele Discrimination/SNP's Real-Time analysis:

Amplification Plots

Plate Sample Values

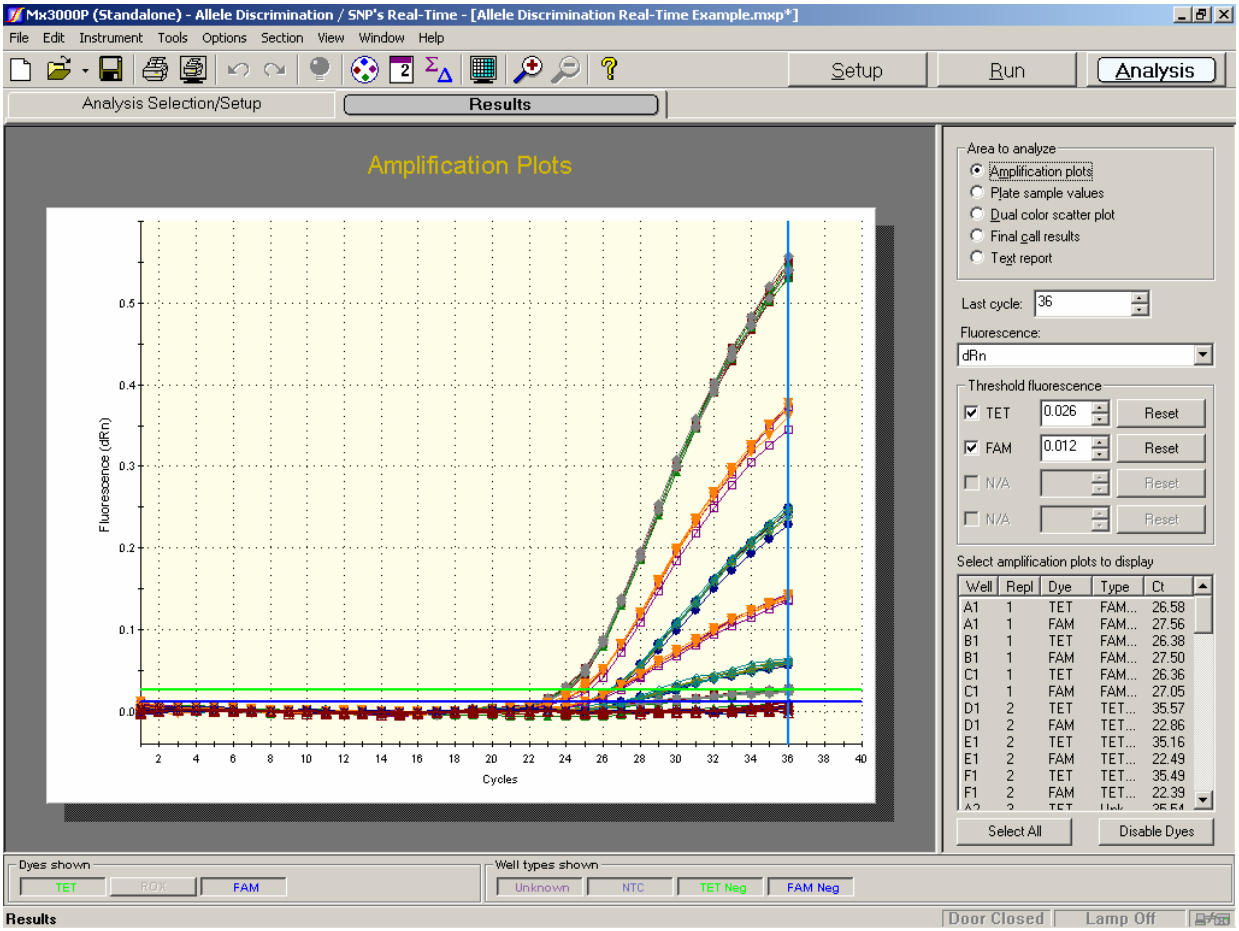
Dual Color Scatter Plot

Final Call Results

Text Report

Allele Discrimination/SNP's Real-Time Amplification Plots

The **Amplification Plots** screen, accessed through the **Results** tab, shows a plot of cycles versus fluorescence for a ramp or plateau on which data are gathered. Select the ramp or plateau for which data should be analyzed using the **Analysis Selection/Setup** screen.



Analysis Options

Fluorescence Data Type Plotted

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are: **R** (raw fluorescence), **dR** (baseline-corrected raw fluorescence), **Rn** (normalized fluorescence), and **dRn** (baseline-corrected normalized fluorescence).

Threshold Fluorescence

When basing plots on normalized fluorescence (**dR** or on **dRn**), the **Threshold fluorescence** value for each dye is listed on the command panel and is marked on the graph by a horizontal line. (Note that if a dye is hidden using the **Dyes shown** controls or if the check box next to the dye is cleared, the line corresponding to threshold fluorescence for that dye will not be shown on the graph.)

Threshold fluorescence is calculated by the Mx3000P software according to the settings provided in **Analysis Term Settings**. If desired, the threshold fluorescence may be adjusted manually. (To restore the software-calculated values, click **Reset**).

Baseline correction is required to establish a threshold fluorescence. Thus selections related to threshold fluorescence are unavailable for **R** or **Rn** data plots.

Last Cycle Setting

The **Last cycle** setting specifies the cycle from which the final (last) fluorescence values will be reported in the **Text Report** and **Plate Sample Values** screens. The position of the last cycle is marked on the graph with a solid vertical line.

This setting also affects the way Ct values are reported on other **Results** screens. While the **Last cycle** is not directly used to determine Ct, any wells with Ct values greater than the **Last cycle** value will be designated as **No Ct** in screens that display Ct.

Excluding Well/Dye Data from Analysis

To exclude the curve for one or more specific well/dye combinations from analysis, use either of the following two methods. (Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen.)

- From the list under **Select amplification plots to display**, select the curve(s) to be excluded and then click **Disable Dyes**.
- Double-click on any data point on the plot to be excluded. This action will exclude the entire data set for the well/dye corresponding to the plot.

To restore a plot that was disabled using either of these methods, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Display Options

To restrict the plots shown to a subset of the selected well/dye combinations, use the **Select amplification plots to display** list near the bottom of the command panel. You may display a single plot or any subset of plots by selecting the desired plots from the list. (While selecting plots with the mouse, use the **Shift** key to select a contiguous group or the **Ctrl** key to select a non-contiguous group.)

To display information about a specific data point on the plots, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

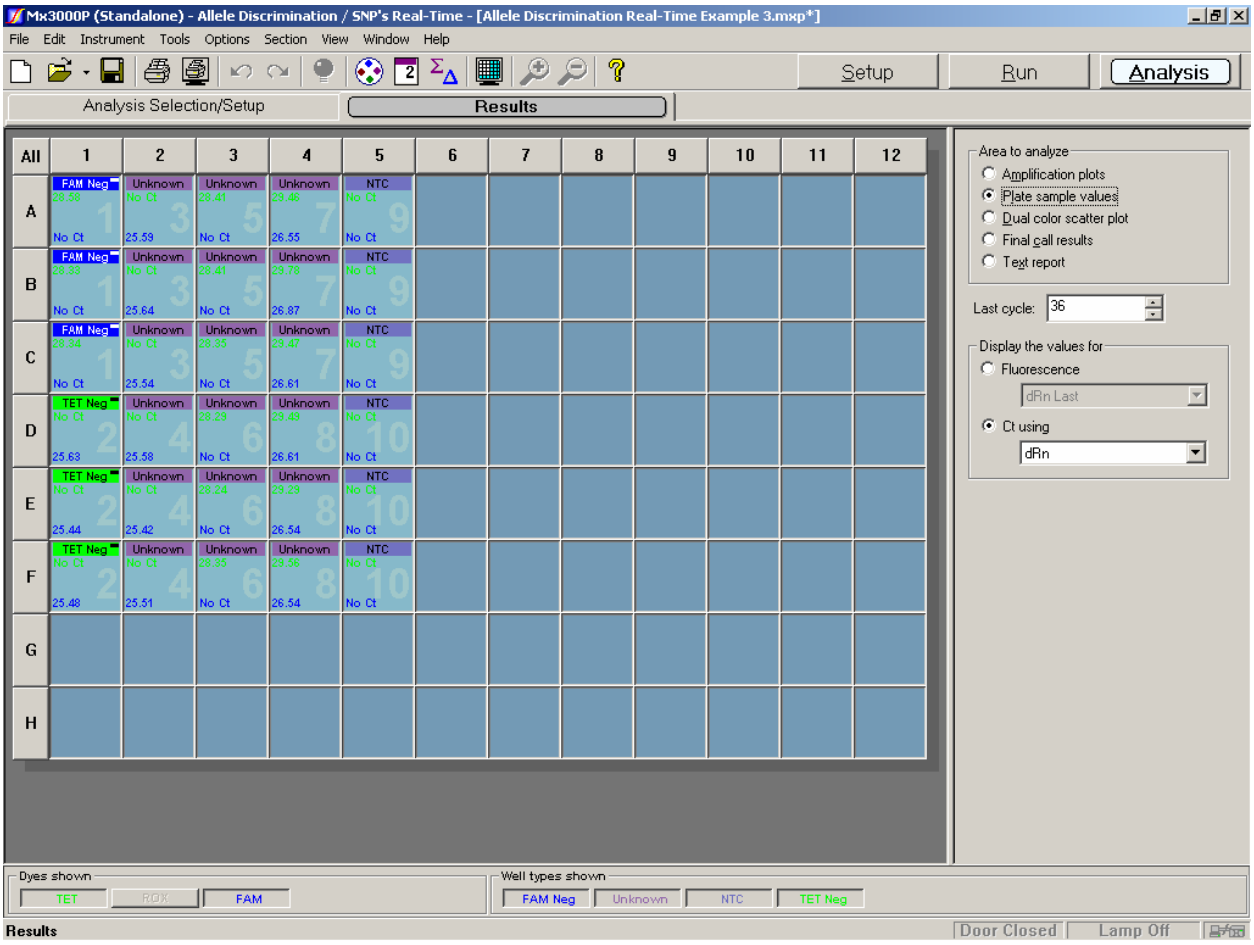
The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** and **Plot Markers** are displayed or hidden. See *Graphs and Graph Properties* for more information.

Allele Discrimination/SNP's Real-Time Plate Sample Values

The **Plate Sample Values** screen displays **Fluorescence** values or **Ct** values for the sampled wells in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Either the fluorescence or the Ct value for each dye for which data was collected
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)



Analysis Options

Display the Values for Fluorescence or Ct

Under **Display the values for**, specify the form of the data to be displayed by selecting either the **Fluorescence** or the **Ct using** radio button. For either of these options, you will need to specify the type of fluorescence data to be used in the analysis by selecting a data type from the menu below the selected radio button. Refer to the tables below for descriptions of the fluorescence data type abbreviations in the menus.

When displaying **Fluorescence** values, the following fluorescence data types are available:

Data Type	How Calculated
R Last	final fluorescence
dR Last	final fluorescence minus the initial fluorescence
Rn Last	normalized final fluorescence
dRn Last	normalized final fluorescence minus the normalized initial fluorescence
R Last/R First	final fluorescence divided by the initial fluorescence

When displaying **Ct** values, Ct calculations may be based on the following fluorescence data types:

Data Type	How Calculated
dR	baseline-corrected fluorescence
dRn	baseline-corrected normalized fluorescence

Last Cycle

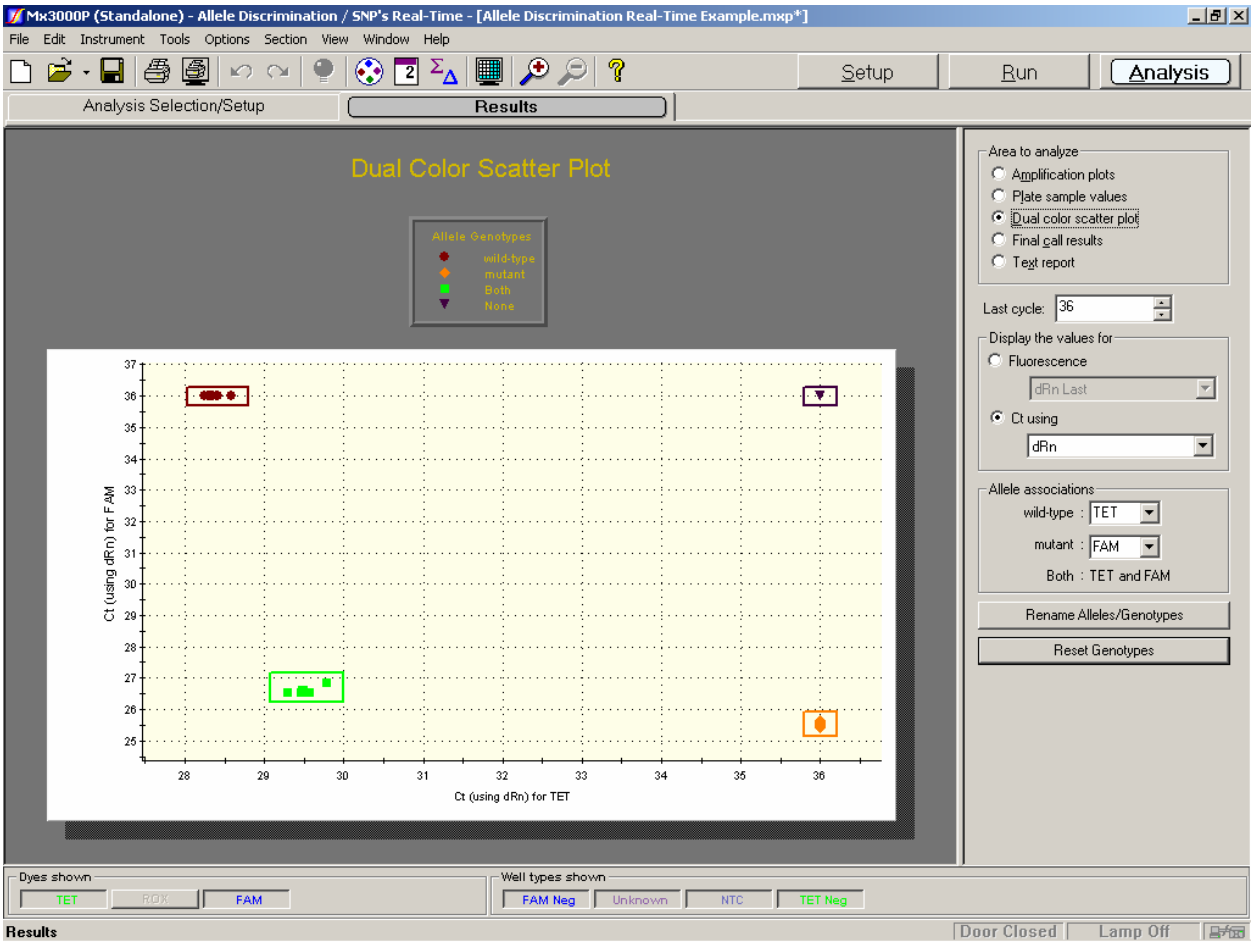
The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

The **Last Cycle** setting also affects the reporting of **Ct**. Any wells having Ct values greater than the **Last cycle** value will be reported as **No Ct**.

Allele Discrimination/SNP's Real-Time Dual Color Scatter Plot

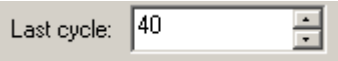
The **Dual Color Scatter Plot** screen is useful to compare the amplification properties of two different targets (represented by two different dyes) in the same well. This form of analysis is especially useful for allele discrimination experiments, where the quantity of each of two possible alleles is indicated by a different dye.

Each plotted point represents the coordinates of either the fluorescence values or threshold cycle (Ct) values for the two dyes in a single well. For example, the X-axis may correspond to TET Ct while the Y-axis corresponds to FAM Ct and the plotted point (x,y) corresponds to the coordinates describing the two Ct values determined for a given well. The position of the data point for a given well on the scatter plot indicates the presence or absence of each allele. Colored rectangles on the plot are used to group wells with a common genotype (allelic composition). The shapes of the genotype-marking rectangles may be changed, and individual data points may also be excluded from the genotype group. The two alleles may be named on this screen, with the user-provided allele names reflected in the genotype descriptions of the wells on this and other **Results** screens.



Analysis Options

Last Cycle Setting

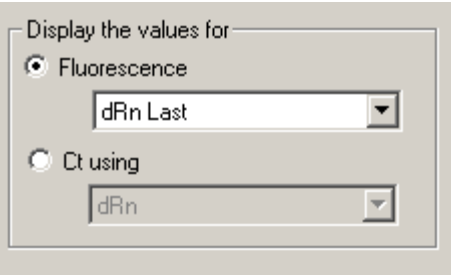


The **Last cycle** setting specifies the cycle from which the fluorescence values will be plotted if **Fluorescence** is selected under **Display the values for**. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

This setting also affects the way Ct values are reported on this and other **Results** screens. While the **Last cycle** is not directly used to determine Ct, any wells with Ct values greater than the **Last cycle** value will be plotted with a Ct value corresponding to the last cycle for which data was collected. The same wells will be designated as **No Ct** in screens that display Ct values.

Plot the Values for Fluorescence or Ct

Under **Display the values for**, specify the form of the data to be plotted by selecting either the **Fluorescence** or the **Ct using** radio button. For either of these options, you will need to specify the type of fluorescence data to be used in the analysis from the menu below the selected radio button. Refer to the tables below for descriptions of the fluorescence data type abbreviations in the menus.



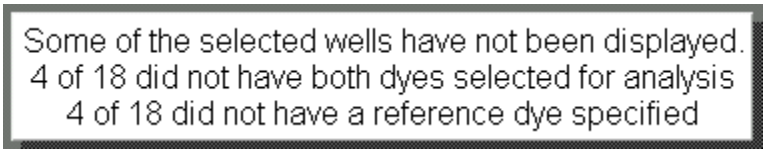
When plotting **Fluorescence** values, the following fluorescence data types are available:

Data Type	How Calculated
R Last	final fluorescence
dR Last	final fluorescence minus the initial fluorescence
Rn Last	normalized final fluorescence
dRn Last	normalized final fluorescence minus the normalized initial fluorescence
R Last/R First	final fluorescence divided by the initial fluorescence

When plotting **Ct** values, Ct calculations may be based on the following fluorescence data types:

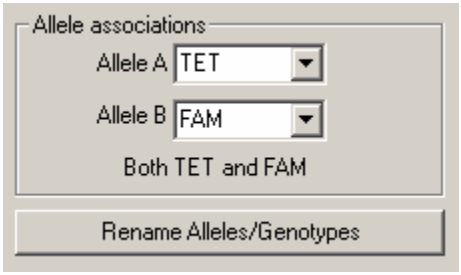
Data Type	How Calculated
dR	baseline-corrected fluorescence
dRn	baseline-corrected normalized fluorescence

Note that if no reference dye has been assigned to the wells and **dRn**, **Rn Last** or **dRn Last** is selected, a message like the following will appear below the graph:

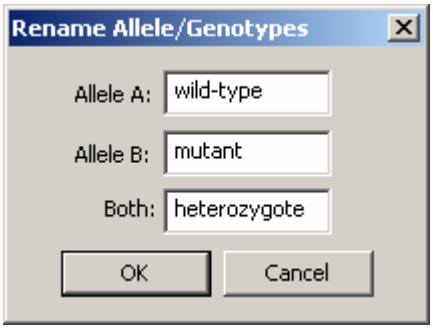


Allele Associations

The **Allele associations** section of the command panel is used to identify the association between each of the plotted dyes and the allele detected by the dye. The allele/dye specified in the top menu will be plotted on the X axis, and the allele/dye specified in the bottom menu will be plotted on the Y axis. Any two dyes assigned during **Plate Setup** may be selected in these menus.



By default, alleles are named *Allele A* and *Allele B*. The allele names also correspond to the genotypes assigned to wells in which a single allele is detected. *Both* is the default name for the genotype group in which both alleles are detected. Each of these allele/genotype names may be changed to user-specified names by clicking **Rename Alleles/Genotypes**. When the following dialog box appears, enter the desired allele/genotype names and then click **OK**.



The new allele names will appear in the **Allele associations** section of the command panel. The specified allele or genotype names are also displayed on some other **Results** screens.

Display Options

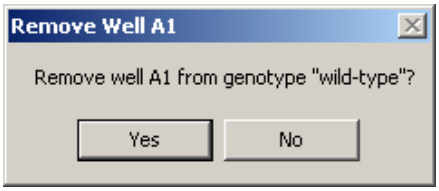
Display of Genotype Groups on the Scatter Plot

Wells with a common genotype (allelic composition) are grouped on the scatter plot in color-coded rectangles. The genotype corresponding to each group is indicated in the graph legend (see *Graph Properties* below). If a single allele was detected in a well, the genotype designation corresponds to the allele name. If both alleles were detected, the genotype is designated *Both* by default; this designation may be changed by clicking **Rename Alleles/Genotypes** on the command panel. If neither allele was detected, the genotype is designated *None.*

The size and shape of each rectangle may be changed by dragging any side of the rectangle with the mouse. The color of the rectangle and the enclosed group of data points may be changed for any genotype by double-clicking on the genotype's entry in the legend.

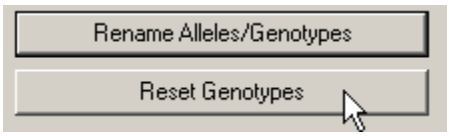
Note *The size and shape of a rectangle does not convey information about dye intensity, data quality, or any other measured data attribute. The rectangles are only intended to identify and group the data points which fit the positive/negative calling result criteria that define each genotype.*

Any well may be manually excluded from the genotype groups by double-clicking on the data point for the well. The following dialog box will appear.



Once **Yes** is selected, the symbol for the affected data point will change to the color and point style assigned to excluded data points. In addition, the **Genotype** for the well will change in the **Text Report**.

To restore any manually removed data points to their original genotype assignments, click **Reset Genotypes** on the command panel.



Graph Properties

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the legend is shown or hidden and the attributes of the axes. See *Graphs and Graph Properties* for more information.

To display information about a specific data point on the scatter plot, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

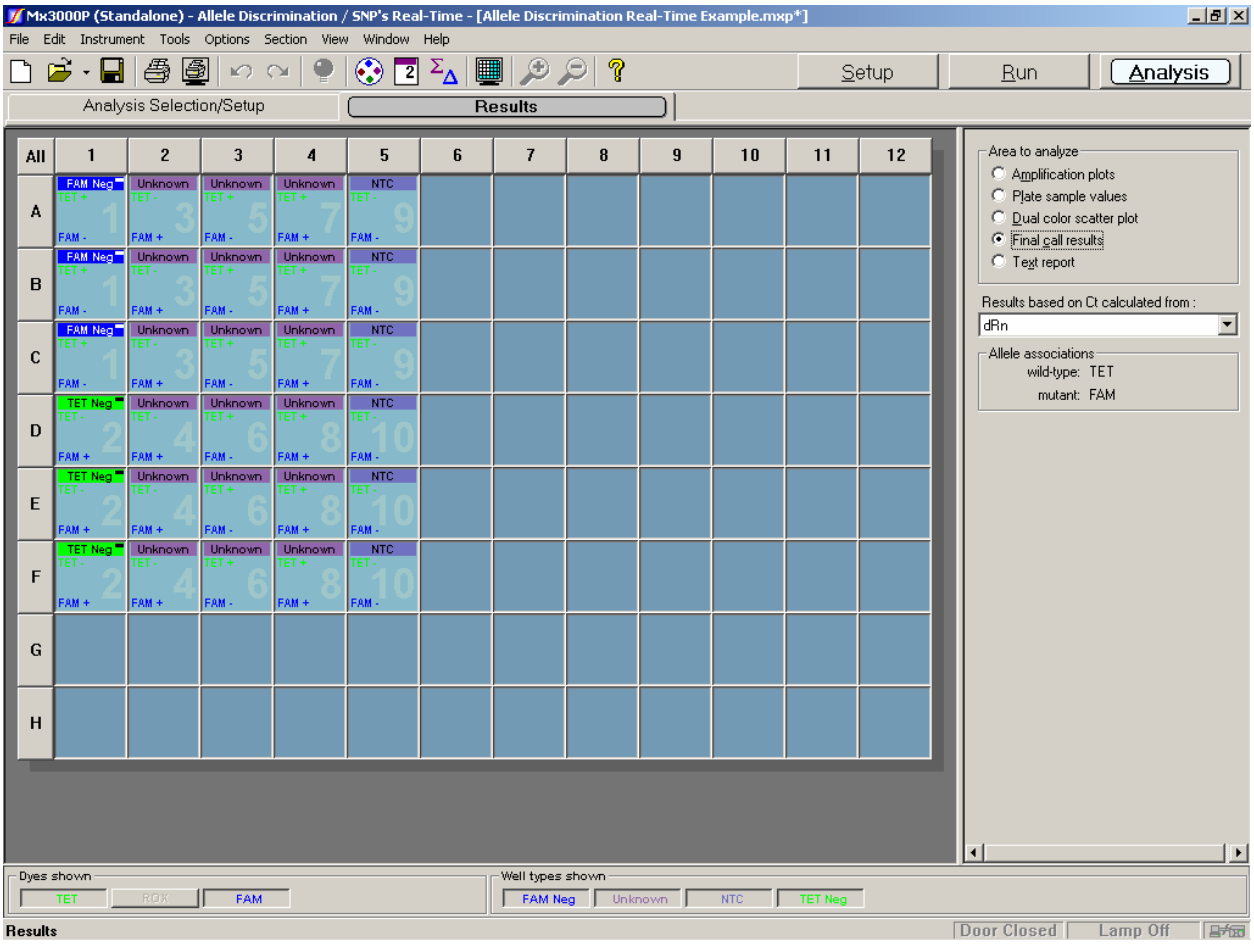
Allele Discrimination/SNP's Real-Time Final Call Results

The **Final Call Results** screen provides a simple depiction of whether product was accumulated for each dye in each well in a plate format. A plus sign (**+**) signifies accumulation of product for the indicated dye, while a minus sign (**–**) signifies that product accumulation was not detected. Calling is based on Ct, where **+** is returned if the Ct is less than the last cycle for which data was collected and **–** is returned if the Ct is equal to the last cycle for which data was collected, indicating that the threshold fluorescence value was not attained during amplification.

In each well with a well type assigned, the following information is shown:

- Well type
- The final call for each dye for which data was collected. [For all well types, the reference dye (**Ref**) is not associated with a final call]
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)

The allele associated with each dye is shown under **Allele associations** on the command panel. Allele names may be changed on the **Dual color scatter plot** screen.



Analysis Options

Fluorescence Data Type for Ct Determinations

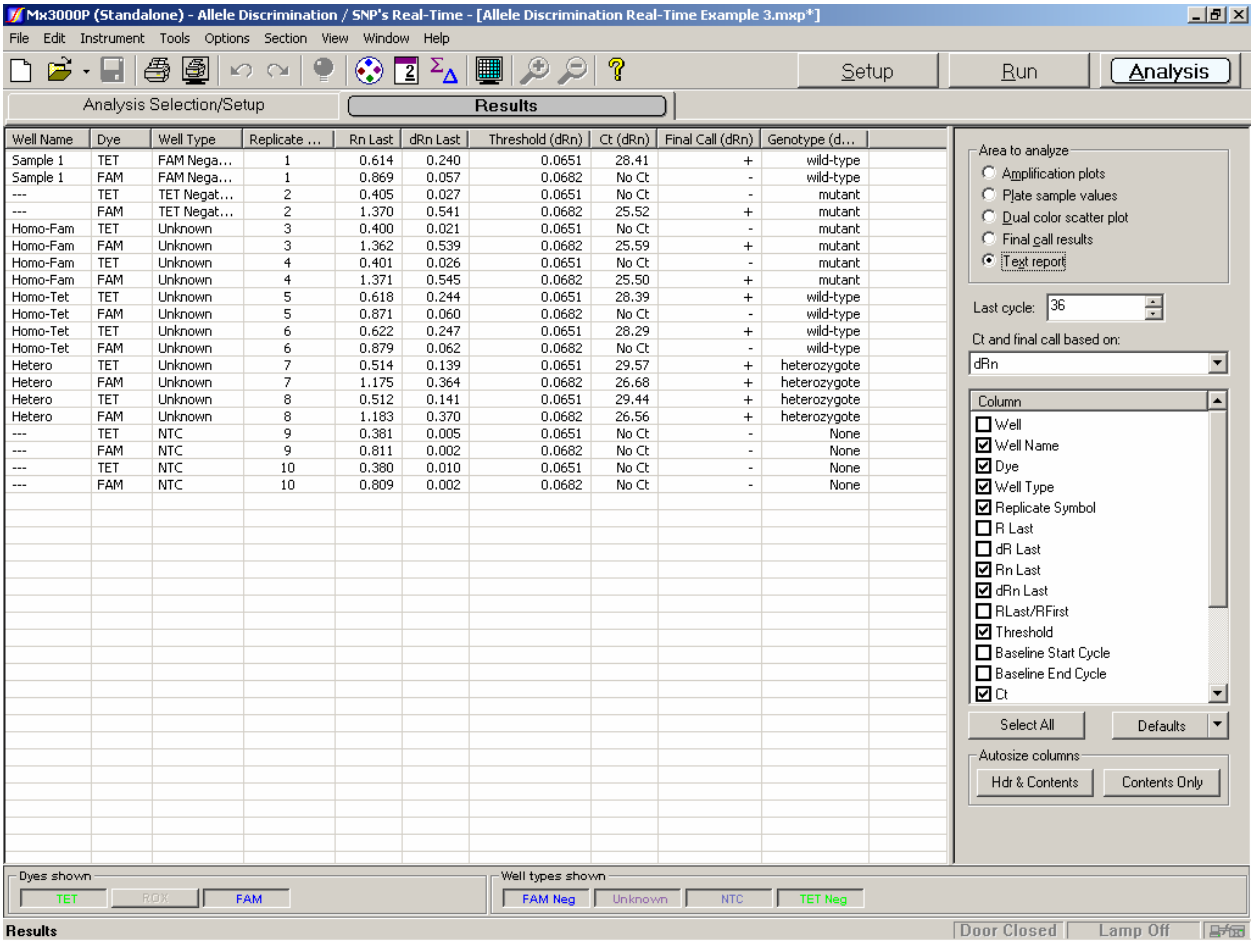
Select the type of fluorescence data to be used to calculate the Ct values to be used for interpolation from the **Results based on Ct calculated from** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected raw fluorescence), and **dRn** (baseline-corrected normalized fluorescence). If dRn is selected, but no reference dye was assigned during **Plate Setup**, the final call result will be replaced with the error indicator of **No Ref**.

Effect of Threshold Fluorescence

Positive and negative calls are based on threshold cycle (Ct), the cycle at which the fluorescence signal first exceeds or equals the threshold fluorescence for the experiment. The settings that determine how threshold fluorescence is calculated may be accessed and adjusted on the **Threshold Fluorescence** tabbed page of the **Analysis Term Settings** dialog box. Threshold fluorescence may also be adjusted manually.

Allele Discrimination/SNP's Real-Time Text Report

The **Text Report** screen shows the data from a run in text format.



Analysis Options

Last Cycle

The **Last cycle** setting specifies the cycle from which the fluorescence values will be reported when fluorescence value columns (such as **dRn Last**) are selected for display. By default, the cycle shown is the last cycle of the ramp or plateau being analyzed.

The **Last cycle** setting also affects the reporting of **Ct** and the **Final Call**. Wells having Ct values greater than the **Last cycle** value will be reported as **No Ct** and will be called as negative (**–**).

Ct and Final Call Based On

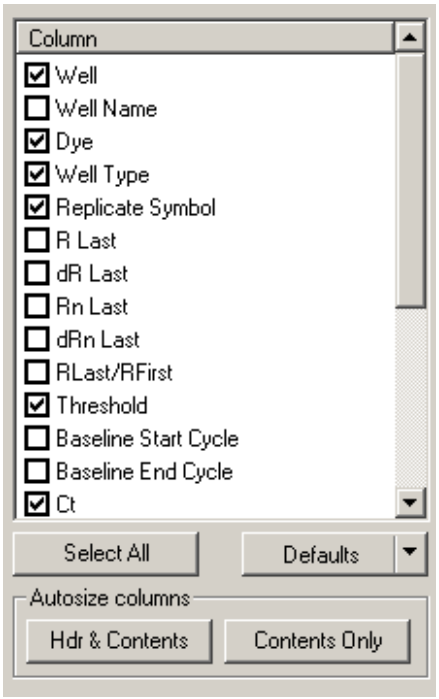
Select the type of fluorescence data that the reported **Ct** and **Final Call** should be based on from the **Ct and final call based on** menu on the command panel. Available fluorescence data types are: **dR** (baseline-corrected fluorescence) and **dRn** (baseline-corrected normalized fluorescence).

Display Options

Columns Included in the Report

Select the **Columns** to be included in the text report by checking the boxes next to the column names. To exclude columns that are selected by default, click on the check box to deselect the column name. To select all columns to display, click **Select All**. The default settings for column selection can be restored by expanding the **Defaults** menu and then clicking **Get**.

If the included columns will not fit in the screen, a horizontal scroll bar appears at the bottom of the window to allow horizontal scrolling.



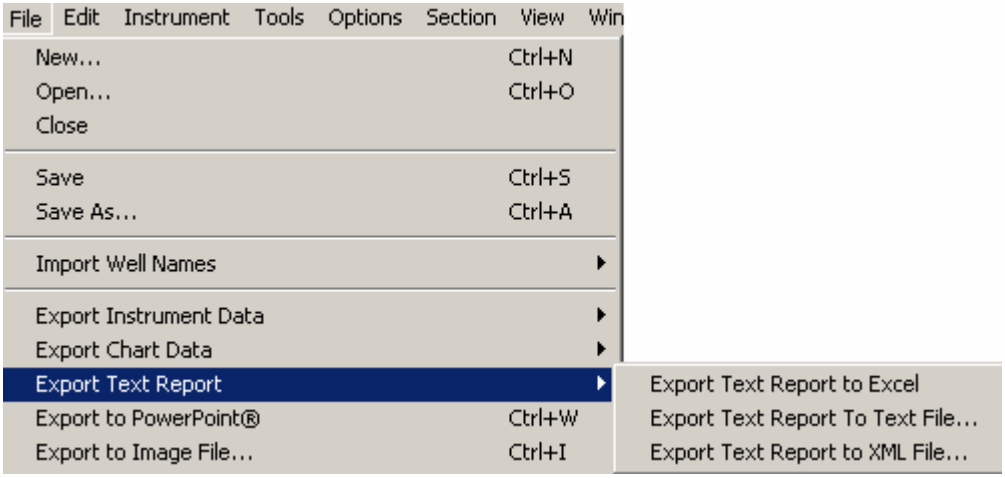
Column Width

To facilitate viewing data, columns can be automatically sized. Clicking the **Contents Only** button will cause each column to be automatically resized to the width of the widest data cell in that column (some column headers may be only partially displayed using this setting). Clicking the **Hdr & Contents** button will cause each column to be resized to the widest cell in that column, including the header cell.

Columns can also be resized manually by dragging the column dividers with the mouse.

Exporting Text Report Data

Text report data can be exported from the application in text, Microsoft Excel spreadsheet or XML format for analysis. Text report exporting options are available from the **File** menu. See *Exporting Data to Other Applications* in the *File Menu* section for more information.



Mx3000P System Experiments: Molecular Beacon Melting Curve

The Molecular Beacon Melting Curve Experiment Type

After a molecular beacon is manufactured, its melting characteristics are verified by melting curve analysis to determine the molecular beacon's target specificity, experimental melting temperature (Tm), and the appropriate annealing temperature for subsequent PCR experiments. Melting curves are typically performed with the molecular beacon alone (**MB**) and in the presence of either a perfectly-matched (**MBO**) or a mismatched (**MBMO**) oligonucleotide target in order to determine the temperature that results in maximum specificity.

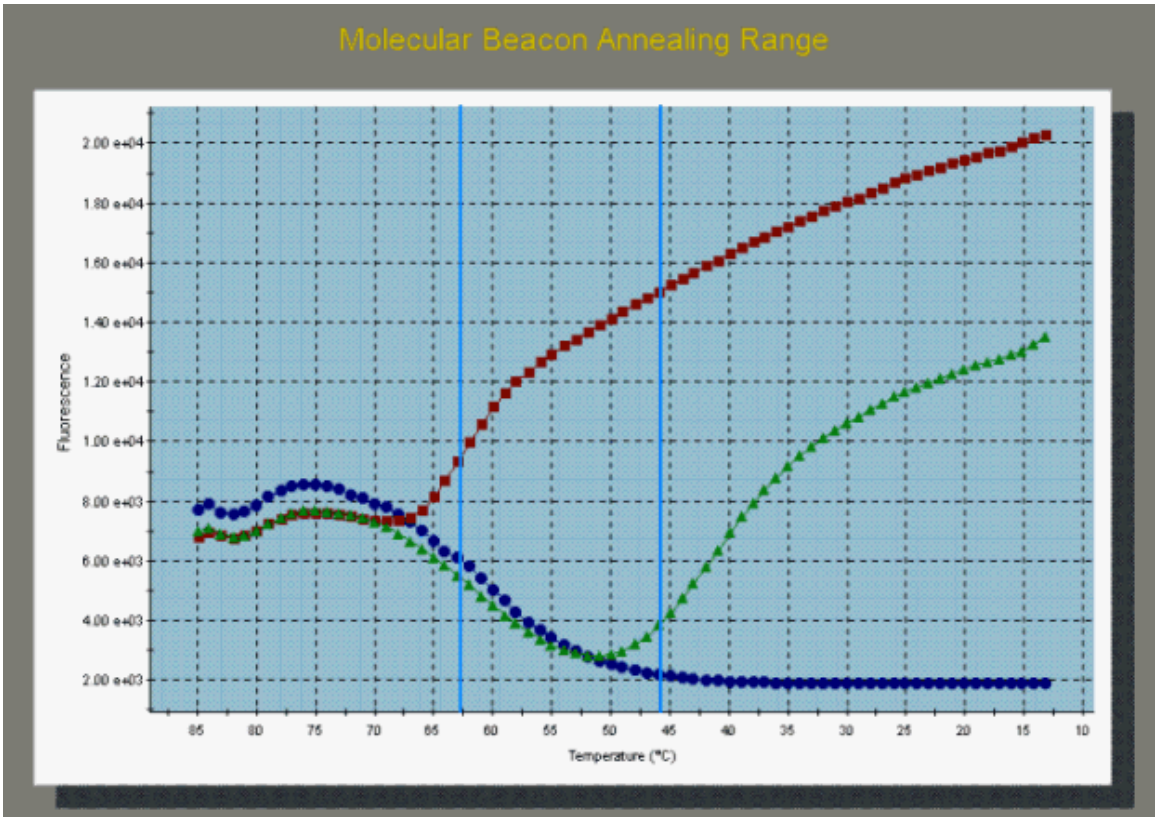
A melting curve displays the fluorescence of the molecular beacon at various temperatures in the presence or absence of the diagnostic single-stranded oligonucleotide targets. At high temperatures, the molecular beacon will assume an unstructured conformation and will fluoresce. In the absence of the single-stranded oligonucleotide target, as the temperature decreases the molecular beacon will assume a hairpin conformation, and fluorescence will be quenched. The temperature at which half-maximum fluorescence is obtained gives the Tm for that molecular beacon.

In the presence of either single-stranded oligonucleotide, as temperature decreases the molecular beacon will hybridize to the target, physically separating the fluorophore and quencher, and allowing fluorescence. At higher temperatures in the curve, the perfectly matched oligonucleotide (**Well type** of **MBO**) is expected to demonstrate greater binding (and therefore greater fluorescence) compared to the mismatched oligonucleotide (**Well type** of **MBMO**). The temperature window displaying a significant difference in **Fluorescence** between the **MBO** and **MBMO** wells represents the range of acceptable annealing temperatures to achieve discrimination between the two target oligonucleotides.

Selecting the Optimal Annealing Temperature

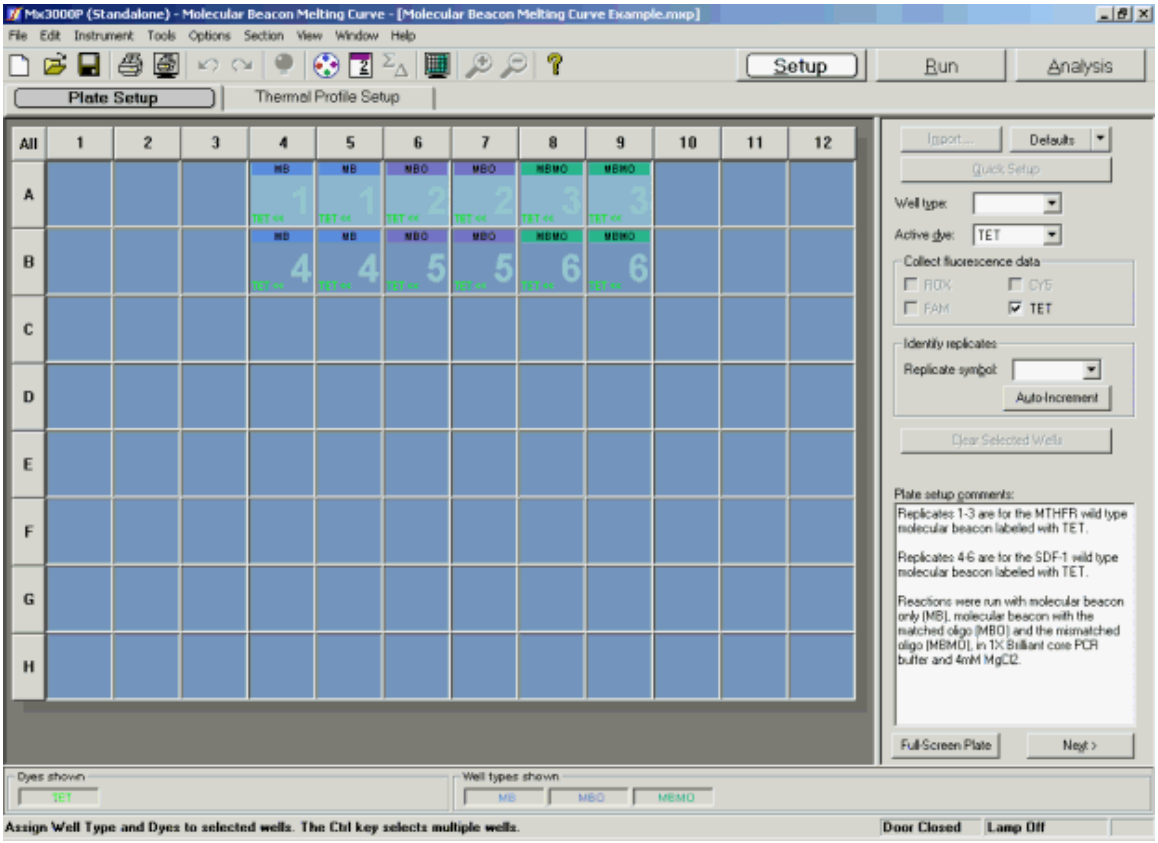
An annealing temperature should be chosen at which the molecular beacon will bind efficiently to its complementary target, and at which the molecular beacon will adopt a stem-loop conformation when unbound to target. For allele discrimination or other assays requiring specificity, the annealing temperature should be chosen according to the temperature at which the molecular beacon binds to its completely complementary target but binds minimally to a mismatched target.

The figure below illustrates a typical molecular beacon melting profile performed in the development of an allele discrimination assay. Three melting curves are shown: molecular beacon alone (**MB**) (blue curve), molecular beacon plus its complementary single stranded oligonucleotide target (**MBO**) (red curve), and molecular beacon plus a single stranded oligonucleotide target containing a single mismatch (**MBMO**) (green curve). To ensure PCR assay specificity in discrimination, an annealing temperature at which the molecular beacon will bind to its completely complementary target but not to a mismatched target should be chosen. In this example, an annealing temperature of 60°C will provide low background fluorescence, high fluorescence when the perfectly matched target is present and low fluorescence when a target containing a single mismatch is present.



Molecular Beacon Melting Curve Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical **Molecular Beacon Melting Curve** plate setup. In this example, the melting curve for a single molecular beacon labeled with TET is analyzed.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) by using the **Well type** menu on the command panel. The well types available in a Molecular Beacon Melting Curve experiment are described in the table below.

Well Type	Description
MBO	Contains the molecular beacon plus its perfectly matched oligonucleotide.
MB	Contains the molecular beacon only.
MBMO	Contains the molecular beacon plus a single-base mismatched oligonucleotide.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.

The **Active dye** menu is used to specify which dye will be displayed in the **Analysis** screens. If the experiment is analyzing the melting curve for more than one molecular beacon, select the dye corresponding to the molecular beacon that you want to analyze first. After the experiment has been run, the **Active dye** assignment may be changed in this menu to allow analysis of the other molecular beacons labeled with other dyes.

Select the dye(s) for which data should be collected for the selected wells using the **Collect fluorescence data** check boxes. Typically, only the dye(s) used to label the molecular beacon(s) will have data collected.

Assign replicate set numbers to selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

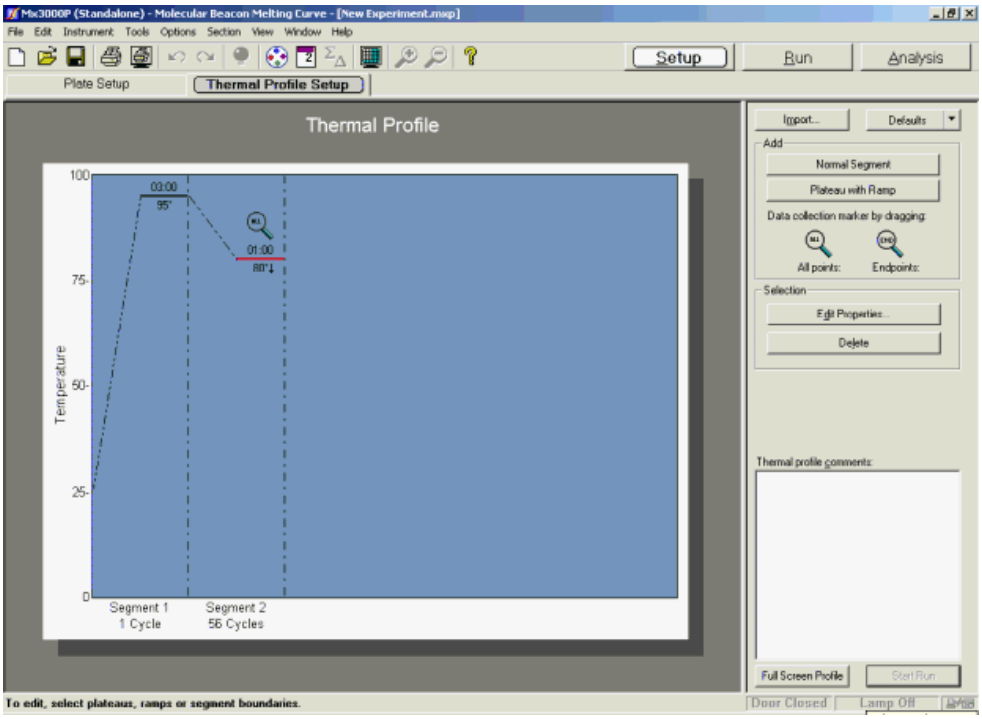
Once plate setup is complete, click **Next** to proceed to the **Thermal Profile Setup** screen.

Molecular Beacon Melting Curve Thermal Profile Setup

When the **Thermal Profile Setup** tab is selected in a new Molecular Beacon Melting Curve experiment, a default thermal profile opens. The default thermal profile should be modified to the specific requirements of the new experiment, as discussed below. It is also possible to **Import** the thermal profile from an existing experiment into the new experiment.

Shown in the figure below is the default thermal profile for a Molecular Beacon Melting Curve when using the software's **Original Settings**. (If you are using a user-defined default set, the default thermal profile stored in that default set will appear when the **Thermal Profile Setup** screen is opened. See *Preferences-Defaults* for more information about the use and management of **Defaults**.)

Segment 1 contains a plateau of 95°C for 3 minutes, which is used to denature the molecular beacon prior to the melting curve. The melting curve, in Segment 2, consists of 56 successive 1-minute plateaus in which the temperature is decreased by 1.0°C for each plateau (from 80°C to 25°C). This cycle-by-cycle decrease in temperature is indicated by the downward pointing arrow adjacent to the data collection marker for this plateau.



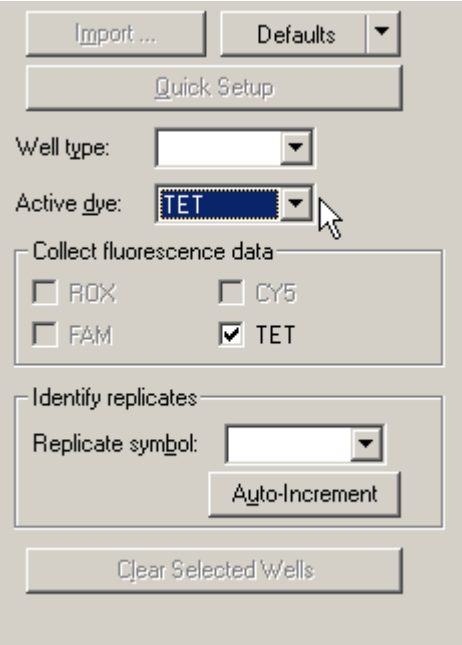
Data are collected throughout each plateau during the melting curve (Segment 2) in the default thermal profile. These settings are suitable for a typical Molecular Beacon Melting Curve.

If necessary, modify the default thermal profile according to the specific requirements of the current experiment. The properties of any of the plateaus, ramps, and segments may be adjusted. See *Thermal Profile Setup* in the *How-To (Detailed Protocols)* section for more information about changing the default thermal profile and data collection options.

Molecular Beacon Melting Curve Data Analysis

Analysis Setup

If more than one molecular beacon was assayed in the experiment, navigate to the **Plate Setup** screen and select the dye to be analyzed from the **Active Dye** menu.

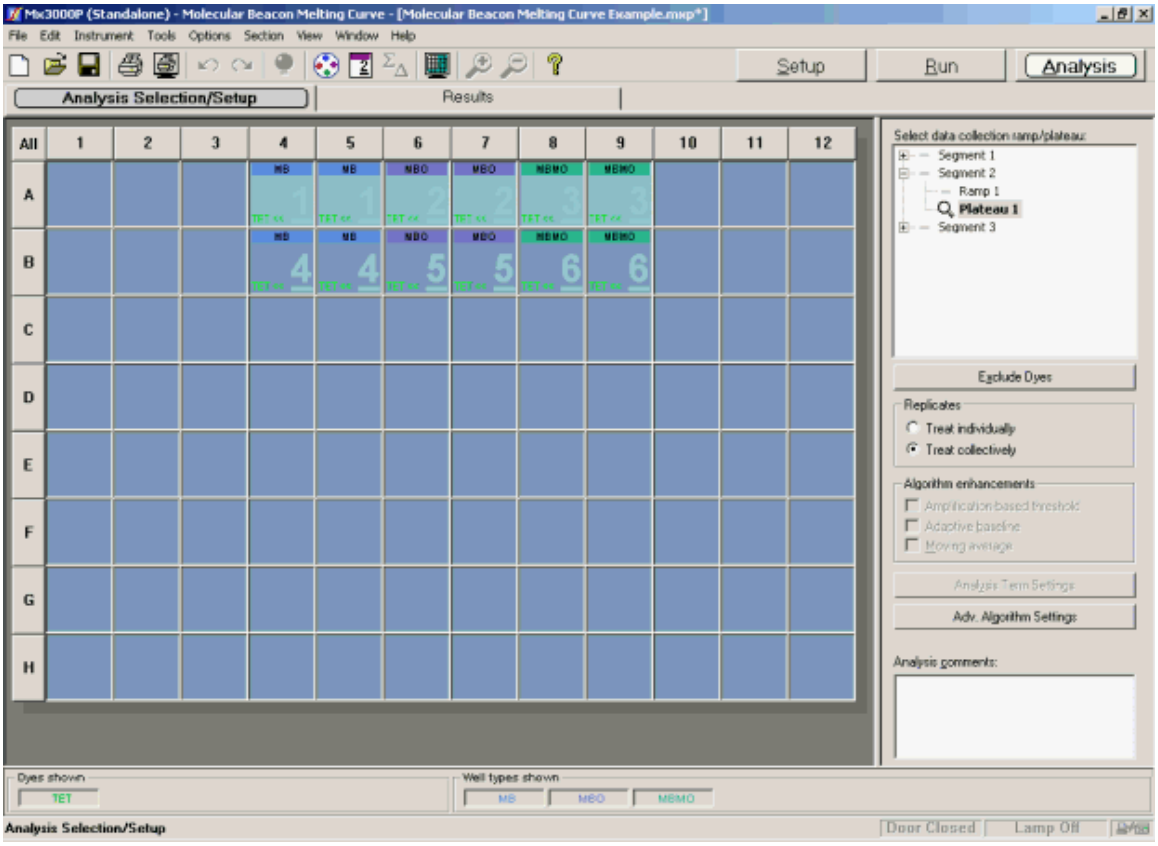


On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.

2. **Select** the **data collection ramp/plateau** from which data should be analyzed.

3. Specify the desired analysis settings on the command panel. For Molecular Beacon Melting Curve experiments, the only analysis settings that are available are **Replicates** (controlling replicate treatment) and **Adv. Algorithm Settings** (controlling the statistical basis for reporting read data). The remainder of the settings on the command panel concern analysis of amplification data only.



See **Analysis Selection/Setup** for more detailed instructions on using the features on this screen.

When all selections and settings are made, click the **Results** tab. One of two available results screens will appear. Use the radio buttons under **Area to analyze** to navigate between analysis screens. The following screens are available for Molecular Beacon Melting Curve analysis:

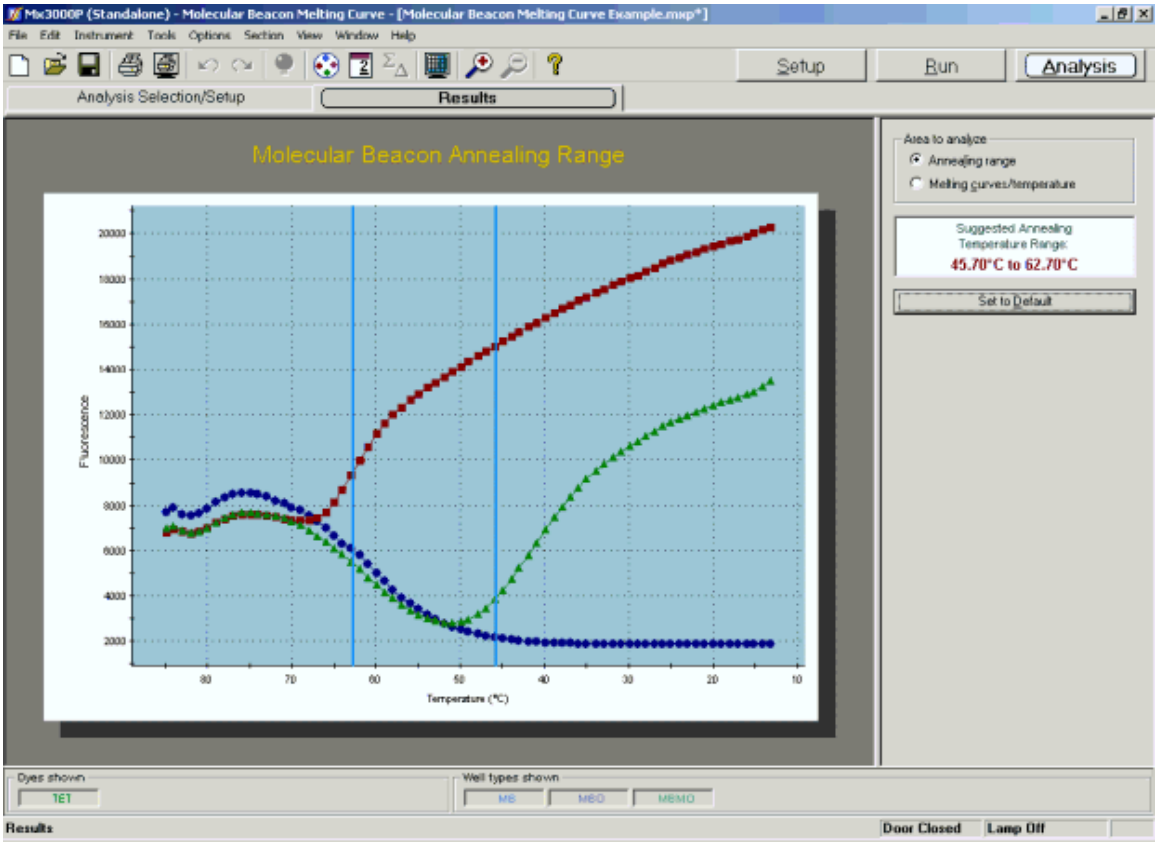
Molecular Beacon Melting Curve Annealing Range

Molecular Beacon Melting Curves/Temperature

Molecular Beacon Melting Curve Annealing Range

The **Annealing Range** screen displays the optimal annealing range for the beacon(s) tested. The optimal range is the temperature range in which the molecular beacon plus perfectly-matched oligo (MBO) has a greater fluorescence than the beacon plus mismatched oligo (MBMO) and in which the background fluorescence is low.

The **Suggested Annealing Temperature Range**, calculated using the algorithm described below, is reported on the command panel at the right of the screen, and is indicated on the plot by two vertical lines.



Suggested Annealing Temperature Range Calculations

The Mx3000P system software uses the algorithm below to calculate the annealing temperature range.

Note *The annealing temperature algorithm does not incorporate data points collected below 40°C or above 85°C.*

Suggested Minimum Annealing Temperature

1. The minimum fluorescence value of the MBMO melting curve (green curve in the plot above) is determined.

2. The temperature at this minimum MBMO fluorescence value is determined.

3. The difference between the MBO (red curve in the plot above) and the MBMO curve fluorescence values at the temperature corresponding to the MBMO minimum is calculated.

4. 10% of the difference between the MBO and the MBMO fluorescence values (MBO value minus MBMO value) is added to the MBMO minimum fluorescence value.

5. This adjusted fluorescence value corresponds to two temperatures on the MBMO curve. The lower of these two temperatures is the minimum temperature of the **Suggested Annealing Temperature Range**.

Suggested Maximum Annealing Temperature

1. Following steps 1–3 above, the difference between the MBO curve (red curve above) and the MBMO (green curve above) fluorescence values at the temperature corresponding to the MBMO minimum is calculated.

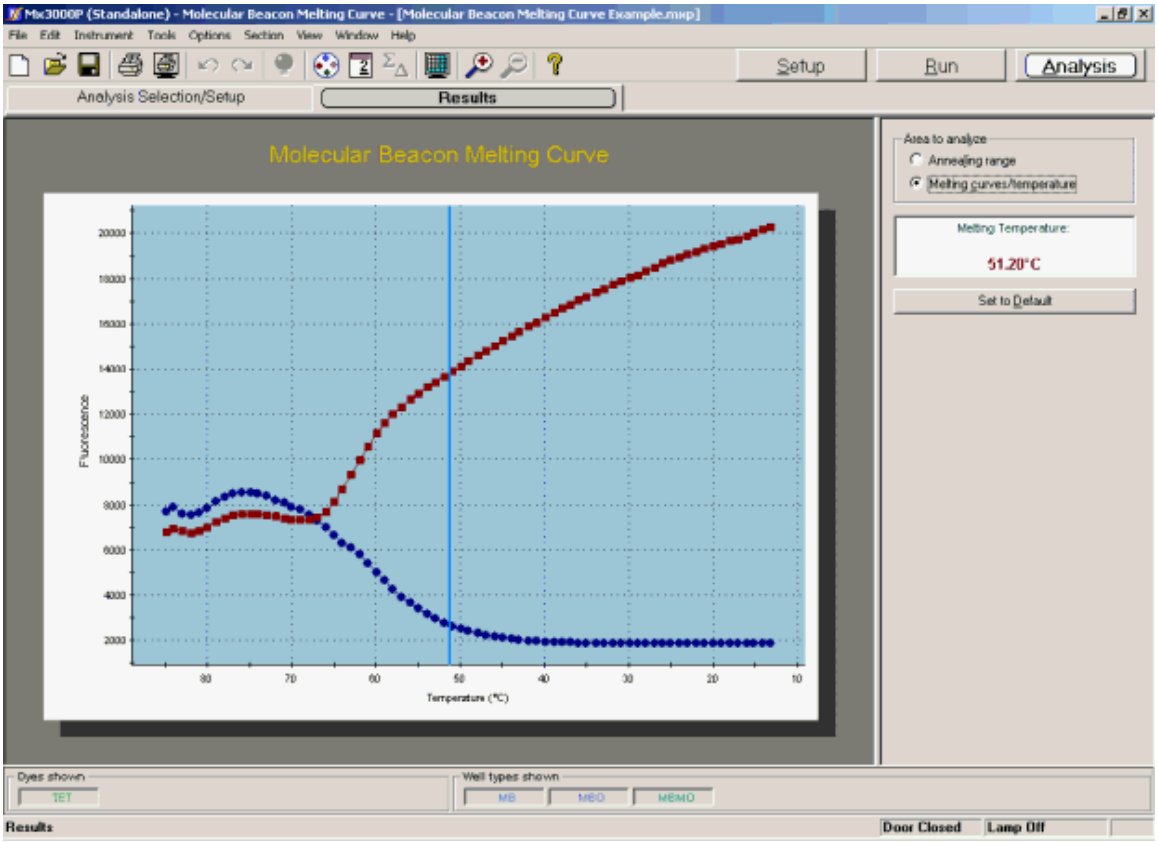
2. 60% of the difference between the MBO and the MBMO fluorescence values is added to the MBMO minimum fluorescence value.

3. This adjusted fluorescence value corresponds to one temperature on the MBO curve. This is the maximum temperature of the **Suggested Annealing Temperature Range**.

Molecular Beacons Melting Curve Melting Curves/Temperature

The **Melting Curves/Temperature** screen calculates and displays the melting temperature for the beacon(s) tested.

The **Melting Temperature**, calculated using the algorithm described below, is reported on the command panel at the right of the screen, and is indicated on the plot by a vertical line.



Calculating the Suggested Melting Temperature

The Mx3000P system software uses the algorithm below to calculate the melting temperature.

1. The range of fluorescence of the MBO curve (red curve in the plot above) is calculated.
2. The value from step 1 is multiplied by 0.5.
3. The resulting value is added to the MBO curve minimum fluorescence value.
4. The temperature at which the fluorescence value of the MBO curve equals the result of step3 is the reported **Melting Temperature**.

Mx3000P System Experiments: Quantitative Plate Read

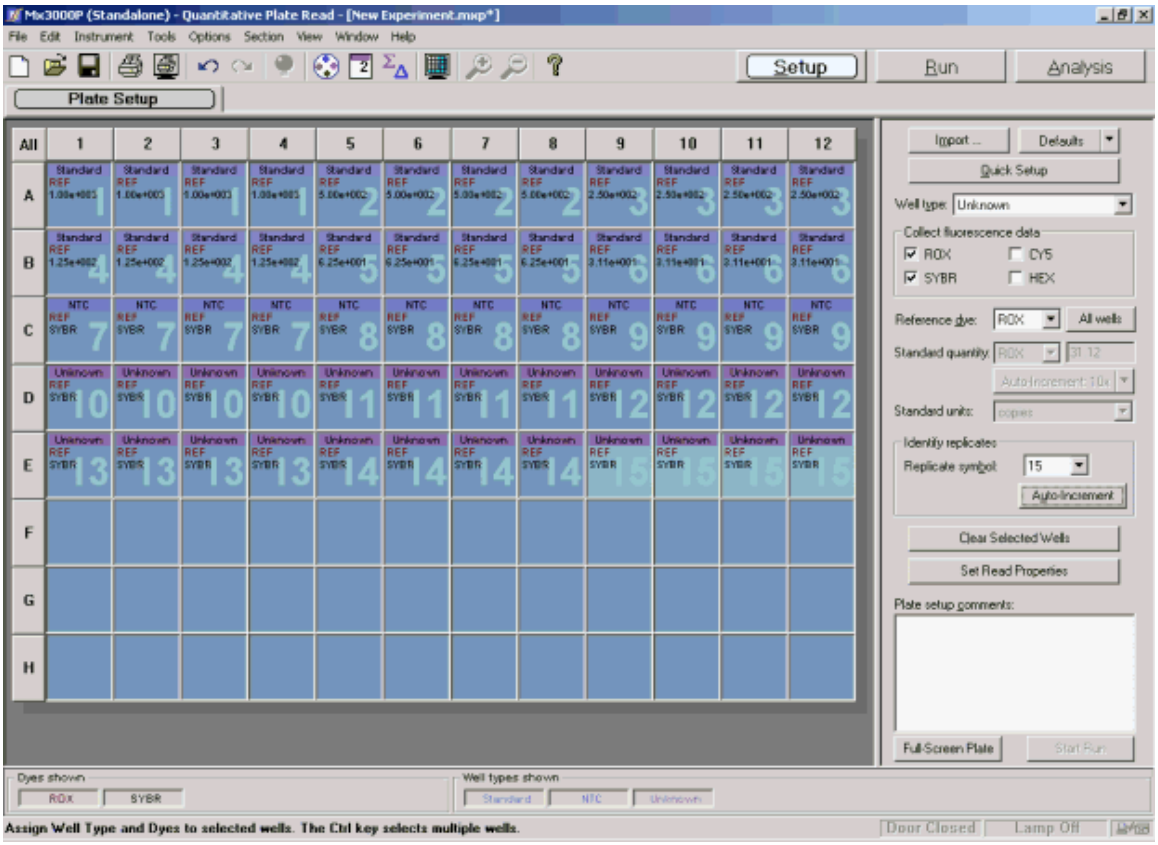
The Quantitative Plate Read Experiment Type

The **Quantitative Plate Read** experiment type uses a standard curve to estimate the amount of target present in an **Unknown** sample using a fluorescence-labeled probe or SYBR Green dye for detection. A series of **Standard** samples, containing dilutions of a known amount of target, are included in the experiment to generate a **Standard Curve** that relates the quantity of the specific target to the final fluorescence reading. The standard curve is then used to estimate the template quantity in **Unknown** wells based on the fluorescence reading for each well.

In contrast to the Mx3000P system's real-time **Quantitative PCR** experiment type, which uses threshold cycle (Ct) values to calculate initial quantities, **Quantitative Plate Read** experiments use only endpoint fluorescence values for calculating the initial quantities in **Unknowns**. Thus, although this experiment type reports **Initial template quantity** on analysis screens, the plate read measurements are taken at a phase in PCR amplification where the amplification efficiency can be influenced by limiting reagents and other spurious factors and this form of analysis should not be assumed to provide an accurate quantification of starting copy number of a target sequence.

Quantitative Plate Read Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical plate setup for **Quantitative Plate Read**.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Note *In order for the Mx3000P software to calculate p-values for positive or negative calling in* **Unknown** *wells, the Quantitative Plate Read experiment must include at least three* **NTC** *or* **dye-specific negative control** *wells for each dye.*

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) by using the **Well type** menu on the command panel. The well types available in a Quantitative Plate Read experiment are described in the table below.

Well Type	Description
Unknown	Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target.
Buffer	Contains only buffer, used to monitor the background fluorescence attributable to the buffer.
NAC	No amplification control; contains all reaction components except DNA polymerase.
NPC	No probe control; contains all reaction components except the fluorescence-labeled probe.
NTC	No template control; contains all reaction components except the template nucleic acid.
Standard	Contains a complete reaction mixture including a known concentration of target nucleic acid. Used to generate a standard curve, which is then used to relate fluorescence to initial template quantity in **Unknown** wells.
No RT	No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.
Dye-specific Negative Control	Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment is rarely used in Quantitative Plate Read experiments.
Dye-specific Positive Control	Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**. This well type assignment, which qualitatively designates a positive signal for the target without specifying the absolute amount, is rarely used in Quantitative Plate Read experiments.

After assigning the **Well type** to the selected well(s), select the dyes for which data should be collected in the wells using the **Collect fluorescence data** check boxes.

If you are using a passive reference dye in the selected well(s) select the dye from the **Reference dye** menu. If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.

If the selected well(s) is a **Standard**, for each dye for which standard curve data will be collected, enter the amount of template. To do this, first select the dye from the pulldown menu following **Standard quantity.** Next, enter the quantity of template added to the selected well(s) as a decimal number (for example, 400 or 1.234). Specify the units for the quantity in the **Standard units** menu. Instead of assigning quantities to wells on an individual basis, it is also possible to assign quantities to **Standard** wells sequentially using the **Auto-Increment** feature.

Assign the replicate set number to the selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

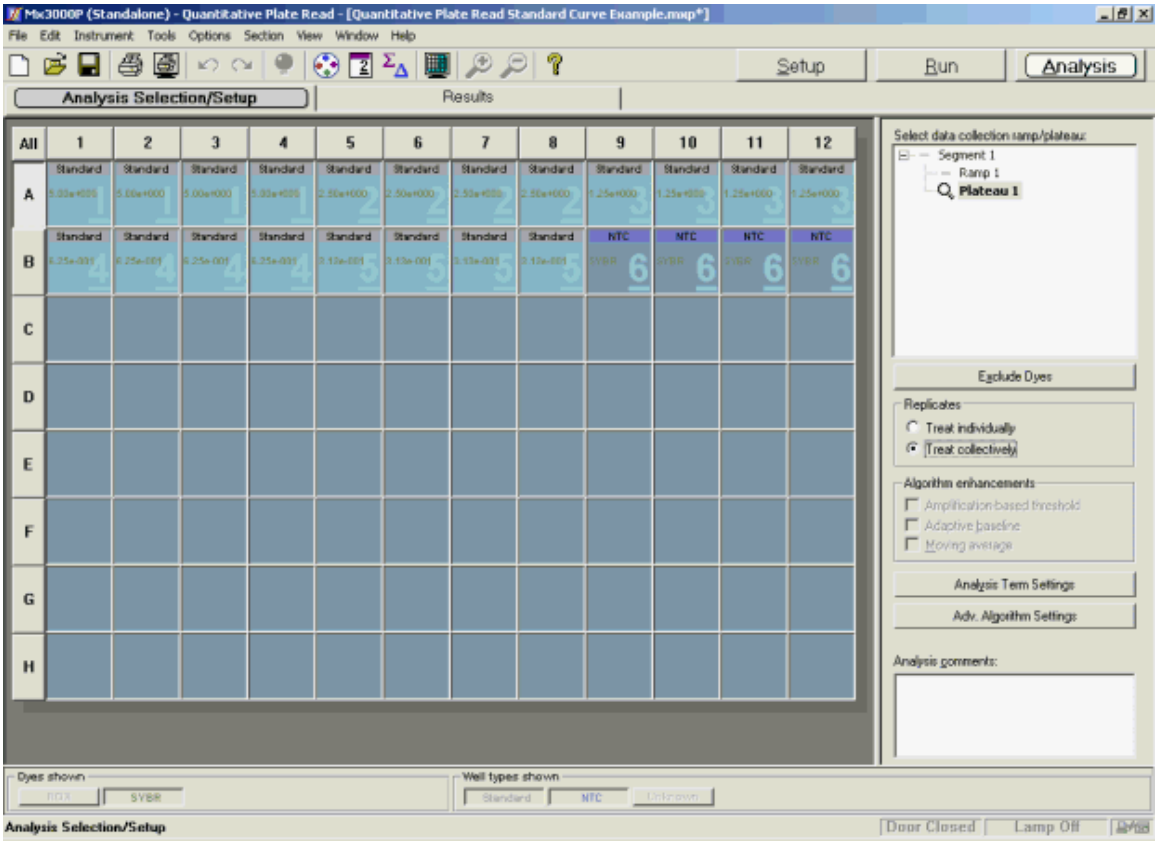
To run the experiment, set the read properties and then click **Start Run** to initiate the run.

Quantitative Plate Read Data Analysis

Analysis Setup

On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.
2. Specify the desired analysis settings on the command panel. (**Algorithm Enhancements** apply only to real-time experiments and the settings in this section are unavailable for plate read experiments.)



See **Analysis Selection/Setup** for more detailed instructions on using the features on this screen.

When all selections and settings are made, click the **Results** tab. One of several available results screens will appear. Use the radio buttons under **Area to analyze** to navigate to different analysis screens. The following screens are available for Quantitative Plate Read analysis:

Plate Sample Values

Standard Curve

Initial Template Quantity

Dual Color Scatter Plot

Fluorescence Intensity Values

Final Call Results

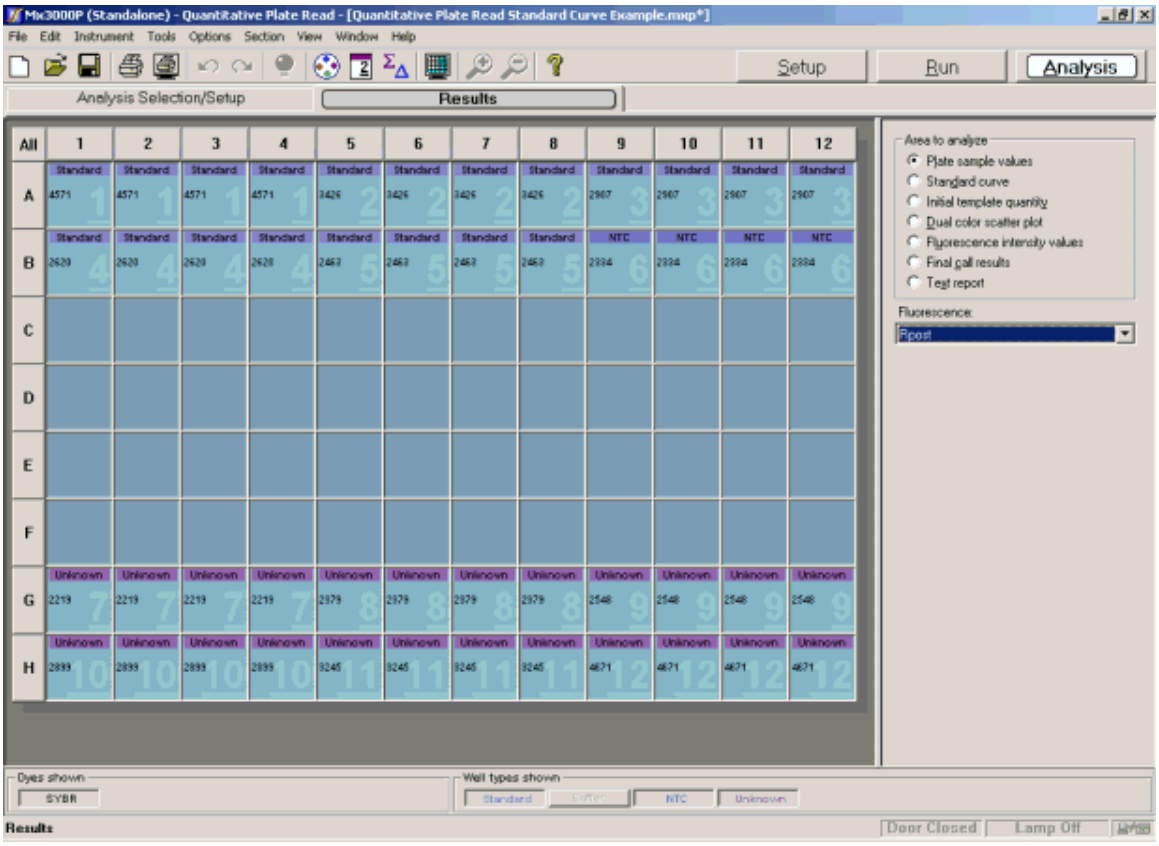
Text Report

Quantitative Plate Read Plate Sample Values

The **Plate Sample Values** screen displays fluorescence values for the sampled wells in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Fluorescence value for each dye for which data was collected
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)



Analysis Options

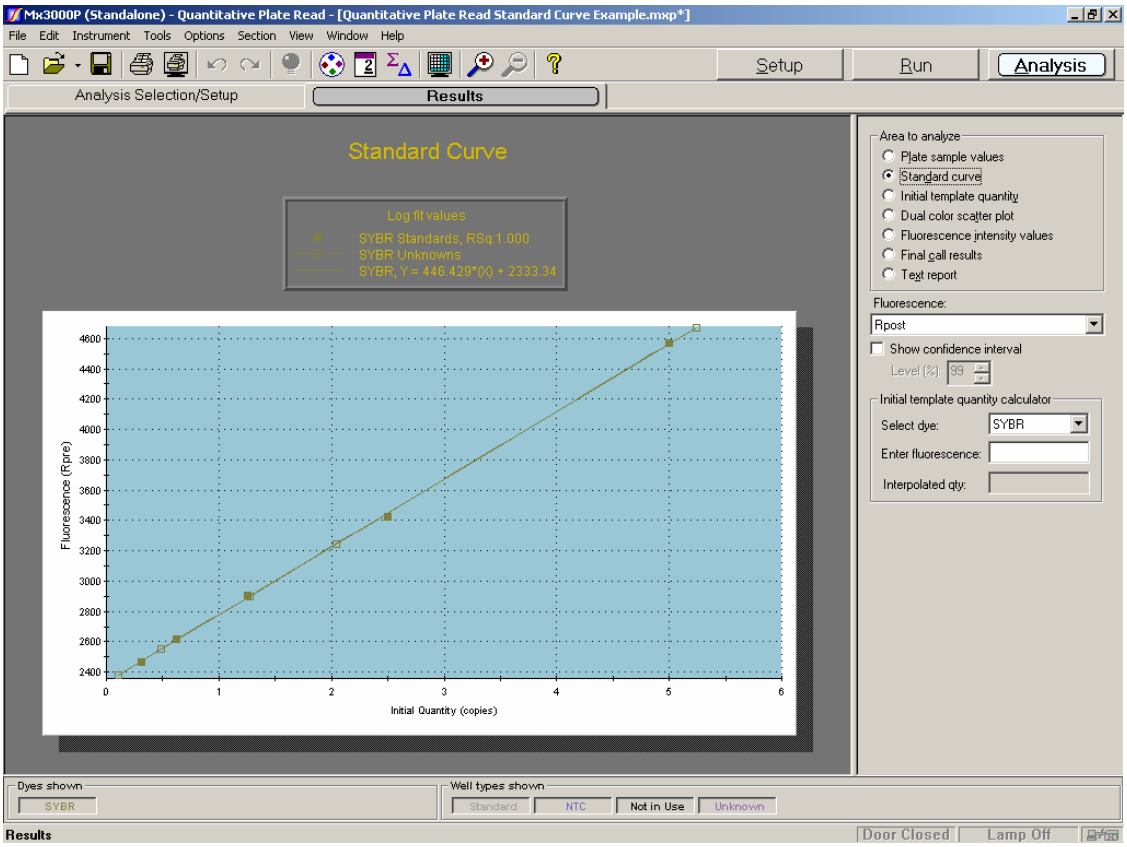
Select the type of fluorescence data to be displayed from the **Fluorescence** menu on the command panel. Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Quantitative Plate Read Standard Curve

The **Standard Curve** is a plot of the initial template quantity in the **Standard** wells (X-axis), versus **Fluorescence**. A least mean squares curve fitting algorithm is used to generate the standard curves displayed. Curves are displayed for each dye for which data was collected in **Standard** wells. **Unknown** wells selected during **Analysis Selection/Setup** are also plotted on the standard curve (using a different data point marker) based on their measured **Fluorescence** values.

If less than two **Standard** wells were selected in **Analysis Selection/Setup**, no data will be plotted on this chart.



Analysis Options

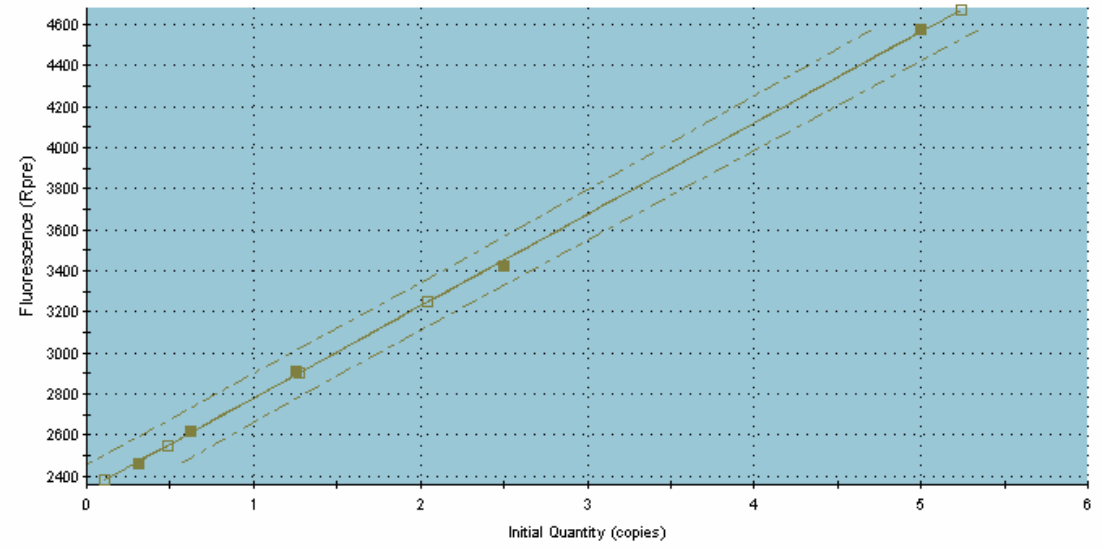
Fluorescence Data Type

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)

Confidence Interval

The confidence interval for each standard curve may be displayed by selecting the **Show confidence interval** checkbox. The resulting graph shows the confidence limits as hashed lines. The lines show the range of Initial Quantity values at a particular Fluorescence that cannot be statistically distinguished from the fit line with more certainty than the confidence level (**Level %**) selected. The width of the confidence interval is an indicator of the quality of the fit of the data to the standard curve. Confidence interval calculations require a **Replicates** setting of **Treat individually**. Confidence level values may be exported to an Excel file, a text file or an XML file by using the **Export Chart Data** commands on the **File** menu.



Display Options

To display information about a specific data point on the plot, place the cursor over the point of interest to display the following information: the well location, the well type, the dye associated with the well, the initial template quantity and the fluorescence. If the well is a **Standard**, the initial template quantity is the amount assigned to the well during plate setup. If the well is an **Unknown**, the initial template quantity is the interpolated amount based on the standard curve.

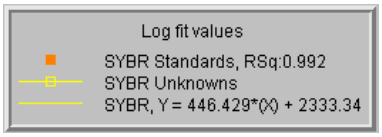
Note *If* ***Display tooltips*** *is cleared (deselected) on the* **Display** *tab of the* ***Preferences*** *dialog box, this well information will not be displayed.*

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the **Legend** is displayed or hidden. See *Graphs and Graph Properties* for more information.

To exclude a specific well/dye data point, double click on the data point on the chart. (Disabling a particular well/dye combination requires a replicate treatment setting of **Treat individually,** specified on the **Analysis Selection/Setup** screen). To restore an excluded data point, go to the **Analysis Selection/Setup** screen, click on **Exclude Dyes**, and then press the button corresponding to the specific dye/well combination, restoring the button to a depressed state.

Interpretation and Use of Curve Attributes

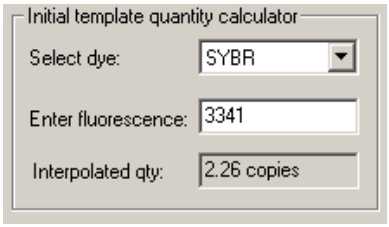
The graph legend displays information about each curve plotted. (If no legend is displayed on the screen, open the **Graph Properties** dialog box by double-clicking on the graph, and then select the **On** radio button under **Legend**.) Besides correlating plot markers with dyes and well types, the legend displays the R Squared (**RSq**) value and the equation for the line (y = mx + b).



The **RSq** value is an indication or the quality of the fit of the standard curve to the **Standard** data points plotted. The value will always be between 0 and 1. The closer the value is to 1, the better the fit of the line.

Using the Initial Template Quantity Calculator

To use the displayed standard curve to calculate an interpolated quantity of template in another sample from the run based on the fluorescence reading, choose the dye from the **Select dye** menu, then enter the **fluorescence** value. The software will calculate and display the interpolated initial template quantity (**Interpolated qty**). Note that initial template quantities are automatically determined, based on the standard curves, for all **Unknowns** in the same experiment, and these results may be viewed on the Initial Template Quantity results screen.



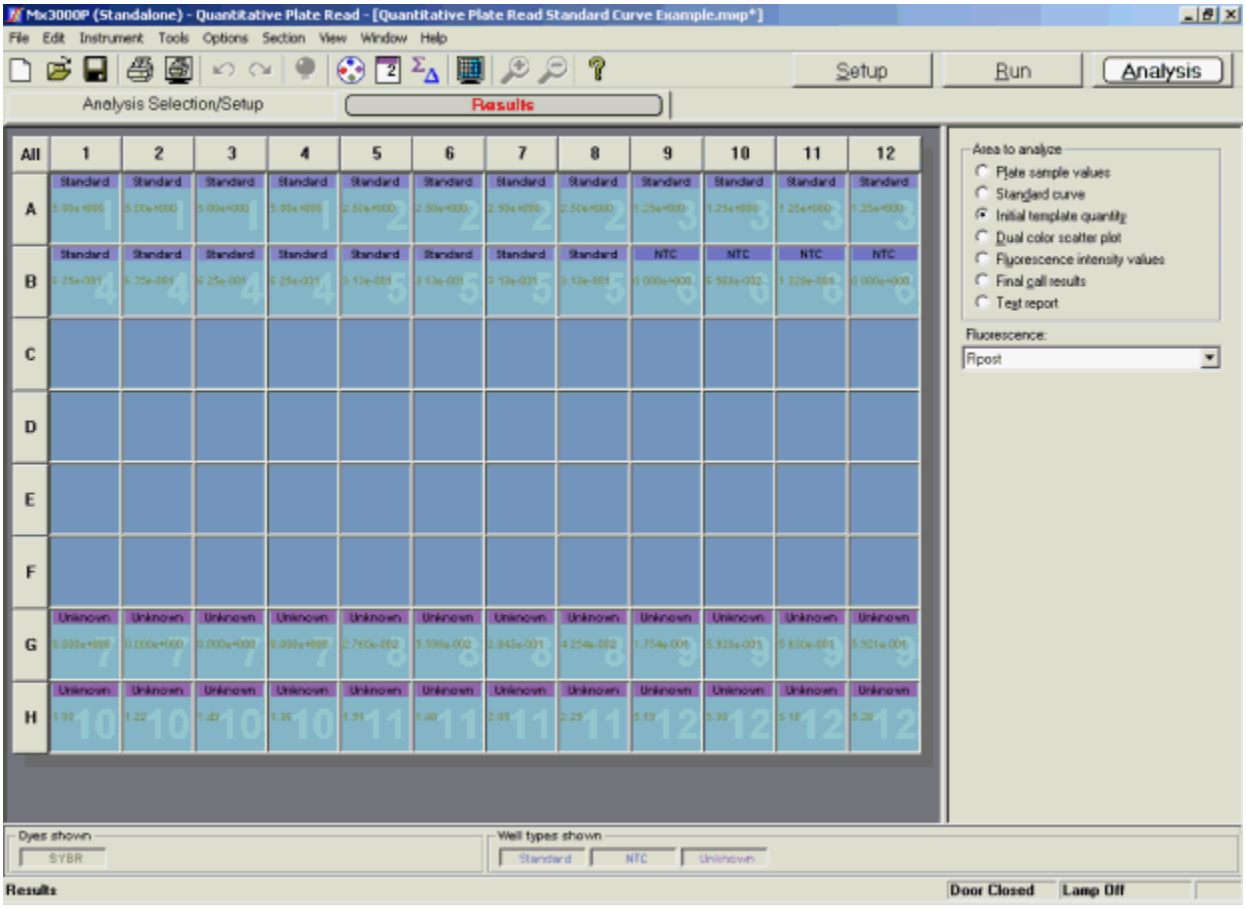
Quantitative Plate Read Initial Template Quantity

The **Initial Template Quantity** screen displays interpolated quantities of template added to **Unknown** wells before thermal cycling in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Initial template quantity (for **Unknowns**, the value interpolated from the standard curve based on the fluorescence measured for the **U**nknown; for **Standards**, the amounts entered during plate setup)
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)

If less than two **Standard** wells were selected in **Analysis Selection/Setup**, a standard curve cannot be plotted and an error message will appear in place of results in the **Unknown** wells.



Analysis Options

Fluorescence Data Type

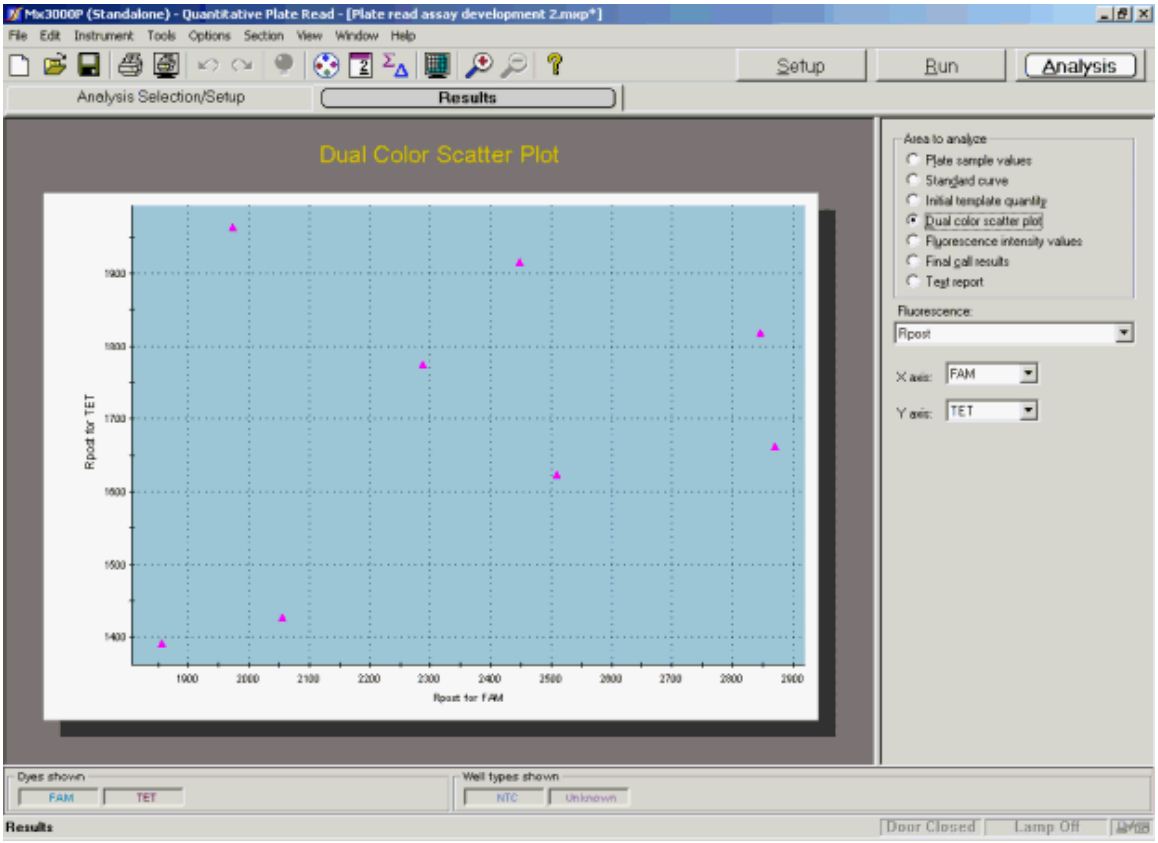
Select the type of fluorescence data to be used for interpolation from the **Fluorescence** menu on the command panel. Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)

Quantitative Plate Read Dual Color Scatter Plot

The **Dual Color Scatter Plot** screen is useful to compare the final quantities of two different targets (represented by two different dyes) in the same well.

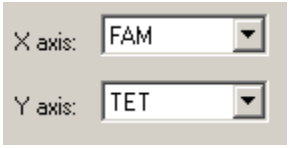
Each plotted point represents the coordinates of the fluorescence values for two dyes in a single well. For example, the X-axis may correspond to FAM fluorescence while the Y-axis corresponds to TET fluorescence and the plotted point (x,y) corresponds to the coordinates describing the two fluorescence values determined for a given well.



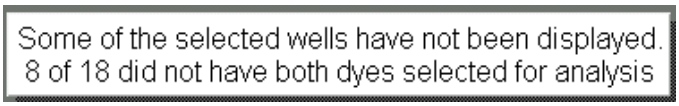
Analysis Options

Dye Selection

Specify the dye for which data should be plotted on each axis by selecting the dyes of interest from the **X axis** and **Y axis** menus on the command panel. Any two dyes assigned during **Plate Setup** may be specified.



A well will not be included in the scatter plot if the two dyes selected for the plot are not assigned to that well. An error message similar to the example shown below will appear if wells selected during **Analysis Selection/Setup** cannot be displayed.

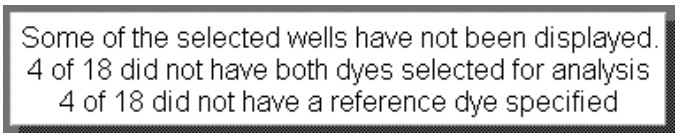


Fluorescence Data Type Plotted

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Note that if no reference dye has been assigned to the wells and a normalized data type (**Rn**) is selected, a message like the following will appear below the graph:



Display Options

To display information about a specific data point on the scatter plot, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

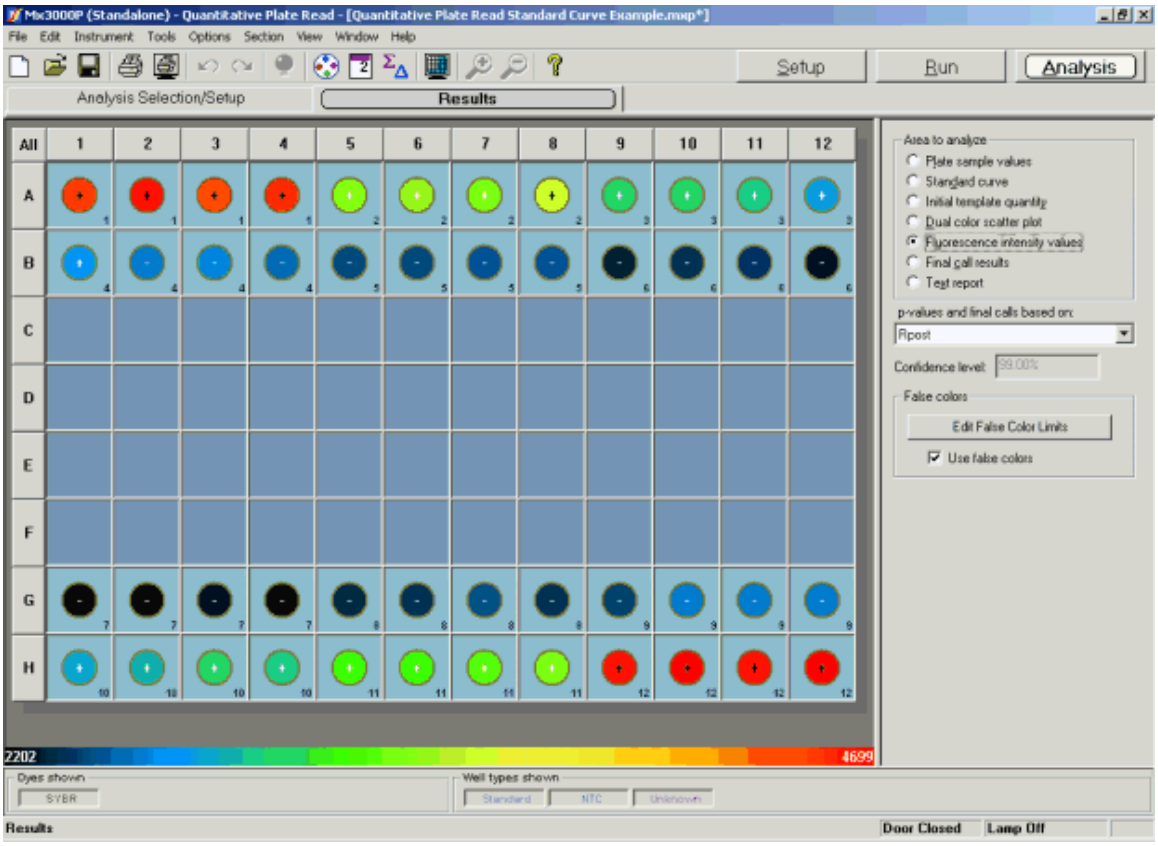
The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including the attributes of the axes. See *Graphs and Graph Properties* for more information.

Quantitative Plate Read Fluorescence Intensity Values

The **Fluorescence Intensity Values** screen displays a colorized image of final calls and fluorescence intensities. A plus sign (+) indicates a positive call and a minus sign (–) indicates a negative call.

A spectrum of colors, displayed at the bottom of the screen, is used to indicate the relative fluorescence readings for each dye in each well.

The circle for each well is divided into arcs which correspond to the dyes selected for analysis in the **Analysis Selection/Setup** screen. An **R** in a portion of the circle indicates that portion refers to a reference dye. The replicate set number, if any, appears in the lower right corner of the well.



Interpreting the Intensity Values Display

The accumulation of product can be interpolated by comparing the color in the arc to the horizontal color bar at the bottom of the screen. On the color bar, as fluorescence values increase, the corresponding color shifts from blue towards red. Note that the bar only covers the fluorescence range measured on the plate. For example, in the example shown above, the range of fluorescence measured in the run was between 2202 and 4699, and this is the range that is covered by the color bar.

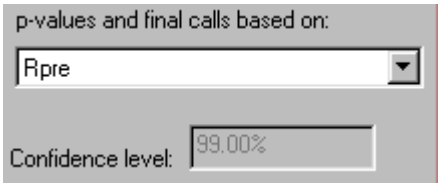
Interpreting Final Calls

The **p-value** is the probability that the mean of one set of sample data is different from the mean of another set of sample data. The first set of sample data is always the control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an **Unknown** well). When replicates are being treated collectively, the second set of sample data consists of all of the replicates. If the **p-value** exceeds the user-specified confidence level, the well/dye is called as positive and signified with a plus sign (**+**); otherwise, the well is called as negative (no difference detected) and signified with a minus sign (**–**). The confidence level for positive calling is displayed on the command panel. This value may be changed using the **Analysis Term Settings** dialog box that may be accessed by clicking the corresponding button on the **Analysis Selection/Setup** screen.

Analysis Options

Fluorescence Data Type Displayed and Used for Calculations

Under **p-values and final calls based on,** specify the form of the fluorescence data to be displayed and used in calculating the p-values used for making final calls.



Available fluorescence data types are:

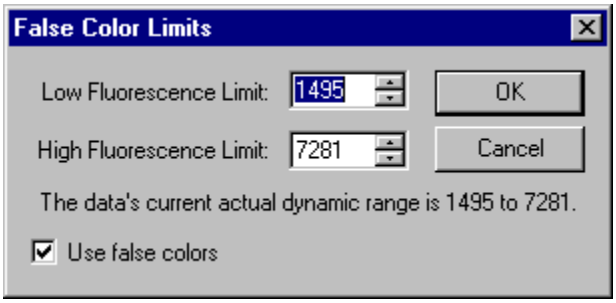
Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Display Options

Adjusting the Fluorescence Intensity Range

The range of fluorescence intensity covered by the color spectrum can be manually specified by clicking the **Edit False Color Limits** button to open the **False Color Limits** dialog box.

The minimum value for the **Low Fluorescence Limit** is 0 and the maximum value for the **High Fluorescence Limit** is 65535. A range of at least 4 must be specified. Manually specifying a narrow range of values is helpful when attempting to discern small differences in fluorescence values among samples.



False Colors Versus Grayscale

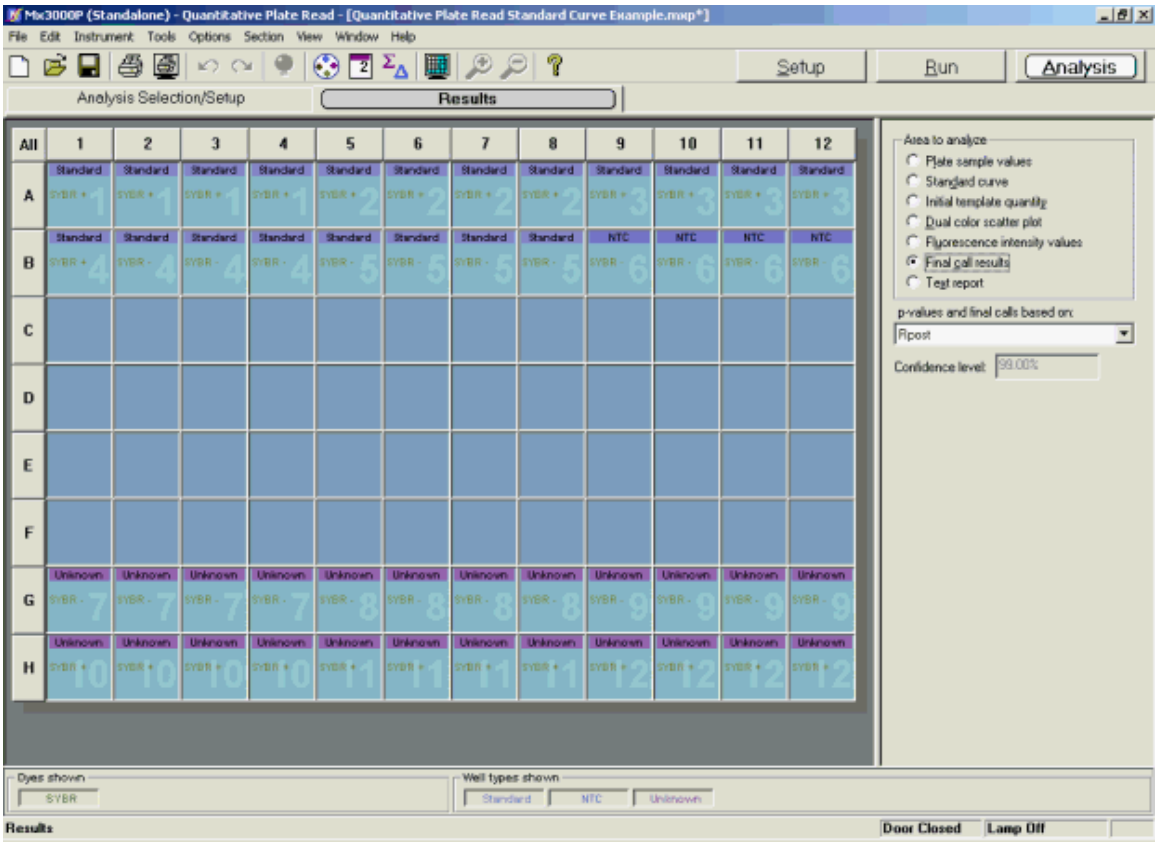
By default, the Mx3000P software uses a multi-hued spectrum of colors to depict differences in fluorescence values on this screen. The depiction may be changed to grayscale by deselecting the **Use false colors** checkbox. All colors will turn to shades of gray and the color bar will display a gradient of gray from black (lowest fluorescence) to white (highest fluorescence).

Quantitative Plate Read Final Call Results

The **Final Call Results** screen provides a simple depiction of whether product was accumulated for each dye in each well in a plate format. A plus sign (**+**) signifies detection of product accumulation for the indicated dye, while a minus sign (**–**) signifies that product accumulation was not detected. See below for more information on how final calls are made.

In each well with a well type assigned, the following information is shown:

- Well type
- The final call for each dye for which data was collected. [For all well types, the reference dye (**Ref**) is not associated with a final call]
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)



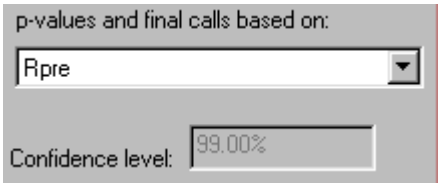
Interpreting Final Calls

The **p-value** is the probability that the mean of one set of sample data is different from the mean of another set of sample data. The first set of sample data is always the control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an **Unknown** well). When replicates are being treated collectively, the second set of sample data consists of all of the replicates. If the **p-value** exceeds the user-specified confidence level, the well/dye is called as positive and signified with a plus sign (+); otherwise, the well is called as negative (no difference detected) and signified with a minus sign (–). The **Confidence level** is displayed on the command panel. This value may be changed using the **Analysis Term Settings** dialog box..

Analysis Options

p-Values and Final Calls Based On

Under **p-values and final calls based on,** specify the form of the fluorescence data to be used in calculating the p-values used for making final calls.



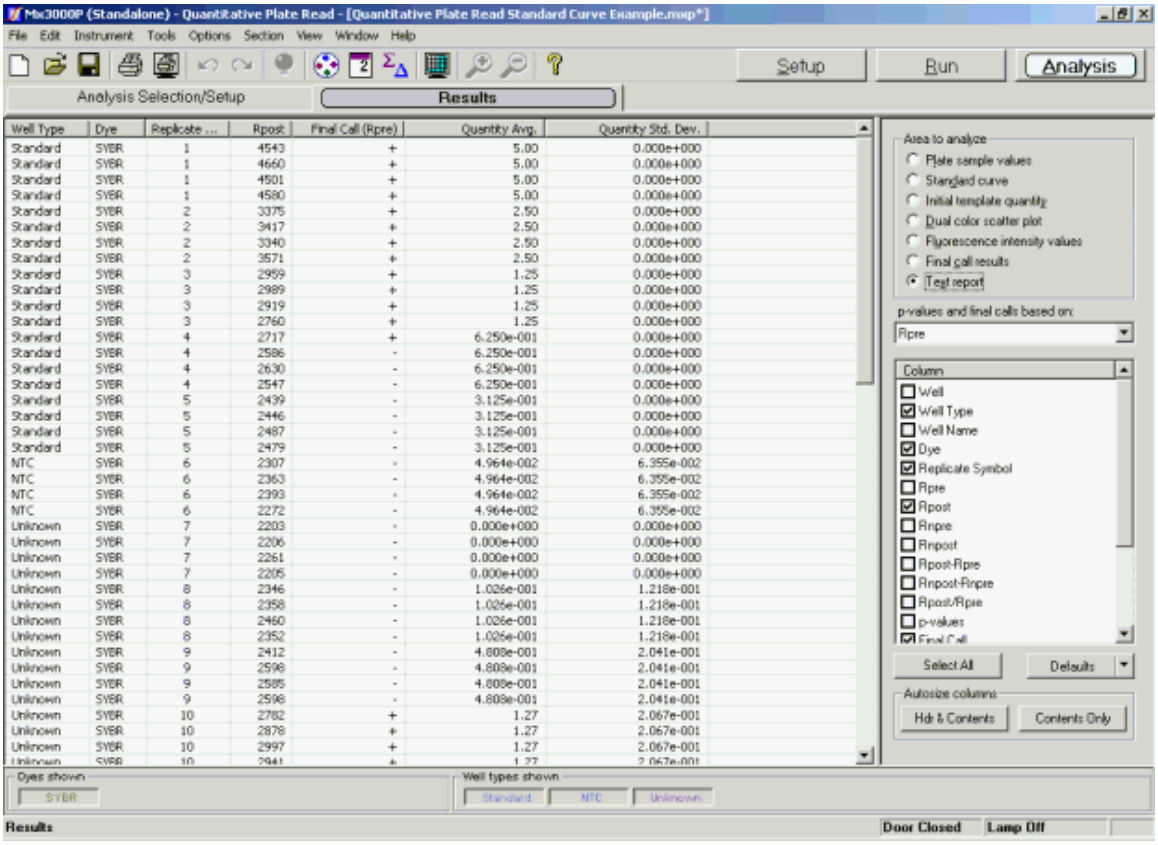
Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Note that if no reference dye has been assigned to the wells and a normalized data type (**Rn**) is selected, a **No Ref** error designation will replace the final call entry on the plate.

Quantitative Plate Read Text Report

The **Text Report** screen shows the data from a run in text format.



Analysis Options

p-Values and Final Calls Based On

Under **p-values and final calls based on,** specify the form of the fluorescence data to be used in calculating the p-values used for making final calls.

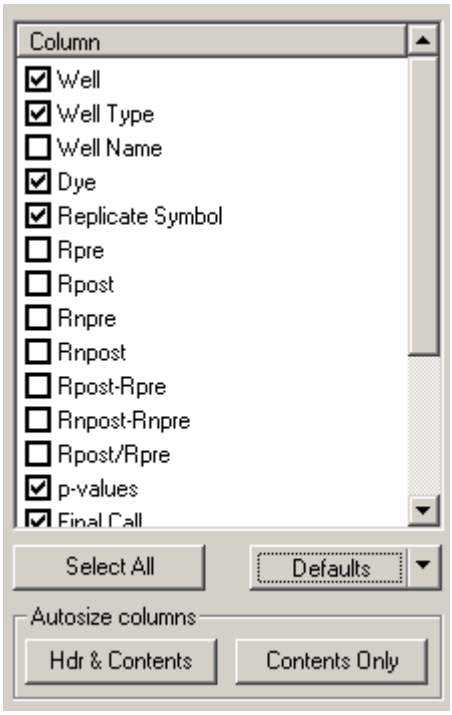
Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Note that if no reference dye has been assigned to the wells and a normalized data type (**Rn**) is selected, a **No Reference** error designation will replace the p-value and final call entries in the report.

Display Options

Columns Included in the Report



Select the **Columns** to be included in the text report by checking the boxes next to the column names. To exclude columns that are selected by default, click on the check box to deselect the column name. To select all columns to display, click **Select All**. The default settings for column selection can be restored by expanding the **Defaults** menu and then clicking **Get**.

If the included columns will not fit in the screen, a horizontal scroll bar appears at the bottom of the window to allow horizontal scrolling.

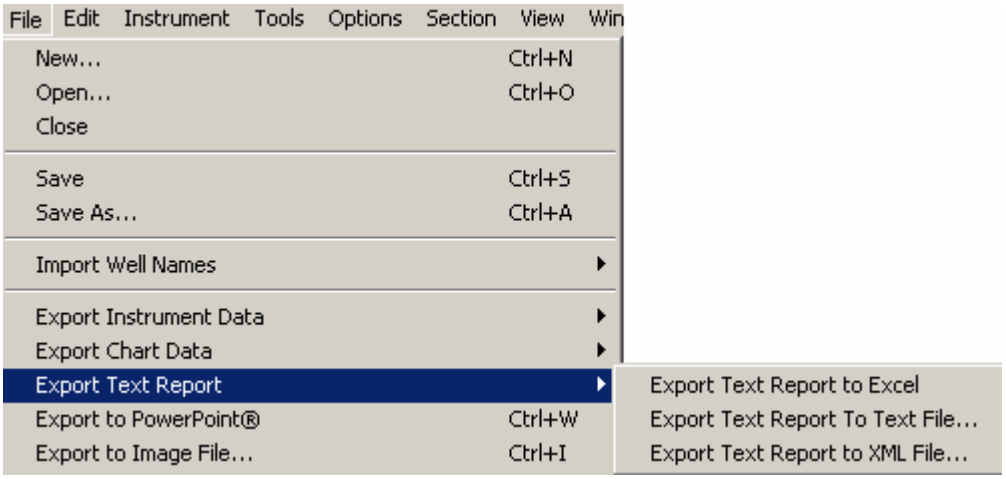
Column Width

To facilitate viewing data, columns can be automatically sized. Clicking the **Contents Only** button will cause each column to be automatically resized to the width of the widest data cell in that column (some column headers may be only partially displayed using this setting). Clicking the **Hdr & Contents** button will cause each column to be resized to the widest cell in that column, including the header cell.

Columns can also be resized manually by dragging the column dividers with the mouse.

Exporting Text Report Data

Text report data can be exported from the application in text, Microsoft Excel spreadsheet or XML format for analysis. Text report exporting options are available from the **File** menu. See *Exporting Data to Other Applications* in the *File Menu* section for more information.



Mx3000P System Experiments: Plate Read/Allele Discrimination

The Plate Read/Allele Discrimination Experiment Type

The **Plate Read/Allele Discrimination** experiment type is used both for general plate-reading tasks and for allele discrimination assays with endpoint detection. In both cases, samples may be called as positive or negative for the presence of a specific sequence based on whether the endpoint fluorescence readings meet user-defined statistical criteria.

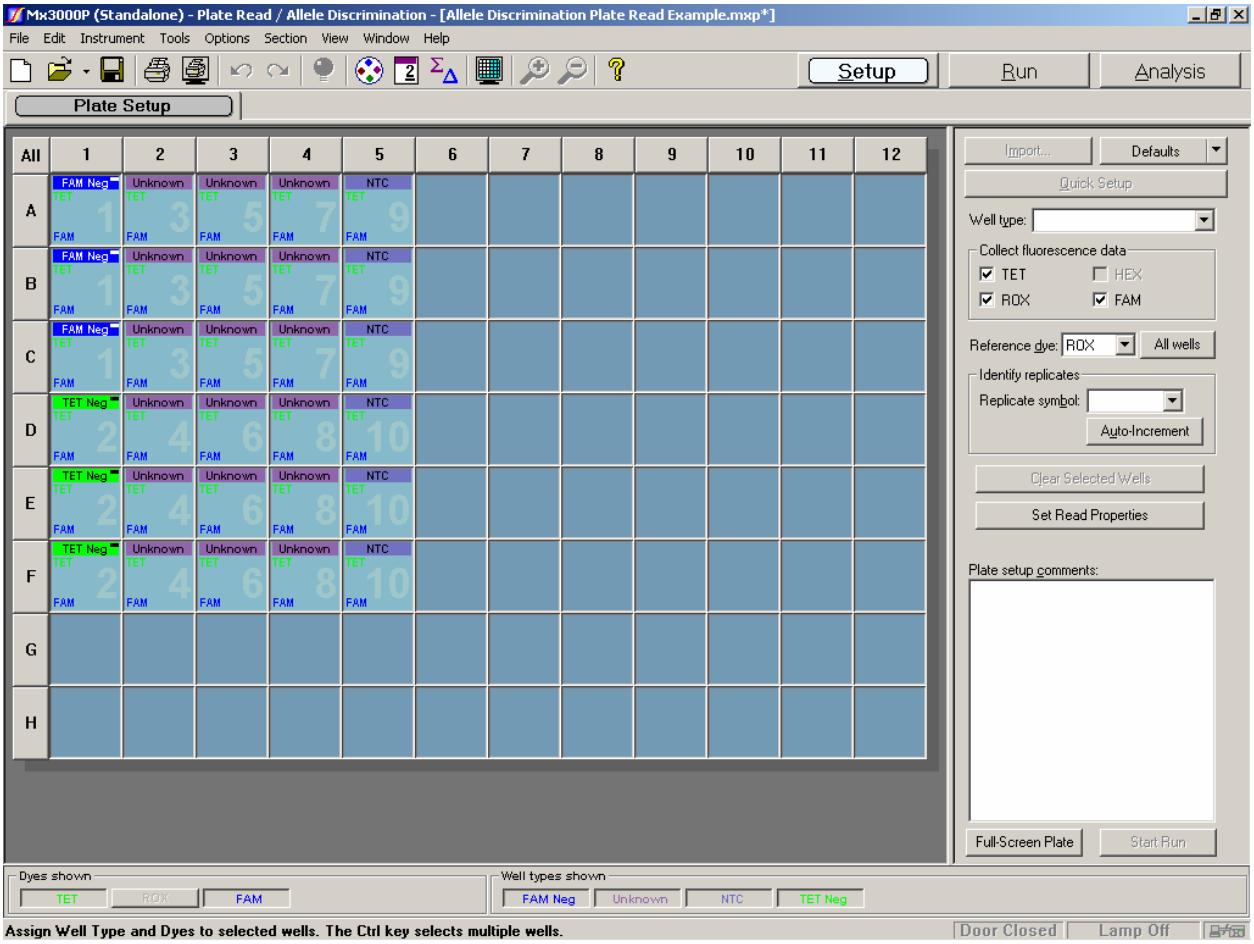
This experiment type may be used for any application that requires a single round of plate readings or pre- and post-cycling readings collected at a single temperature. The simplest application of the plate read function of the Mx3000P instrument is qualitative detection, or detection of the presence or absence of a specific PCR product. In experiments of this type, a fluorogenic probe is added to PCR reactions along with the other PCR components. PCR is carried out on any thermal cycler and then the fluorescence is detected at the end of cycling using the plate read mode of the Mx3000P instrument. This reading provides a measure of the amount of specific product that has accumulated at the end of the PCR reaction and can be used to detect the presence or absence of a particular sequence. Note that plate read measurements are taken at a phase in PCR amplification where the amplification efficiency can be influenced by limiting reagents, small differences in reaction components, or cycling conditions and do not provide an accurate quantification of starting copy number of a target sequence.

The **Allele Discrimination** assay with endpoint detection is a commonly used application of the plate read function of the Mx3000P system. For example, two fluorogenic probes, labeled with two spectrally distinct dyes, might be used to discriminate between the wild-type allele and a mutant allele. If **Fluorescence** of the unknown DNA sample is statistically higher than background for the dye identifying the wild-type allele but not for the dye identifying the mutant allele, the sample can be designated as wild-type homozygous. If **Fluorescence** is above background for the dye identifying the mutant allele but not for the dye identifying the wild-type allele, the sample can be designated as mutant homozygous. If the sample generates intermediate values for both dyes, it is designated as heterozygous for the two alleles. Results are conveniently displayed to highlight the allelic composition of the samples on the **Dual Color Scatter Plot**, the **Fluorescence Intensity Values** and the **Final Call Results** analysis screens.

The Mx3000P system also features the Allele Discrimination/SNP's Real Time experiment type, which should be used for allele discrimination assays that require maximum accuracy and sensitivity.

Plate Read/Allele Discrimination Plate Setup

In the **Setup** section of the software, open the **Plate Setup** tab. Shown below is a typical plate setup for an allele discrimination assay performed using a **Plate Read/Allele Discrimination** experiment.



The Mx3000P software features several shortcuts to expedite plate setup including the ability to **Import** an existing setup, to use a **Default** setup, and to rapidly specify collection of data for all dyes in all wells by using **Quick Setup**.

Note *In order for the Mx3000P software to calculate p-values for positive or negative calling in* **Unknown** *wells, the Plate Read/Allele Discrimination experiment must include at least three* **NTC** *or* **dye-specific negative control** *wells for each dye.*

Plate Setup is accomplished using the selection boxes and commands on the command panel at the right of the screen. The command panel is designed to be followed in a top-to-bottom order, beginning with **Well type** assignment. All other selections are unavailable until after **Well type** has been assigned.

Select the well(s) to be assigned using the mouse then assign the well(s) by using the **Well type** menu on the command panel. The well types available in an Allele Discrimination Plate Read experiment are described in the table below.

Well Type	Description
Unknown	Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target.
Buffer	Contains only buffer, used to monitor the background fluorescence attributable to the buffer.
NAC	No amplification control; contains all reaction components except DNA polymerase.
NPC	No probe control; contains all reaction components except the fluorescence-labeled probe.
NTC	No template control; contains all reaction components except the template nucleic acid.
No RT	No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.
Not in Use	Optional designation for wells that are not in use. It is **not** necessary to define any unused wells. This well type is equivalent to an unassigned well.
Dye-specific Negative Control	Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**.
Dye-specific Positive Control	Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**.

After assigning the **Well type** to the selected well(s), select the dyes for which data should be collected in the wells using the **Collect fluorescence data** check boxes.

If you are using a passive reference dye in the selected well(s) select the dye from the **Reference dye** menu. If all sample wells on the plate will contain the same passive reference dye, depress the **All wells** button.

Assign the replicate set number to the selected well(s) by using the **Replicate symbol** menu. Instead of assigning replicate numbers to wells on an individual basis, it is also possible to assign replicates sequentially using the **Auto-Increment** feature.

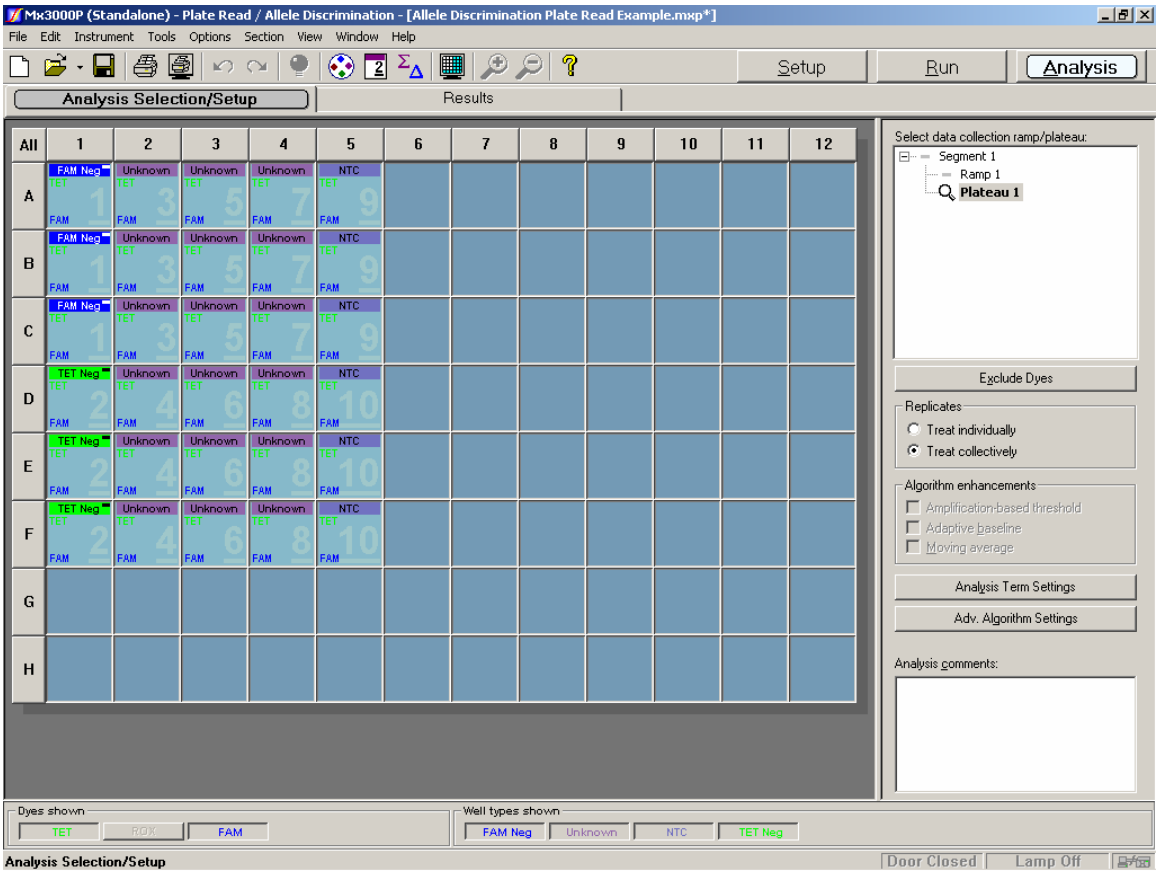
To run the experiment, set the read properties and then click **Start Run** to initiate the run.

Plate Read/Allele Discrimination Data Analysis

Analysis Setup

On the **Analysis Selection/Setup** screen:

1. Select the wells to be included in the analysis.
2. Specify the desired analysis settings on the command panel. (**Algorithm Enhancements** apply only to real-time experiments and the settings in this section are unavailable for plate read experiments.)



See **Analysis Selection/Setup** for more detailed instructions on using the features on this screen.

When all selections and settings are made, click the **Results** tab. One of several available results screens will appear. Use the radio buttons under **Area to analyze** to navigate to different analysis screens. The following screens are available for Plate Read/Allele Discrimination analysis:

Plate Sample Values

Dual Color Scatter Plot

Fluorescence Intensity Values

Final Call Results

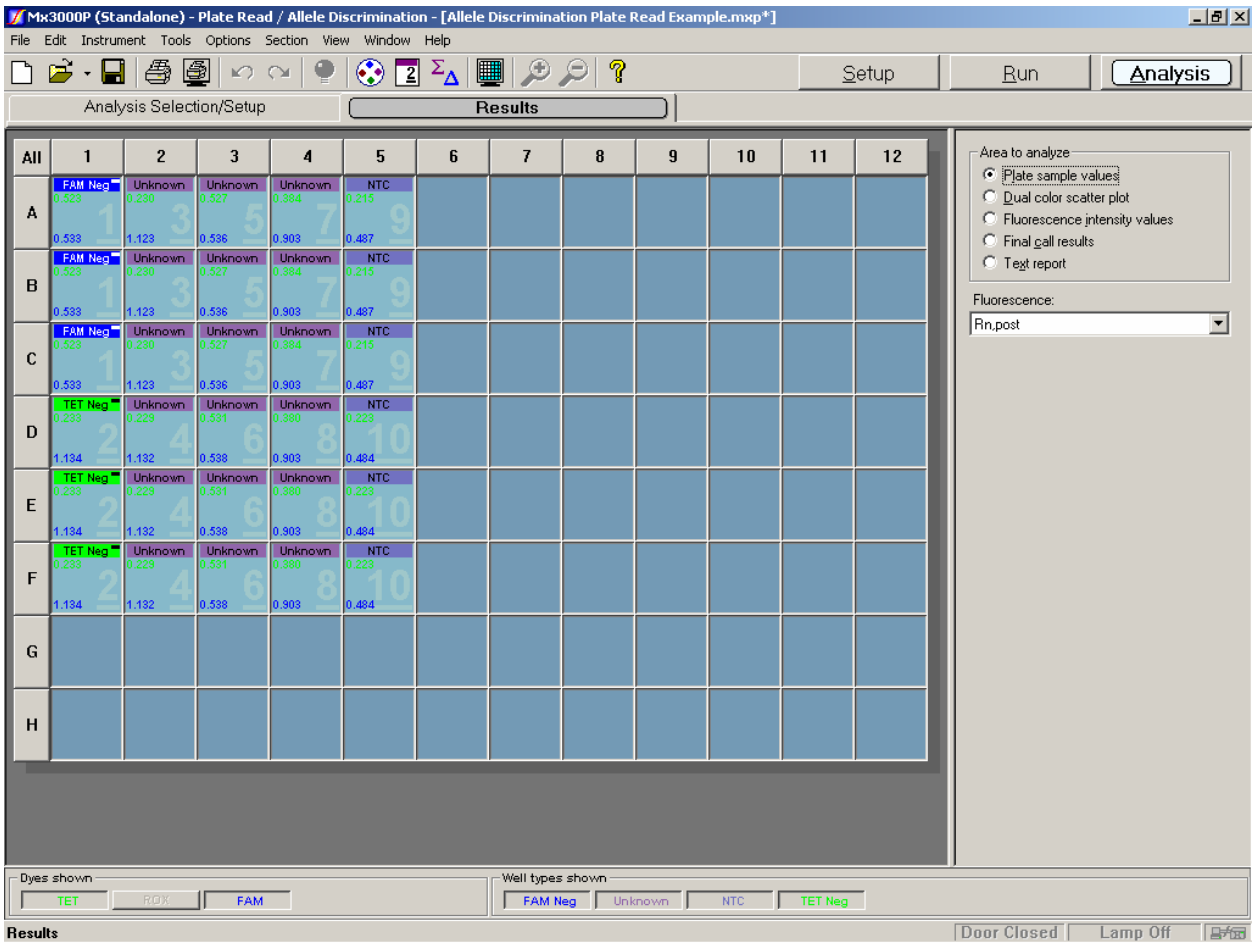
Text Report

Plate Read/Allele Discrimination Plate Sample Values

The **Plate Sample Values** screen displays fluorescence values for the sampled wells in a plate format.

In each well with a well type assigned, the following information is shown:

- Well type
- Fluorescence value for each dye for which data was collected
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)



Analysis Options

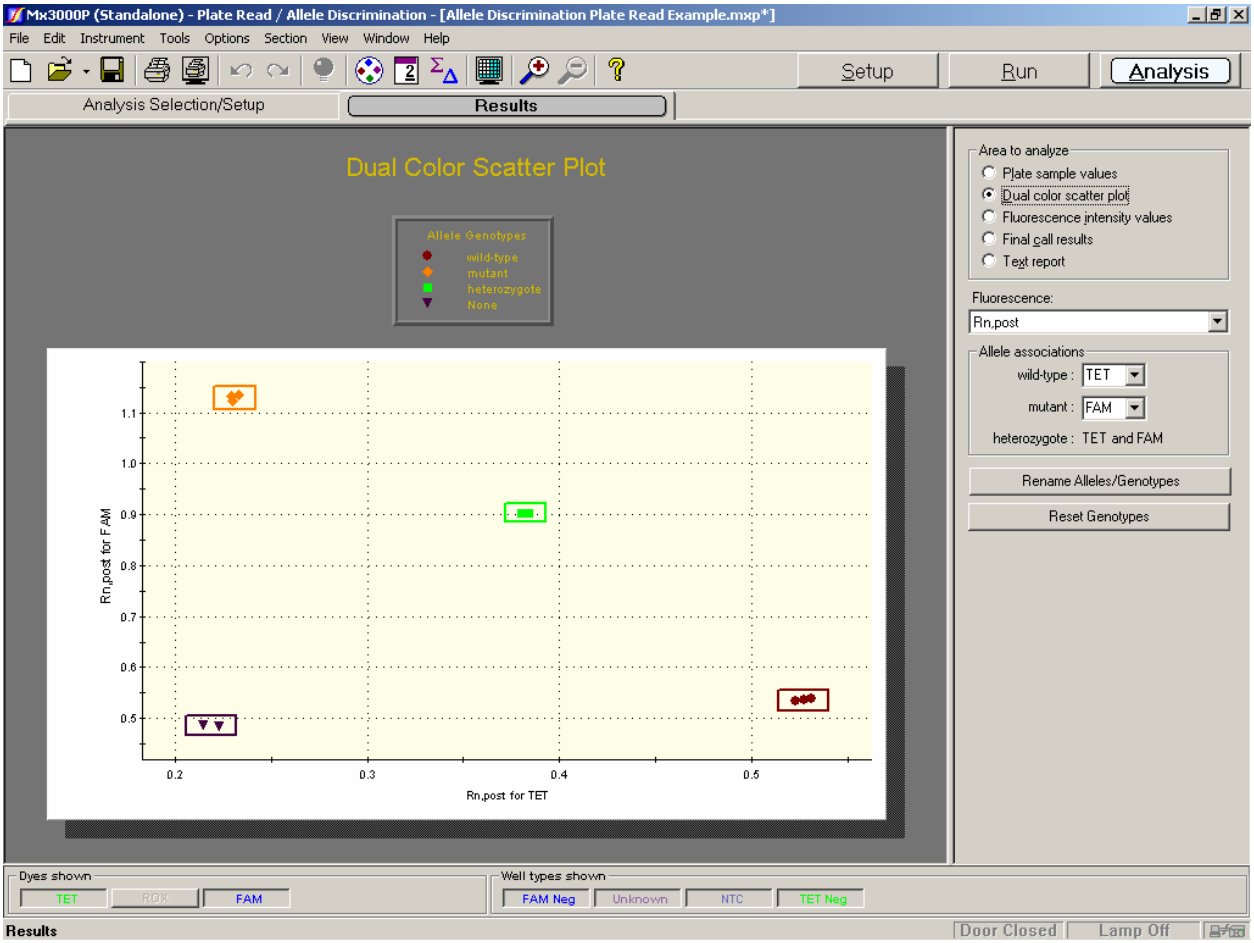
Select the type of fluorescence data to be displayed from the **Fluorescence** menu on the command panel. Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Plate Read/Allele Discrimination Dual Color Scatter Plot

The **Dual Color Scatter Plot** screen is useful to compare the final quantities of two different targets (represented by two different dyes) in the same well. This form of analysis is especially useful for allele discrimination experiments, where the quantity of each of two possible alleles is indicated by a different dye.

Each plotted point represents the coordinates of the fluorescence values for two dyes in a single well. For example, the X-axis may correspond to TET fluorescence while the Y-axis corresponds to FAM fluorescence and the plotted point (x,y) corresponds to the coordinates describing the two fluorescence values determined for a given well. The position of the data point for a given well on the scatter plot indicates the presence or absence of each allele. Colored rectangles on the plot are used to group wells with a common genotype (allelic composition). The shapes of the genotype-marking rectangles may be changed, and individual data points may also be excluded from the genotype group. The two alleles may be named on this screen, with the user-provided allele names reflected in the genotype descriptions of the wells on this and other **Results** screens.



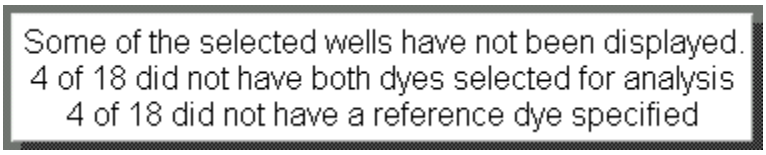
Analysis Options

Fluorescence Data Type Plotted

Select the type of fluorescence data to be plotted from the **Fluorescence** menu on the command panel. Available fluorescence data types are:

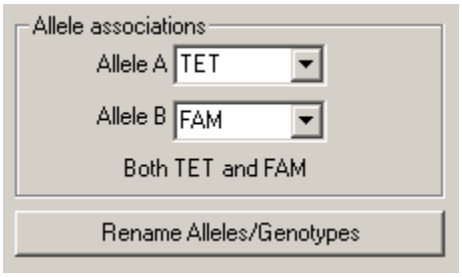
Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Note that if no reference dye has been assigned to the wells and a normalized data type (**Rn**) is selected, a message like the following will appear below the graph:



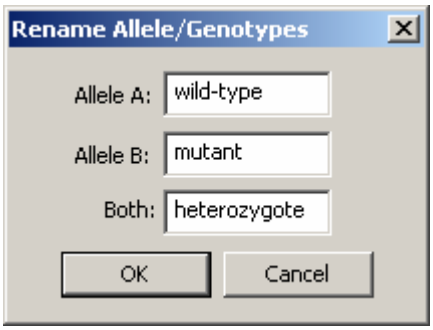
Allele Associations

The **Allele associations** section of the command panel is used to identify the association between each of the plotted dyes and the allele detected by the dye. The allele/dye specified in the top menu will be plotted on the X axis, and the allele/dye specified in the bottom menu will be plotted on the Y axis. Any two dyes assigned during **Plate Setup** may be selected in these menus.



By default, alleles are named *Allele A* and *Allele B*. The allele names also correspond to the genotypes assigned to wells in which a single allele is detected. *Both* is the default name for the genotype group in which both alleles are detected. Each of these allele/genotype names may be

changed to user-specified names by clicking **Rename Alleles/Genotypes**. When the following dialog box appears, enter the desired allele/genotype names and then click **OK**.



The new allele names will appear in the **Allele associations** section of the command panel. The specified allele or genotype names are also displayed on some other **Results** screens.

Display Options

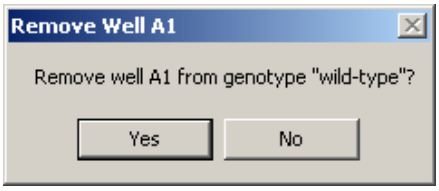
Display of Genotype Groups on the Scatter Plot

Wells with a common genotype (allelic composition) are grouped on the scatter plot in color-coded rectangles. The genotype corresponding to each group is indicated in the graph legend (see *Graph Properties* below). If a single allele was detected in a well, the genotype designation corresponds to the allele name. If both alleles were detected, the genotype is designated *Both* by default; this designation may be changed by clicking **Rename Alleles/Genotypes** on the command panel. If neither allele was detected, the genotype is designated *None.*

The size and shape of each rectangle may be changed by dragging any side of the rectangle with the mouse. The color of the rectangle and the enclosed group of data points may be changed for any genotype by double-clicking on the genotype's entry in the legend.

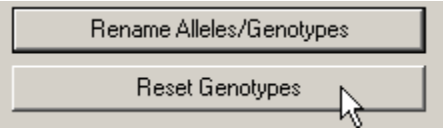
Note *The size and shape of a rectangle does not convey information about dye intensity, data quality, or any other measured data attribute. The rectangles are only intended to identify and group the data points which fit the positive/negative calling result criteria that define each genotype.*

Any well may be manually excluded from the genotype groups by double-clicking on the data point for the well. The following dialog box will appear.



Once **Yes** is selected, the symbol for the affected data point will change to the color and point style assigned to excluded data points. In addition, the **Genotype** for the well will change in the **Text Report**.

To restore any manually removed data points to their original genotype assignments, click **Reset Genotypes** on the command panel.



Graph Properties

The **Graph Properties** dialog box, accessed by double-clicking on the graph, controls several display features including whether the legend is shown or hidden and the attributes of the axes. See *Graphs and Graph Properties* for more information.

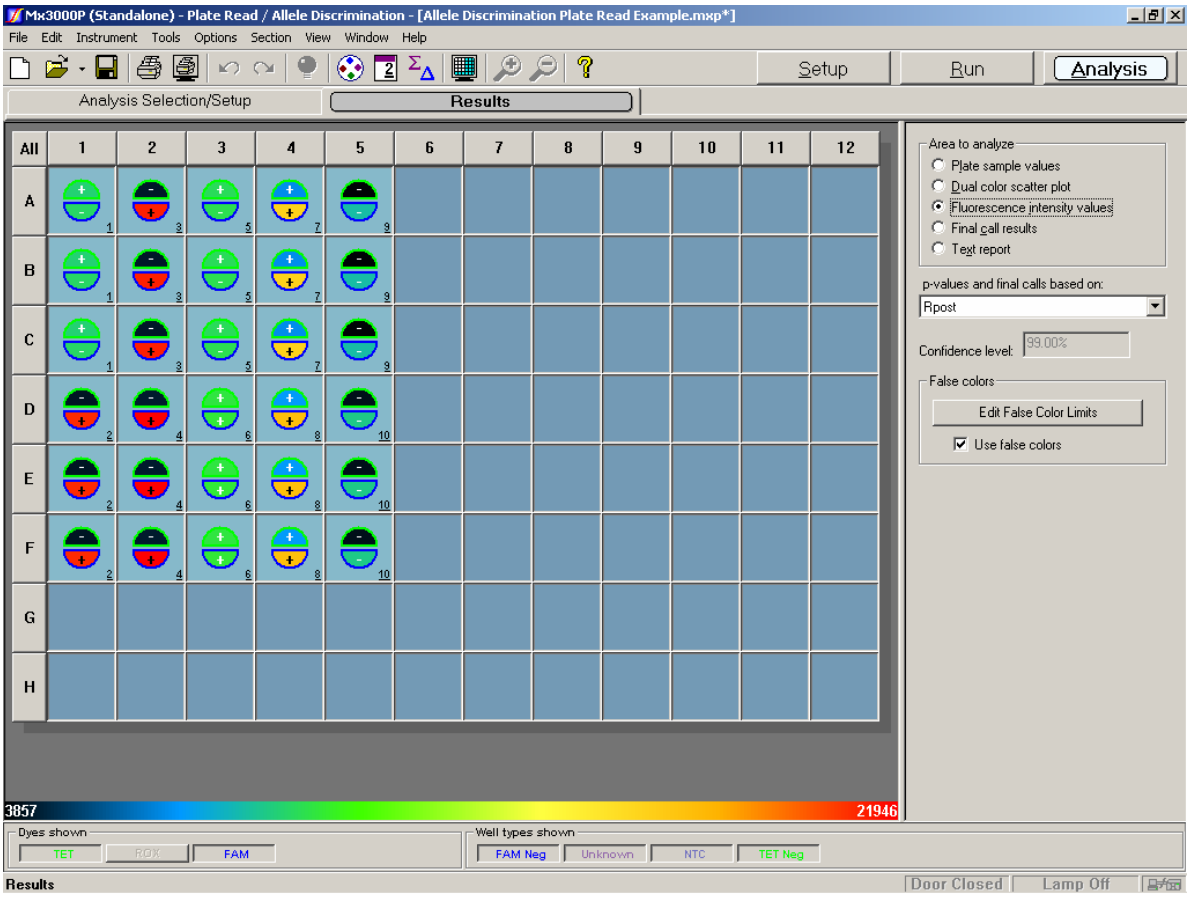
To display information about a specific data point on the scatter plot, place the cursor over the point of interest. (Note that **Display tooltips** must be selected on the **Display** tab of the **Preferences** dialog box in order for the well information to be displayed.)

Plate Read/Allele Discrimination Fluorescence Intensity Values

The **Fluorescence Intensity Values** screen displays a colorized image of final calls and fluorescence intensities. A plus sign (+) indicates a positive call and a minus sign (–) indicates a negative call.

A spectrum of colors, displayed at the bottom of the screen, is used to indicate the relative fluorescence readings for each dye in each well.

The circle for each well is divided into arcs which correspond to the dyes selected for analysis in the **Analysis Selection/Setup** screen. An **R** in a portion of the circle indicates that portion refers to a reference dye. The replicate set number, if any, appears in the lower right corner of the well.



Interpreting the Intensity Values Display

The accumulation of product can be interpolated by comparing the color in the arc to the horizontal color bar at the bottom of the screen. On the color bar, as fluorescence values increase, the corresponding color shifts from blue towards red. Note that the bar only covers the fluorescence range measured on the plate. For example, in the example shown above, the range of fluorescence measured in the run was between 3857 and 21946, and this is the range that is covered by the color bar.

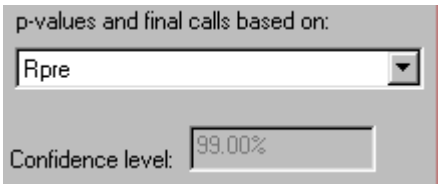
Interpreting Final Calls

The **p-value** is the probability that the mean of one set of sample data is different from the mean of another set of sample data. The first set of sample data is always the control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an **Unknown** well). When replicates are being treated collectively, the second set of sample data consists of all of the replicates. If the **p-value** exceeds the user-specified confidence level, the well/dye is called as positive and signified with a plus sign (**+**); otherwise, the well is called as negative (no difference detected) and signified with a minus sign (**–**). The confidence level for positive calling is displayed on the command panel. This value may be changed using the **Analysis Term Settings** dialog box that may be accessed by clicking the corresponding button on the **Analysis Selection/Setup** screen.

Analysis Options

Fluorescence Data Type Displayed and Used for Calculations

Under **p-values and final calls based on,** specify the form of the fluorescence data to be displayed and used in calculating the p-values used for making final calls.

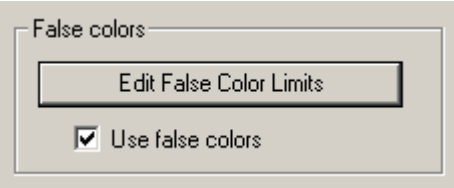


Available fluorescence data types are:

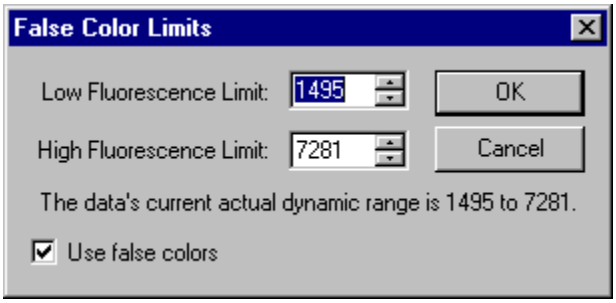
Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Display Options

Adjusting the Fluorescence Intensity Range



The range of fluorescence intensity covered by the color spectrum can be manually specified by clicking the **Edit False Color Limits** button to open the **False Color Limits** dialog box. The minimum value for the **Low Fluorescence Limit** is 0 and the maximum value for the **High Fluorescence Limit** is 65535. A range of at least 4 must be specified. Manually specifying a narrow range of values is helpful when attempting to discern small differences in fluorescence values among samples.



False Colors Versus Grayscale

By default, the Mx3000P software uses a multi-hued spectrum of colors to depict differences in fluorescence values on this screen. The depiction may be changed to grayscale by deselecting the **Use false colors** checkbox. All colors will turn to shades of gray and the color bar will display a gradient of gray from black (lowest fluorescence) to white (highest fluorescence).

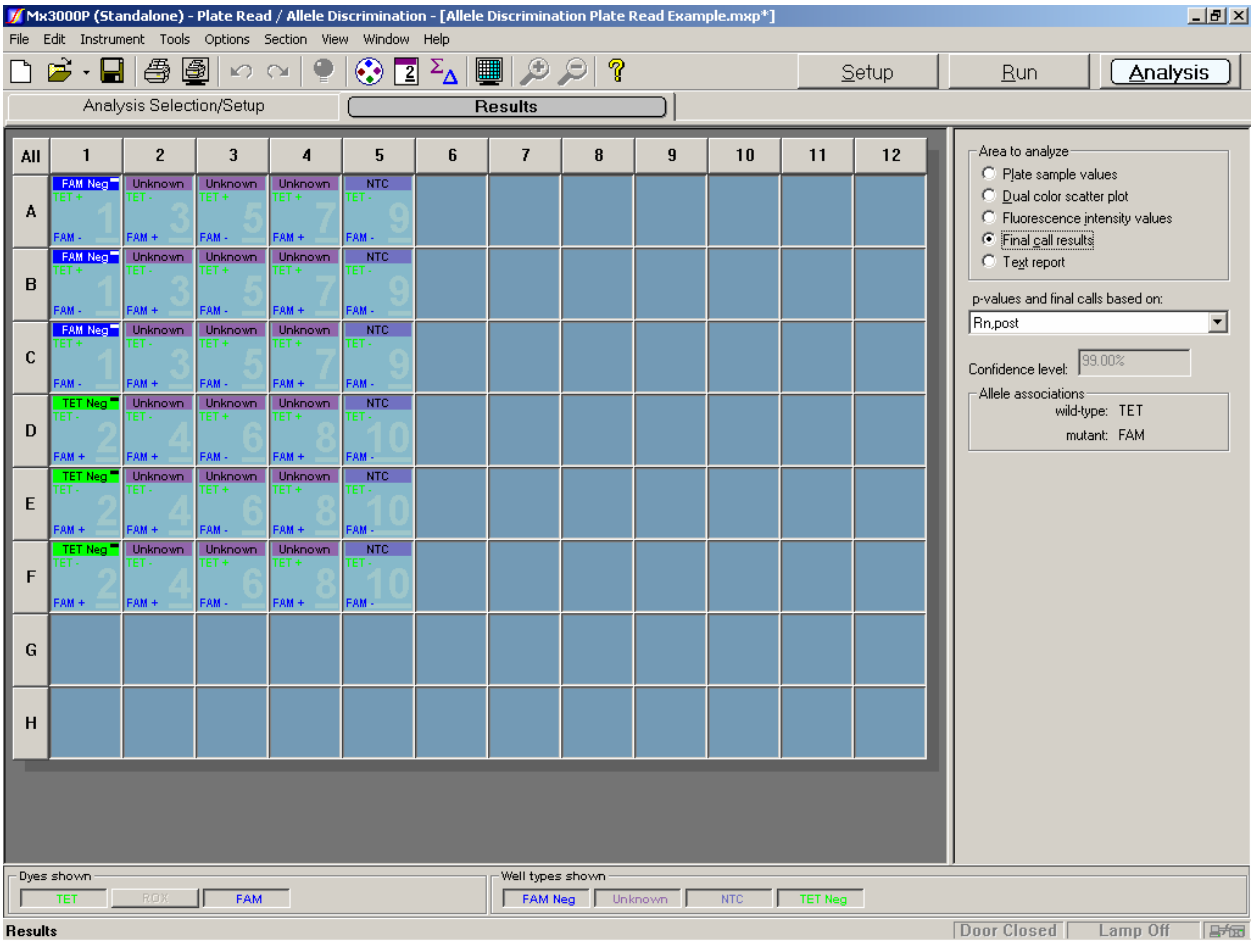
Plate Read/Allele Discrimination Final Call Results

The **Final Call Results** screen provides a simple depiction of whether product was accumulated for each dye in each well in a plate format. A plus sign (**+**) signifies detection of product accumulation for the indicated dye, while a minus sign (**–**) signifies that product accumulation was not detected. See below for more information on how final calls are made.

In each well with a well type assigned, the following information is shown:

- Well type
- The final call for each dye for which data was collected. [For all well types, the reference dye (**Ref**) is not associated with a final call]
- Replicate symbol, if used (when replicates are treated collectively, the replicate symbol is underlined)

The allele associated with each dye is shown under **Allele associations** on the command panel. Allele names may be changed on the **Dual color scatter plot** screen.



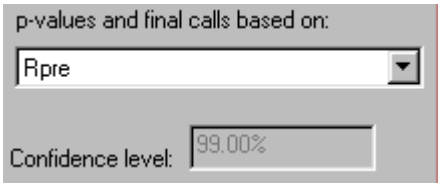
Interpreting Final Calls

The **p-value** is the probability that the mean of one set of sample data is different from the mean of another set of sample data. The first set of sample data is always the control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an **Unknown** well). When replicates are being treated collectively, the second set of sample data consists of all of the replicates. If the **p-value** exceeds the user-specified confidence level, the well/dye is called as positive and signified with a plus sign (+); otherwise, the well is called as negative (no difference detected) and signified with a minus sign (–). The **Confidence level** is displayed on the command panel. This value may be changed using the **Analysis Term Settings** dialog box..

Analysis Options

p-Values and Final Calls Based On

Under **p-values and final calls based on,** specify the form of the fluorescence data to be used in calculating the p-values used for making final calls.



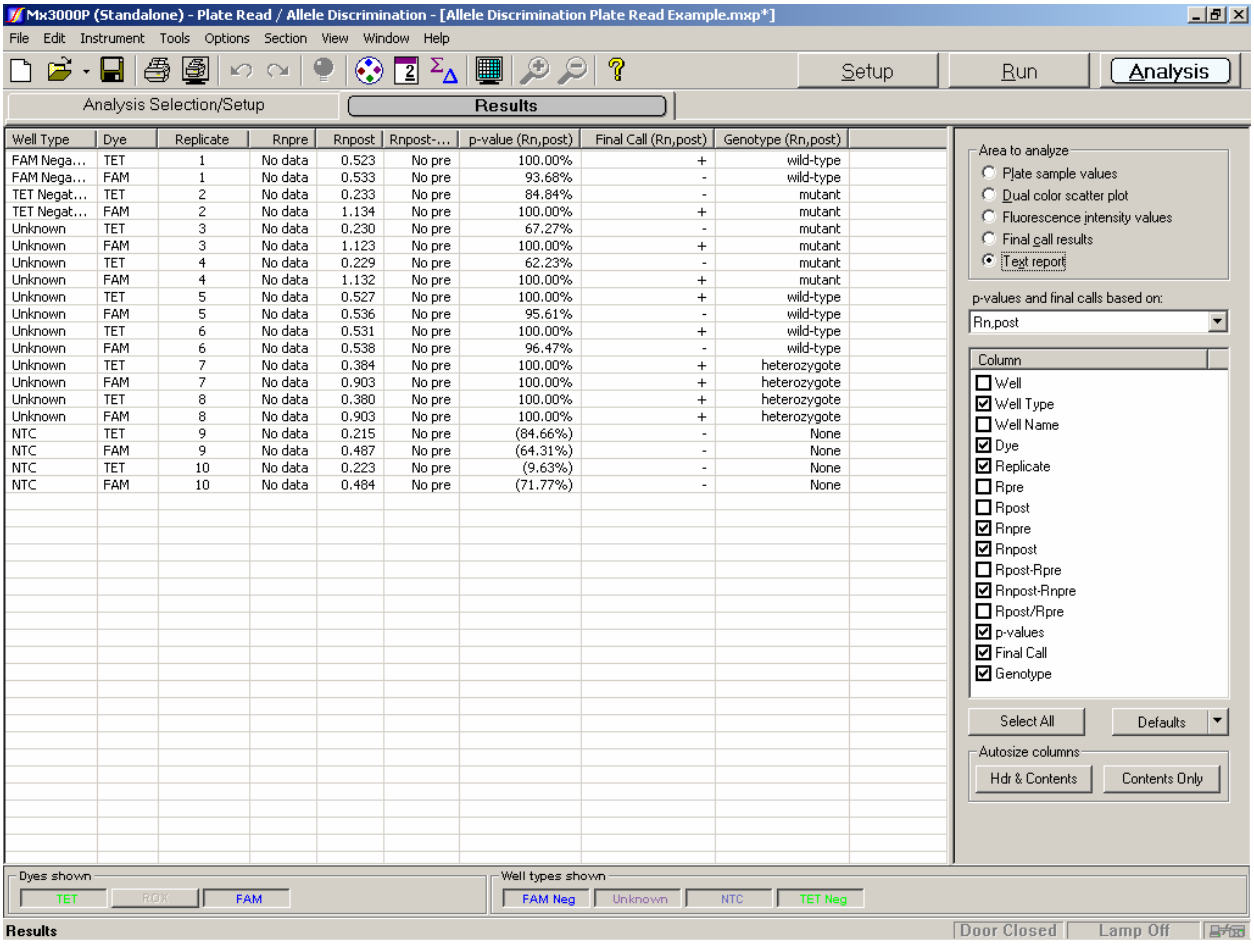
Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Note that if no reference dye has been assigned to the wells and a normalized data type (**Rn**) is selected, a **No Ref** error designation will replace the final call entry on the plate.

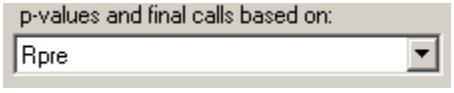
Plate Read/Allele Discrimination Text Report

The **Text Report** screen shows the data from a run in text format.



Analysis Options

p-Values and Final Calls Based On



Under **p-values and final calls based on,** specify the form of the fluorescence data to be used in calculating the p-values used for making final calls.

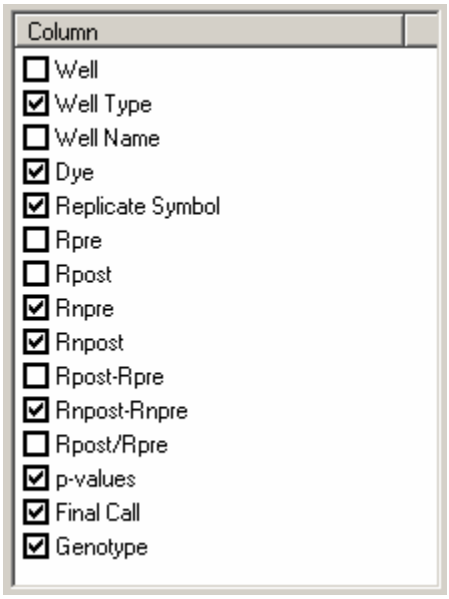
Available fluorescence data types are:

Data Type	How Calculated
Rpre	initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rpost	final fluorescence (use also for single reading initiated with **Post-Read**)
Rn,pre	normalized initial fluorescence (use also for single reading initiated with **Pre-Read**)
Rn,post	normalized final fluorescence (use also for single reading initiated with **Post-Read**)
Rpost–Rpre	total change in fluorescence (final fluorescence minus initial fluorescence)
Rn,post–Rn,pre	total change in normalized fluorescence (normalized final fluorescence minus normalized initial fluorescence)
Rpost/Rpre	fold-change in fluorescence (final fluorescence divided by initial fluorescence)

Note that if no reference dye has been assigned to the wells and a normalized data type (**Rn**) is selected, a **No Reference** error designation will replace the p-value and final call entries in the report.

Display Options

Columns Included in the Report



Select the **Columns** to be included in the text report by checking the boxes next to the column names. To exclude columns that are selected by default, click on the check box to deselect the column name. To select all columns to display, click **Select All**. The default settings for column selection can be restored by expanding the **Defaults** menu and then clicking **Get**.

If the included columns will not fit in the screen, a horizontal scroll bar appears at the bottom of the window to allow horizontal scrolling.

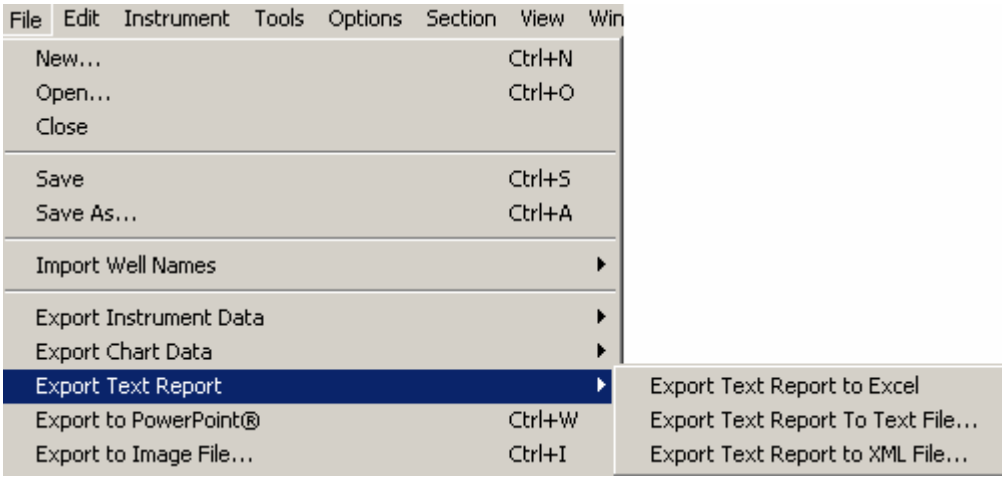
Column Width

To facilitate viewing data, columns can be automatically sized. Clicking the **Contents Only** button will cause each column to be automatically resized to the width of the widest data cell in that column (some column headers may be only partially displayed using this setting). Clicking the **Hdr & Contents** button will cause each column to be resized to the widest cell in that column, including the header cell.

Columns can also be resized manually by dragging the column dividers with the mouse.

Exporting Text Report Data

Text report data can be exported from the application in text, Microsoft Excel spreadsheet or XML format for analysis. Text report exporting options are available from the **File** menu. See *Exporting Data to Other Applications* in the *File Menu* section for more information.



Maintaining the Mx3000P Instrument

Hardware Information

Replacement Parts

It is important to use only Stratagene-supplied replacement parts in the maintenance of the Mx3000P system (excluding fuses).

Fuses

The Mx3000P instrument uses a single slow-acting fuse. The fuse housing is located adjacent to the power cord inlet in the power entry module.

To replace the fuse, disconnect the Mx3000P instrument from the power source, open the fuse housing cover and remove the fuse holder from the power entry module. Replace the fuse with the appropriate fuse type from the table below, and then replace the fuse holder.

Instrument Type (Voltage)	Replacement Fuse
110-V	10-A, 250-V, 5×20-mm slow-acting fuse
230-V	5-A, 250-V, 5×20-mm slow-acting fuse

Warning: High voltage present. Turn off the power switch and remove the power cord from the power entry module prior to replacing the fuse.

Changing the Lamp

The lamp of the Mx3000P system has an average life expectancy of 2000 hours. Replacement bulb assemblies are available from Stratagene (Catalog #401411).

Important: Never touch the glass portions of the quartz-tungsten halogen lamp!

The lamp is located in the excitation housing, on the left-hand side of the instrument. Before changing the lamp, be sure to turn off the instrument. The lamp is replaced, along with the lamp housing, as follows:

1. Remove the two screws securing the excitation housing cover panel and remove the panel.
2. Disconnect the wire connector for the lamp housing. This is located near the bottom of the module towards the front of the instrument.
3. Loosen the thumbscrew that holds the lamp housing in the excitation module.
4. Remove the old lamp housing and lamp.
5. Insert the new lamp housing and lamp. A keyway on the lamp housing allows the housing to be placed in its holder in only one orientation. Push the housing in as far as it will go.
6. Tighten the thumbscrew.
7. Connect the wire connector.
8. Replace the cover panel and the two cover screws.
9. After the lamp has been changed, reset the lamp timer to 0. To do this, access the **Lamp Reset and Utilities** dialog box (from the **Instrument** menu) and click the **Reset** button. The **Total time** will be set to 0 and the current date will be entered in the **Last date reset** field.

Cleaning the Instrument

Clean the exterior housing of the Mx3000P system using only mild soap and warm water on a soft cloth. Do not use alcohol or solvent-based cleaners.

The wells of the thermal block should be cleaned with cotton swabs and isopropyl alcohol. Ensure the block is at room temperature before cleaning.

Appendix

Mx3000P System Troubleshooting

Observation	Suggestion
"Communication Error" appears in software	Instrument has not finished warming up, wait for both front panel LED's to become steady lights and for the instrument communication icon in the bottom right corner of the screen to turn from red to green. Verify that the Mx3000P instrument is powered on. Verify that the RS-232 cable between the PC and Mx3000P instrument is attached. If the "Communication Error" warning persists, connect the cable to the other USB port on the PC.
Lamp On or other instrument-related software commands are not available	Make sure the software version running is not a Standalone version. If using software installed for analysis only or if multiple copies of the software are open, *Standalone* will appear in the upper left corner of the Mx3000P software title bar. When communication between the PC and the instrument is established, the communication icon in the lower right corner is green.
Software locks up or shuts down during a run	Turn off the sleep mode for the monitor and disable the screen saver. This can be accessed in the Microsoft Windows software Control Panel.
Lower LED power indicator light never comes on	Verify that the instrument is plugged into the power source or surge protector and that power is getting to the instrument. Check fuse on back panel near the power cord. If fuse is burned out, replace the fuse. See *Fuses* for more information.
After switching on the instrument, the upper LED status indicator light does not indicate Ready status (steadily lit)	During instrument power-up, the upper LED should remain off for 2-3 minutes while the instrument warms up. The instrument will perform a self-test, which is indicated by a blinking upper LED. If the LED does not light continuously after the self-test is completed, reboot the instrument (power off the instrument, wait 30 seconds, and then switch the instrument power on). If this does not address the problem, contact Stratagene Technical Services.
Very low amplification signal	May indicate that the lamp is starting to fail. Check lamp, and replace if necessary. See *Changing the Lamp*.

Sudden amplification signal loss or downward drift during run	Check lamp, replace if necessary. See *Changing the Lamp*.
Signal fluctuates during run	Check lamp, replace if necessary. See *Changing the Lamp*. Verify that the fluorescence signal is below the recommended maximum of 35000. If necessary, adjust the **Filter Gain Settings** to bring the signal into the recommended range.
Consistently unexpected results in one sample	Check the sample container for contaminating material. Check optical clarity of sample container cap. Clean the thermal block.
Decreased volume in sample containers at end of run	Sample containers are not vapor tight. Ensure caps are tightly sealed and containers are not malformed.
Software font difficult to read/Poor resolution of text	Microsoft Windows software display settings are incorrect. Required minimum settings are 16 bit high color and 1024x768 resolution.
Chart printing is low quality	Check the status of the **High resolution chart printing** option on the Print Page Setup dialog box. Chart printing using most printers will be enhanced by selecting the **High resolution chart printing** option. Limitations of certain printer drivers cause incompatibilities with this setting; try deselecting the **High resolution chart printing** checkbox.
Difficulty in manually setting baseline	When running samples with a broad dynamic range, use the adaptive baseline algorithm.
Increased signal noise for run	Use the moving average algorithm.
Ct value reported for NTC (no-target control) sample is less than the total number of cycles but the amplification plot curve is horizontal	Examine variation in fluorescence intensity during the run. Review amplification plot and adjust the threshold accordingly.

Low increase in fluorescence with cycling	The probe or beacon is not binding the target efficiently. Lower annealing temperature and verify melting temperature. Target PCR product is too long, redesign primers to yield a PCR product <150 bp in length. Magnesium concentration is too low; run a titration to optimize concentration. Insufficient or non-specific product, verify product formation through gel electrophoresis.
Increase in fluorescence in control reactions without template	The reaction has been contaminated.
Excess condensation in reaction tubes	When using single tubes or strip tubes, install the Perfect Fit frame on the thermal block to optimize contact between the sample tubes and the hot top.

Getting Support for the Mx3000P Real-Time PCR System

Email: tech_services@stratagene.com

QPCRSystemsSupport@stratagene.com

World Wide Web: www.stratagene.com

United States and Canada

Stratagene
11011 North Torrey Pines Road
La Jolla, CA 92037
USA
Telephone: (858) 535-5400
Order Toll Free: (800) 424-5444
Technical Services: (800) 894-1304

Europe

Location	Telephone	Fax	Technical Services
Austria	0800 292 499	0800 292 496	0800 292 498
Belgium	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 15775	0800 15740	0800 15720
France	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 919 288	0800 919 287	0800 919 289
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 182 8232	0800 182 8231	0800 182 8234
Netherlands	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 023 0446	+31 (0)20 312 5700	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 563 080	0800 563 082	0800 563 081
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 917 3282	0800 917 3283	0800 917 3281

Japan Technical Services

Telephone: 03-5159-2070
Email: jtech@stratagene.com

All Other Countries

Please contact your local distributor. A complete list of distributors is available at *www.stratagene.com*.

QPCR Glossary

Experiment Type and QPCR Detection Chemistry Terms

Real-Time Experiments: Experiments that monitor and report the accumulation of PCR product, as detected by increased fluorescence, during thermal cycling. This allows data collection during the early exponential phase of PCR so that results may be calculated from **Ct** values.

Plate-Read Experiments: A one-time fluorescence reading taken on a plate. Experiments can be performed including pre- and post- thermal cycling readings in the same experiment. Results are evaluated as + or –.

Quantitative PCR Experiment Type: Experiments of this type typically use a standard curve to quantitate the amount of target present in an **Unknown** sample with high accuracy using a fluorescence-labeled probe for detection. A series of **Standard** samples, containing dilutions of a known amount of target, are amplified to generate a curve that relates the initial quantity of the specific target to the **Ct**. The standard curve is then used to derive the initial template quantity in **Unknown** wells based on their **Ct** values. This method is sometimes referred to as absolute quantitation or as standard-curve quantitation in the literature. This experiment type is also useful for primer/probe optimization experiments in the absence of a standard curve.

Comparative Quantitation Experiment Type: This experiment type is a form of relative quantitation, comparing the levels of a target gene in test samples (referred to as **Unknowns**) relative to a sample of reference (referred to as the **Calibrator**). For example, the **Calibrator** sample might contain RNA from untreated cells, while the **Unknowns** might contain RNA from cells treated with different agents of interest. This experiment type provides an efficient method for comparing levels of RNA or DNA across samples when information about the absolute amounts of target in any sample is not required. This method is used for establishing relative quantitation without the need for repeatedly performing a dilution series standard curve.

SYBR Green Experiment Type: Experiments of this type typically use a standard curve to quantitate the amount of target present in an **Unknown** sample using SYBR Green I dye for detection. This experiment type is also useful for SYBR Green assay optimization experiments in the absence of a standard curve. The thermal profile includes a dissociation curve, used to discriminate between specific and non-specific PCR products.

Allele Discrimination/SNP's Real Time Experiment Type: .This experiment type uses fluorescence-labeled probes to determine the allelic composition of DNA samples. Two fluorogenic probes, labeled with two spectrally distinct dyes, are used to discriminate between two different alleles that may differ by as little as a single nucleotide. The presence or absence of a given allele is based on the Ct value determined for the allele-specific probe.

Molecular Beacon Melting Curve Experiment Type: This experiment type analyzes the melting characteristics of a molecular beacon for the determination of the experimental melting temperature (Tm) and optimal annealing temperature for subsequent PCR experiments. Melting curves are typically performed with the molecular beacon alone and in the presence of both perfectly-matched and mismatched oligonucleotide targets in order to determine the temperature that exhibits the best discrimination.

Plate Read/Allele Discrimination Experiment Type: This experiment type is used both for general plate-reading tasks and for allele discrimination assays with endpoint detection. In both cases, samples may be called as positive or negative for the presence of a specific sequence based on whether the endpoint fluorescence readings meet user-defined statistical criteria.

Quantitative Plate Read: This experiment type uses a standard curve to estimate the amount of target present in an **Unknown** sample using a fluorescence-labeled probe for detection. A series of **Standard** samples, containing dilutions of a known amount of target, are included in the experiment to generate a curve that relates the quantity of the specific target to the final fluorescence reading. The standard curve is then used to estimate the template quantity in **Unknown** wells based on their fluorescence readings.

Reference Dye: Passive dye used for normalization of the fluorescence signal of the reporter fluorophore. The reference dye fluoresces at a constant level during the reaction.

Reporter Dye: Fluorescent dye that increases in fluorescence signal as the amount of PCR product increases.

Normalizer: Available only in **Comparative Quantitation** to designate the dye corresponding to the target used for normalization of the fluorescence signal across samples.

Molecular Beacon Probes: Hairpin-shaped fluorescence-labeled probes that can be used to monitor PCR product formation either during or after the amplification process. The free probe will maintain its hairpin structure, which causes the quenching of the fluorophore. In the bound form the fluorophore is separated from the quencher and is thereby able to fluoresce.

TaqMan Probes: linear FRET fluorescence-labeled probes that can be used to monitor PCR product formation either during or after the amplification process. As the DNA polymerase extends the upstream primer and encounters the downstream probe, the exonuclease activity of the polymerase cleaves the probe. In this event, the reporter fluorophore is released into the reaction solution and is able to fluoresce.

Quencher: a moiety that absorbs the energy of the reporter dye in its excited state. The quencher can emit its own fluorescence signal (TAMRA) or emit no fluorescence signal (DABCYL, BHQ).

Well-Types

Unknown: Contains a complete reaction mixture including a test template that contains an unknown amount of the specific target.

Buffer: Contains only buffer, used to monitor the background fluorescence attributable to the buffer.

NAC: No amplification control; contains all reaction components except DNA polymerase.

NPC: No probe control; contains all reaction components except the fluorescence-labeled probe.

NTC: No template control; contains all reaction components except the template nucleic acid.

Standard: Contains a complete reaction mixture including a known concentration of target nucleic acid. Used to generate a standard curve, which is then used to relate the threshold cycle (**Ct**) to initial template quantity in **Unknown** wells.

No RT: No reverse transcriptase control; contains all QRT-PCR reaction components except reverse transcriptase.

Negative Control: Contains all reaction components except the template or target sequence detected with the specified dye. Negative control well types available correspond to the dyes defined in **Optics Configuration**. In allele discrimination experiments, **Negative Control** wells are used in combination with **NTC** wells to calculate positive and negative calls.

Positive Control: Contains all reaction components including template known to be positive for the target sequence that is detected with the specified dye. Positive control well types available correspond to the dyes defined in **Optics Configuration**. Typically used in allele discrimination experiments to validate the reaction mixture.

Calibrator: Available only in **Comparative Quantitation** as the reference sample to which **Unknowns** are compared. Contains a complete reaction mixture including a characterized target. The level of a gene of interest in the **Calibrator** wells is set to 1.0 for comparison to the relative quantities in **Unknown** samples.

MBO: Available only in **Molecular Beacon Melting Curve** experiments. Sample type that contains the molecular beacon plus an oligonucleotide corresponding to its perfectly matched target.

MB: Available only in **Molecular Beacon Melting Curve** experiments. Sample type that contains the molecular beacon only (in the absence of target).

MBMO: Available only in **Molecular Beacon Melting Curve** experiments. Sample type that contains the molecular beacon plus an oligonucleotide corresponding to a target with a single-base mismatch.

Analysis Terms

Standard Curve: The Standard Curve is a plot of the initial template quantity added to standard wells on the X-axis, versus the **Ct** (threshold cycle) on the Y-axis. A best-fit curve is displayed for each dye with data collected in **Standard** wells.

Amplification Plot: The Amplification Plots view shows a plot of cycles versus fluorescence for each ramp or plateau on which data are gathered.

Dual Color Scatter Plot: The Dual Color Scatter Plot view is used to compare the amplification properties of two different targets (represented by two different dyes) in the same well. Each plotted point represents the coordinates of either the fluorescence values or threshold cycle (**Ct**) values for the two dyes in a single well.

Initial Template Quantity: Provides interpolated quantities of template added to **Unknown** wells before thermal cycling. The quantities are interpolated from a standard curve based on the calculated Ct values determined for the known quantities of template in the **Standard** wells.

Baseline Correction: For each well and each path the raw fluorescence data are fit over the specified range of cycles using a linear least mean squares algorithm to produce a baseline. The value of the baseline function is calculated for every cycle and subtracted from the raw fluorescence to produce the baseline corrected fluorescence (**dR**).

Threshold Cycle (Ct): The cycle at which fluorescence is determined to be statistically significant above background signal contributed by the fluorescently labeled oligonucleotides within the PCR reaction. The threshold cycle is inversely proportional to the log of the initial copy number.

Background Cycle Range: Specifies the range of cycles of fluorescence data the software uses to calculate the background noise level when using the **Background-based threshold** algorithm to set the threshold fluorescence. The region specified is typically in the cycle range before exponential amplification occurs. The standard deviation of the raw fluorescence for the specified cycles is calculated and is multiplied by the constant Sigma multiplier for threshold fluorescence.

Replicates: Allows specifying sets of replicated wells as the basis for the software to average results from those wells when the **Treat Collectively** setting is used. (Selecting **Treat Individually** in the **Analysis Selection/Setup** screen will cause the program to analyze each well independent of any replicate definitions.)

Collective Replicate Treatment: Will cause the program to analyze all wells with the same replicate symbol as a group, effectively treating the measurements as all coming from the same well.

R squared: The **RSq** value is an indication of the fit of the standard curve to the standard data points plotted. The value will always be between 0 and 1. The closer the value is to 1, the better the fit of the line.

Sigma: Measurement of the variability (standard deviation) of the fluorescence measured from all wells and more than one cycle. Typically its value is determined from the first few cycles, before the PCR reaction starts to affect the measurement. The Sigma multiplier is a user-defined number that is used to multiply by sigma to create a threshold value for determination of **Ct**.

p-value: The probability that the mean of one set of sample data is different than the mean of another set of sample data. The first set of sample data is always the control wells in the analysis selection. When replicates are being treated individually, the second set of sample data consists of a single well (usually an **Unknown** well). When replicates are being treated collectively, the second set of sample data consists of all of the replicates. If the p-value exceeds the user-specified confidence level, the well/dye is given a (+) call, whereas if the p-value does not exceed the user-specified confidence level, the well/dye is given a (–) call.

Confidence Level: The user-defined confidence level for calls is the statistical probability required before the algorithm will call amplification occurrence in a well. The default is 99%.

Multicomponent: A term used for distinguishing the contribution that each dye and the background makes to the total fluorescence spectra detected.

Fluorescence Data Terms

R: fluorescence reading in arbitrary units

dR: baseline subtracted fluorescence reading

Rn: fluorescence reading normalized to the reference dye

dRn: baseline subtracted fluorescence reading normalized to the reference dye

R Last: the final fluorescence reading in a real-time experiment

dR Last: the final fluorescence reading minus the initial fluorescence reading in a real-time experiment

Rn Last: the final fluorescence normalized to a reference dye in a real-time experiment

dRn Last: the normalized final fluorescence reading minus the normalized first fluorescence reading in a real-time experiment

R Last/R First: the final fluorescence reading divided by the initial fluorescence reading in a real-time experiment

Rpre: initial fluorescence reading in a plate-read experiment

Rpost: final fluorescence reading in a plate-read experiment

Rn, pre: fluorescence before thermal cycling normalized to the reference dye in a plate-read experiment

Rn, post: fluorescence after thermal cycling normalized to the reference dye in a plate-read experiment

Rpost–Rpre: the total change in fluorescence in a plate-read experiment

Rn, post–Rn, pre: the total change in normalized fluorescence in a plate-read experiment

Rpost/Rpre: the final fluorescence reading divided by the initial fluorescence reading in a plate-read experiment

Selected QPCR Links

Stratagene Webpages

www.Mx3000P.com

Stratagene QPCR Links (Provides links for primer/probe design applications and links for other QPCR-related topics.)

Stratagene Lab Tools (See the **PCR Designer** section.)

Stratagene QPCR Products

Other Websites

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

http://www.pitt.edu/~rsup/oligocalc.html

http://searchlauncher.bcm.tmc.edu/

http://www.ncbi.nlm.nih.gov/blast/

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